Effects of ODC on Polyamine Metabolism, Hormone Levels, Cell proliferation and Apoptosis in Goose Ovarian Granulosa Cells

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Abstract

Background: Ornithine decarboxylase (ODC) plays an indispensable role in the process of polyamine biosynthesis. Polyamines are a pivotal part of living cells and have diverse roles in the regulation of cell proliferation and apoptosis, aging and reproduction. However, to date, there have been no reports about ODC regulating follicular development in goose ovaries. Here, we constructed *ODC* siRNA and overexpression plasmids and transfected them into goose primary granulosa cells (GCs) to elucidate the effects of *ODC* interference and overexpression on the polyamine metabolism, hormone levels, cell apoptosis and proliferation of granulosa cells.

Results: After interfering with *ODC* in GCs, the mRNA and protein levels of *ODC* and the content of putrescine were greatly decreased ($P < 0.05$). When *ODC* was overexpressed, *ODC* mRNA and protein levels and putrescine content were greatly increased ($P < 0.05$). The polyamine-metabolizing enzyme genes *OAZ1* and *SSAT* were significantly increased, and *SPDS* was significantly decreased when *ODC* was downregulated ($P < 0.05$). *OAZ1*, *SPDS* and *SSAT* were significantly increased when *ODC* was upregulated ($P < 0.05$). In addition, after interference with *ODC*, P4 (progesterone) levels in the culture medium of GCs increased greatly ($P < 0.05$), while the overexpression of *ODC* caused the P4 level to decrease significantly ($P < 0.05$). After *ODC* downregulation, granulosa cell activity was significantly reduced, the apoptosis rate was significantly increased, and the BCL-2/BAX ratio was downregulated ($P < 0.05$). Under *ODC* overexpression, the activity of GCs was notably increased, the apoptosis rate was significantly reduced, and the BCL-2/BAX protein ratio was upregulated ($P < 0.05$).

Conclusions: Our study successfully induced ODC interference and overexpression in goose ovarian GCs, and ODC regulated mainly putrescine content in GCs with a slight influence on spermidine and spermine. Moreover, ODC participated in the adjustment of P4 levels in the culture medium of GCs, promoted granulosa cell proliferation and inhibited granulosa cell apoptosis.

Background

The egg laying performance of poultry largely depends on the development of the follicles. The granulosa cells (GCs) in ovarian follicles participate in the regulation of follicular development through the synthesis of hormones. Polyamines mainly include putrescine, spermidine and spermidine, which are indispensable components of living cells. They have diverse roles in the adjustment of gene expression, RNA translation, cell proliferation and apoptosis, as well as in the regulation of animal gametogenesis, embryo implantation, development and other reproductive functions [1–5].

ODC is a critical rate-limiting enzyme in polyamine biosynthesis that can catalyze the decarboxylation of ornithine to putrescine in cells and plays a pivotal role in polyamine metabolism [6, 7]. Armstrong research found that ODC activity in chicken ovarian granulosa layers increased with an increase in follicles [8]. Moreover, after blocking the synthesis of ovarian ODC with the irreversible ODC inhibitor α -difluoromethylornithine (DFMO), ovarian and follicular production and luteinization were inhibited.

Studies have found that ODC activity is regulated by ornithine decarboxylase antizyme (OAZ), which regulates the growth and development of animal follicles [9]. After overexpression of *OAZ1* in goose GCs, the concentrations of spermidine and putrescine in cells decreased, the level of spermine increased, and the expression of luteinizing hormone receptor (*LHR*), follicle-stimulating hormone receptor (*FSHR*) and estrogen receptor (*ER*) genes increased significantly. Research has demonstrated that polyamine deletion can inhibit the apoptosis of intestinal epithelial cells in rats by reducing the activity of CASPASE 3 and 9 and the transfer of BAX to mitochondria, thereby reducing cytochrome c efflux [10]. It was found that in many mammals (rats and pigs), ODC activity briefly increases before ovulation [11]. Older mice showed decreased ovarian ODC and putrescine levels [12, 13]. Putrescine supplementation in drinking water before obtaining oocytes and supplementing putrescine in oocyte maturation medium in vitro can reduce oocyte aneuploidy in aged mice [13]. It is worth noting that putrescine supplementation in ovulating mice can significantly improve the quality of embryos and increase the number of blastocyst cells, reducing early embryonic death and increasing the number of live births [12].

Inhibition of ODC activity not only sharply reduces polyamine content but also affects cell proliferation and apoptosis. Studies have found that ODC can directly act on estrogen receptor (ER) and androgen receptor (AR) to regulate human breast cancer cell proliferation and apoptosis [14]. Lee et al [15] showed that during the proliferation stage, the expression of ODC in C2C12 cells was upregulated. After 48 h and 72 h of DFMO treatment, the expression of ODC in C2C12 cells decreased by 40% and 66%, respectively, and cell proliferation decreased. The overexpression of the *ODC* gene promotes the proliferation of C2C12 cells, which indicated that ODC has an important regulatory effect on cell proliferation.

However, there is currently no information available on the influence of ODC on polyamine metabolism, reproductive hormone receptors, cell apoptosis and proliferation-related gene expression in poultry GCs. Focusing on these points, this work used goose primary GCs as the research object to clarify the influence of ODC on polyamine metabolism, reproductive hormone concentration, reproductive hormone receptor gene expression, apoptosis and proliferation in GCs through the interference and overexpression of the *ODC* gene.

Materials And Methods

Animals and ethics statement

The regulations on the protection and use of Sichuan white geese were approved by the Animal Ethics Committee of the College of Animal Science and Technology of Sichuan Agricultural University. In this experiment, Sichuan white geese at the peak of egg production (35–40 weeks) were selected from the poultry breeding farm of Sichuan Agricultural University and euthanized by cervical dislocation.

Cell culture and treatment

According to the methods of Kang et al. [6, 16], follicles were rapidly collected from the ovaries of the geese, and primary granulosa cells were isolated. GCs were cultured in DMEM (HyClone, USA) in a 37 °C,

5% CO₂ incubator.

Construction of ODC interference and overexpression plasmids and cell transfection

To interfere with *ODC* expression, three *ODC* siRNA sequences (si-*ODC*-331, si-*ODC*-511, and si-*ODC*-894) and a negative control (NC) were designed and synthesized (Table 1). siRNAs were transfected when the granulosa cell density reached 70%. si-*ODC*-331, si-*ODC*-511, si-*ODC*-894 and NC were diluted with Opti-MEM (Gibco, USA). Then, Lipofectamine 3000 (Invitrogen, USA) was mixed with the plasmid and added to the GCs. The cells were cultured in an incubator at 37 °C and 5% CO₂ for 24 h, 48 h and 72 h.

The BglIII and KpnI restriction endonucleases (New England Biolabs) for the pEGFP-N1 plasmid (Sangon Biotech, Shanghai, China) were a double enzyme, plastic recycling pEGFP-N1 skeleton DNA sequence. The primer sequences (F: 5'-CGCAAATGGGCGGTAGGCGTG-3' and R: 5'-CGTCGCCGTCCAGCTCGACCAG-3') were verified through bidirectional sequencing. All CDS regions of ODC were correctly inserted into the pEGFP-N1 vector, indicating that the pEGFP-N1-ODC overexpression vector was successfully constructed. The transfection complex was prepared, and the compound was incubated at 25 °C for 15 min and transfected into GCs.

Table 1
List of four siRNA sequences

Name	Sequences(5' – 3')
si- <i>ODC</i> -331	F: GCAAAUCCCUGCAAACAAATT
	R: UUUGUUUGCAGGGAUUUGCTT
si- <i>ODC</i> -511	F: GGAGCUACACUUAAGACAATT
	R: UUGUCUUAAGUGUAGCUCCTT
si- <i>ODC</i> -894	F: GGAGCAAACAGGUUCUGAUTT
	R: AUCAGAACCUGUUUGCUCCTT
NC	F: UUCUCCGAACGUGUCACGUTT
	R: AGCUGACACGUUCGGAGAATT

qRT-PCR was used to detect the expression of genes associated with polyamine metabolism, cell proliferation and apoptosis

Total RNA was extracted from GCs using TRIzol (Invitrogen, USA). The cDNA templates were obtained from total RNA samples using a reverse transcription kit (Takara, Japan). qRT-PCR detection was performed using a SYBR Green qPCR kit (Toyopo, Japan). The reaction system was: 95 °C for 5 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, 39 cycles. The primer sequences are shown in Table 2. Gapdh was used as the internal standard, and three parallel replicates were performed for each sample. The 2^{-ΔΔCt} method was used to calculate the Ct value based on the qRT-PCR.

Table 2
List of primer sequences used for qRT-PCR

Genes	Primer sequences(5' – 3')	Product length(bp)	Annealing temperature(°C)
<i>ODC</i>	F: TGTATCTGCTTGACATTGGTGGTG	146	60
	R: CAGGAAGATACTATGTGCGCATCAGC		
<i>OAZ1</i>	F: ACTTCAGGAACCCTCGCATCAACT	141	65
	R: GCTGCCCTCATCTTTCTAATACGG		
<i>OAZ2</i>	F: AAGCCTCATGTTGTCCACTTC	142	63
	R: GTGCTGATAACCCTTCTTTGC		
<i>SAMDC</i>	F: GCTTGACCCAGTAGTTATGGACCA	180	55
	R: TGAATAGTCCAGTAAGTTCCATCCG		
<i>AZIN1</i>	F: GCTCTTACTGCACATTGCCACA	180	58
	R: TGAATGTACGTTTGCAGTTCCTTG		
<i>SPDS</i>	F: TCTGCTGCCAAGGTGAGTGC	111	55
	R: TAGGGATGGTGCAATAGGCGTA		
<i>SPMS</i>	F: GTGCTGATCCTTGGAGGTGGT	110	58
	R: TTACACCCGTCGATCACCATT		
<i>SSAT1</i>	F: CACCCTTTCTACCACTGTCTG	173	58
	R: CCAATGCCAAGTCCTCTGT		
<i>APAO</i>	F: GAGTTTGAGCAACCCTTCTGG	141	58
	R: TGGCTGGAGGACCACAAA		
<i>SMO</i>	F: CTACCCACGGTGCTGTGCTTT	124	59
	R: GAATCGGGAGTTGGTGGTGTT		
<i>LHR</i>	F: GTAACACTGGAATAAGGGAAT	191	54
	R: GAAGGCTTGACTGTGGATA		
<i>ER</i>	F: ACCCAAACAGACCATTCAACGAA	187	61
	R: CGCCAGACTAAGCCAATCATCAG		
<i>FSHR</i>	F: TCCTGTGCTAACCCTTTCCTCTA	207	59
	R: AACCAGTGAATAAATAGTCCCATC		

Genes	Primer sequences(5' – 3')	Product length(bp)	Annealing temperature(°C)
<i>PR</i>	F: CCAGGATTTCCGAATTTAC	187	55
	R: GACACAGTGAATAGAACGATG		
<i>AR</i>	F: AGGAGTTTGGGTGGCTTCAGA	201	55.7
	R: GCTGGTAAAACCGCCTAGAGC		
<i>CCND1</i>	F: TGTTTACGAGCCTGCCAAGAA	109	55
	R: CTGCTTCGTCCTCTACAGTCTTTG		
<i>PCNA</i>	F: AGAAATGAATGAGCCAGTCCAGC	178	55
	R: TTCAATCTTTGGAGCCAGGTAGT		
<i>SMAD1</i>	F: AAGGGCTGCCGCATGTAATT	146	55
	R: CCGCTTGTAGTGGTAAGGATTGA		
<i>BAX</i>	F: GAAGCATTTACAGTTGCCATTACAG	162	55
	R: CCACAAGCAAGCAAAGAGCC		
<i>BCL-2</i>	F: GATGCCTTCGTGGAGTTGTATG	98	60
	R: GCTCCCACCAGAACCAAAC		
<i>CASPASE 3</i>	F: CTGGTATTGAGGCAGACAGTGG	158	60
	R: CAGCACCTACACAGAGACTGAA		
<i>CASPASE 8</i>	F: GGTGTCGCAGTTCAGGTA	127	57
	R: CATTGTAGTTTCAGGGCTT		
<i>CASPASE 9</i>	F: TTCCAGGCTCTGTCGGGTAA	150	64
	R: GTCCAGCGTTTCCACATACCA		

Western blotting was used to detect ODC expression, cell proliferation and apoptosis-related proteins

GCs were cultured according to the above methods. After washing with cold PBS, RIPA lysate containing protein inhibitor was added. The supernatant was analyzed using a BCA detection kit (Beyotime, Shanghai, China). Proteins were transferred to nitrocellulose membranes by 10% SDS-PAGE. Then, the cells were sealed for 2 h and incubated at 4 °C for 13 h with primary anti-ODC (1: 1000) (Abcam, England). The cell proliferation- and apoptosis-related protein antibodies and dilution ratios were as

follows: anti-PARP (Beyotime, AP102, 1: 1000), anti- β -actin (1: 2000) (TransGen Biotech, HC201-01), anti-Cyclin D1 (1: 1000), anti-Bcl-2 (1: 1000) and anti-Bax (1: 1000) (Abcam, England). The goat anti-rabbit IgG labeled with 1: 2000 diluted horseradish peroxidase (HRP) was incubated at room temperature for 1 h and then washed with TBST. Finally, ECL (enhanced chemiluminescence) reagent (Beyotime, Shanghai, China) was used for development on a gel imaging system instrument. Image Lab (Bio-Rad, USA) was used to analyze the optical density levels and to calculate the relative protein content.

High-performance liquid chromatography (HPLC) was used to detect polyamines

The polyamine content in the goose GCs was determined by HPLC according to the methods of Kang et al. [17]. The general steps were as follows. First, polyamine standard curves were prepared. Goose GCs were ultrasonically lysed, and 1 mL of 5% perchloric acid and 10 μ L of 1,6-hexanediamine standard working solution were added. Then, the samples were sonicated for 10 min and centrifuged, and the supernatant was collected. Then, the samples were extracted again with 1 mL of 5% perchloric acid, the supernatant was removed, an equal volume of 2.5 mol/L NaOH and 7 μ L of benzoyl chloride was added, and the samples were derivatized at 40 °C for 1 h. The samples were adjusted to a neutral pH with 6 mol/L HCl, and a HyperSep C18 extraction column (Thermo Fisher Scientific) was used to extract and separate the derivative products. The extraction column was washed with 15 mL of ultrapure water and 15 mL of 15% (v/v) aqueous methanol solution to purify the derivative products. Chromatographic grade methanol was added to the extraction column for elution. The methanol:ultrapure water ratio was 62: 38 (v/v), and the column temperature of the Hypurity C18 chromatographic separation column was 40 °C. The results were compared with the standard curve of polyamine.

Hormone (E2 and P4) detection in the culture medium

According to the instructions of the goose E2 and P4 ELISA kits (Qisong Biological Technology, Beijing, China), the cell culture medium was collected and centrifuged at 4,000 r/min for 15 min. Then, HRP detection antibody was added to the sample wells and standard product wells, which were incubated at 37 °C for 1 h, filled with washing buffer for 1 min and washed 5 times. Substrates A and B were added to each well, and the OD value was detected. The concentration of each sample was calculated according to the curve equation.

Analysis of cell proliferation in transfected granulosa cells by MTT assay

GCs were cultured in 96-well plates ($n = 6$), and the control and test groups were transfected as described above. Forty-eight or twenty-four hours after transfection of the interference and overexpression plasmids, 0.5 mg/mL MTT reagent (Beyotime, C0009M, China) was added, and the cells were incubated for 4 h before discarding the DMEM. DMSO (150 μ L/ well, Beyotime, Shanghai, China) was added. A microplate reader was used to detect the OD value at 490 nm, and zero adjustment holes were set up

(DMEM, MTT and DMSO). Calculated cell activity = (OD treatment - OD adjustment) / (OD control - OD adjustment).

Flow cytometry was used to detect the apoptosis of transfected GCs

According to the instructions of the Apoptosis Detection Kit (BD Biosciences, USA), after the GCs were transfected, the cell culture medium was removed from the centrifuge tube. The cells were digested with trypsin after washing with precooled PBS. The cell culture medium collected before was added to stop digestion. After centrifugation, the cells were washed with cold PBS once. The cells were resuspended in $1 \times$ binding buffer, and the suspension was filtered through a strainer to adjust the cell concentration to 1×10^6 / mL. Annexin V-FITC and PI staining solution was added to the cell suspension, mixed with $1 \times$ binding buffer, and analyzed with flow cytometry. Analysis was performed by using the flow cytometry software CytExpert 2.0.

Statistical analysis

The MEANS process in SPSS software was used for statistical analysis. ANOVA was used to compare multiple groups, Duncan's multiple comparisons test was used to identify significant relationships, and GraphPad Prism 6 was applied for graphing. The results are shown as the mean $\pm$ SEM. $P < 0.05$ represents a significant difference, and $P < 0.01$ represents an extremely significant difference.

Results

Effects of ODC interference on the levels of ODC mRNA and protein in the ovarian granulosa cells of geese

si-*ODC*-331, si-*ODC*-511 and si-*ODC*-894 were transfected into GCs for 24 h, 48 h and 72 h, respectively. The interference efficiency of *ODC* was the highest when transfected with si-*ODC*-894 plasmid for 48 h, which was much lower than that of the Blank and NC groups ($P < 0.01$) (Fig. 1A). Then, western blot analysis was utilized to further verify the level of ODC protein after the transfection of si-*ODC*-894 at different times. Figure 1B shows that the protein expression decreased significantly when ODC was interfered with for 48 h ($P < 0.05$), which was aligned with the results acquired by qRT-PCR. Therefore, si-*ODC*-894 transfected for 48 h was selected as the subsequent experimental condition.

Effects of ODC overexpression on the levels of ODC mRNA and protein in goose ovarian granulosa cells

As shown in Fig. 2A, when the *ODC* overexpression plasmid was transfected into GCs for 24 h, 48 h and 72 h, *ODC* expression was considerably upregulated by 19.9 times, 19.7 times, and 13.1 times, respectively ($P < 0.01$). Figure 2B shows that after 24 h of overexpression, the protein level of *ODC* was significantly upregulated ($P < 0.05$), which was consistent with the results obtained by qRT-PCR. Thus, the transfection of GCs with the *ODC* overexpression plasmid for 24 h was selected as the subsequent experimental condition.

Effects of ODC on polyamine contents and polyamine metabolism-related gene expression in GCs

Upon *ODC* interference, the putrescine content in GCs was significantly decreased ($P < 0.05$) (Fig. 3A). When *ODC* was overexpressed, the putrescine content in GCs was greatly increased ($P < 0.05$) (Fig. 3B). Both the concentrations of spermidine and spermine were not significantly different under *ODC* interference or overexpression conditions ($P > 0.05$).

Then, we studied the effect of *ODC* interference and overexpression on the gene expression of polyamine-metabolizing enzymes in GCs. The change in the expression of *SPMS* and *SAMDC* was not significant after interference with *ODC* ($P > 0.05$), while *OAZ1*, *OAZ2*, *AZIN1*, *SPDS*, *APAO*, *SSAT* and *SMO* gene expression was significantly upregulated ($P < 0.05$), and *SPDS* expression was remarkably decreased ($P < 0.05$) (Fig. 3C). *OAZ2*, *SPMS* and *APAO* expression was not significantly changed when *ODC* was overexpressed in GCs ($P > 0.05$), and the expression of *OAZ1*, *SPDS*, *AZIN1*, *SSAT* and *SMO* was significantly increased, while *SAMDC* expression was greatly reduced ($P < 0.05$) (Fig. 3D).

Effect of ODC on the gene expression of reproductive hormone receptors in GCs and P4 and E2 levels in culture medium

After interfering with *ODC*, the expression of *ER*, *FSHR* and *LHR* genes in GCs was greatly decreased, while the *AR* gene was markedly increased ($P < 0.05$) (Fig. 4A). The P4 level in the culture medium was remarkably increased ($P < 0.05$) (Fig. 4C), and the E2 content was tremendously decreased ($P < 0.01$) (Fig. 4D). As shown in Fig. 4B, when *ODC* was overexpressed, the expression of the *ER* gene in GCs was markedly downregulated ($P < 0.05$). *LHR*, *FSHR*, *AR* and *PR* were not remarkably impacted ($P > 0.05$). The P4 level was significantly decreased ($P < 0.05$) (Fig. 4E), while the E2 level did not markedly change ($P > 0.05$) (Fig. 4F).

The viability of GCs was analyzed after ODC interference and overexpression

After *ODC* expression interference in goose ovarian GCs, the number of decreased and dead GCs was observed under the microscope and compared with that of the NC group (Fig. 5A). MTT results showed that granulosa cell activity was remarkably reduced ($P < 0.05$) (Fig. 5A). As shown in Fig. 5C, after *ODC* was overexpressed, the number of GCs was significantly increased, and the cell morphology was fuller than that of the NC group. MTT results indicated that the activity of GCs was greatly increased after *ODC* overexpression ($P < 0.01$) (Fig. 5D). Then, we explored the effects of *ODC* on proliferation-related proteins. Figure 5E and 5F show that the protein level of *CCND1* decreased significantly when *ODC* was downregulated ($P < 0.01$), and the protein level of *CCND1* was considerably increased when *ODC* was upregulated ($P < 0.01$).

Effect of ODC interference and overexpression on the apoptosis of GCs

qRT-PCR was applied to detect the expression of cell apoptosis-related genes after *ODC* interference and overexpression. As shown in Fig. 6C, under *ODC* expression interference, the relative expression of *BAX*, *CASPASE 8* and *CASPASE 9* mRNA was greatly upregulated ($P < 0.05$). When *ODC* was overexpressed

(Fig. 6D), the expression of the proapoptotic gene *BAX* was remarkably reduced ($P < 0.05$), and that of *BCL-2*, *CASPASE 3*, *CASPASE 8* and *CASPASE 9* was not significantly changed ($P > 0.05$).

Western blotting was applied to assess the influence of *ODC* interference and overexpression on the protein levels of apoptosis-related genes in GCs. *BAX* and *BCL-2* increased significantly after *ODC* interference ($P < 0.05$) (Fig. 6E). *BAX* was markedly reduced after *ODC* overexpression ($P < 0.05$), whereas *BCL-2* showed no notable change ($P > 0.05$) (Fig. 6F).

Furthermore, flow cytometry was utilized to test the influence of *ODC* interference and overexpression on the apoptosis of GCs. The apoptosis rate of GCs was greatly upregulated after interfering with *ODC* ($P < 0.05$) (Fig. 6A), and the apoptosis rate of GCs was significantly downregulated when *ODC* was overexpressed ($P < 0.05$) (Fig. 6B).

Discussion

ODC is the pivotal rate-limiting enzyme for polyamine biosynthesis, and its activity can directly regulate the content of polyamines in cells. Studies have shown that changes in *ODC* expression and biological activity directly affect the contents of polyamine, which plays a crucial role in cell apoptosis, proliferation and animal reproduction [18–19]. In this study, when GCs were transfected with the si-*ODC*-894 plasmid for 48 h, the mRNA and protein expression levels of *ODC* were remarkably reduced ($P < 0.05$). When the overexpressed *ODC* plasmid was transfected for 24 h, the mRNA and protein expression levels of *ODC* were considerably increased ($P < 0.05$), indicating that *ODC* interference and overexpression in GCs were successfully achieved.

Studies have reported that after *ODC* overexpression, the *ODC* activity in HEK293 cells increased by 100 times, and the content of putrescine increased by 10 times, but the concentrations of spermidine and spermine were only slightly reduced [20]. This experiment found that the content of putrescine in GCs increased significantly after the overexpression of *ODC*. In previous studies that transfected the *ODC* overexpression vector into H9c2 cells, *ODC* activity was significantly increased, and putrescine and spermidine levels were also significantly increased [21]. In this work, interfering with the expression of *ODC* reduced the content of putrescine in GCs, while the content of spermidine and spermine did not change significantly. Furthermore, putrescine content was significantly reduced after interfering with *ODC* expression in MCF7 breast cancer cells [22]. This experiment indicated that *ODC* expression changes mainly affected the putrescine content in GCs and had a small influence on the concentration of spermidine and spermine.

Previous studies found that the level of *OAZ1* mRNA in the ovaries of laying geese was markedly higher than that in the ovaries of prelaying geese. This result indicated that the high expression of *OAZ1* disrupted polyamine homeostasis by suppressing *ODC* activity and inhibited follicular development [23]. At present, there is no direct evidence that *ODC* levels can affect *OAZ1* gene expression, but *ODC* and *OAZ1* are synergistically expressed in GCs, suggesting that enhanced *ODC* expression may cause corresponding changes in *OAZ1* expression. Zhao et al. [24] showed that the overexpression of *ODC* can

promote the expression of *OAZ1* in endometrial cells, which corresponded with the results of this research. *SSAT* is regarded as the rate-limiting enzyme of polyamine catabolism. It has been confirmed in many cell lines that polyamine can promote the expression of *SSAT* [25]. This study found that putrescine content was reduced and *SPDS* gene expression was inhibited after interfering with *ODC*. Zhu et al. [14] interfered with *ODC* gene expression in MCF7 and T47D cells and found that *ODC* protein and mRNA levels were remarkably downregulated while the genes expression of *SMO* and *SSAT* was upregulated, and the protein expression level of *SMO* was increased. This finding was consistent with the results of our experiment. After interfering with the *ODC* gene in goose GCs, in addition to upregulating the expression of the *SMO* and *SSAT* genes, the expression of the *APAO* gene also increased significantly. In this work, *OAZ1* and *OAZ2* expression increased greatly after *ODC* expression interference, and the expression of the *SPMS* and *AZIN* genes also increased significantly. *SAMDC* was considered to be the most important polyamine synthetase after *ODC* [26]. Studies have found that both *ODC* knockout and *SAMDC* knockout are lethal in mouse embryos [27, 28]. In this experiment, the interference or overexpression of *ODC* affected the gene expression of polyamine metabolism enzyme in GCs. Accordingly, we speculated that the protein and mRNA levels of *ODC* were significantly changed, resulting in changes in the content of putrescine in cells and in the stimulation of GCs, which affected the changes in the gene expression of polyamine metabolism enzymes.

ODC activity in mammalian ovaries was reported to transiently increase as oocytes mature in vivo, and oocyte maturation and development are regulated by GCs. In the early pregnancy of hamsters and rats, treatment with progesterone receptor antagonists can result in decreased *ODC* activity and eventually lead to miscarriage, which indicates that *ODC* activity in the ovary is closely related to hormones [29, 30]. This study found that after interfering with *ODC*, the *E2* level was remarkably reduced, and *ER* expression was greatly decreased. After the overexpression of the *ODC* gene, the *E2* level in the medium did not change significantly, and *ER* expression decreased significantly. It is speculated that the expression of *E2* and *ER* was affected not only by *ODC* but also by the content of polyamines in GCs. However, the detailed mechanism remains to be elucidated in the future. Poultry ovaries cannot form a corpus luteum after ovulation, poultry *P4* is mainly produced by follicular GCs [31], and poultry follicles also have the ability to secrete *P4* after ovulation [32]. Studies have shown that low levels of *P4* can mediate an increase in low-frequency LH pulses, prolonging the duration of the dominant follicles and thus affecting the maturation and quality of oocytes [33], while high levels of *P4* can inhibit the synthesis of luteinizing hormone and ultimately cause atresia of the dominant follicles [34]. This study showed that upon *ODC* interference, the level of *P4* hormone in the culture medium of granulosa cells was remarkably increased. Moreover, studies have confirmed that *PR* genes play a critical role in regulating the development and reproduction of animal reproductive systems [35, 36]. Bastida et al. [37] found that after specifically using *DFMO* to inhibit *ODC* in the ovary before ovulation in mice, the level of progesterone in the ovary decreased, and then the level of progesterone in the serum of interestrus decreased. The above research showed that the *ODC* gene can regulate animal reproductive function by mediating progesterone production and its receptor gene expression.

Previous research found that ODC is an indispensable factor in yeast, and the loss of the *ODC* gene in yeast results in growth stagnation [38]. This experiment demonstrated that the expression of *BAX*, *CASPASE 8* and *CASPASE 9* was considerably increased under *ODC* interference, and cell activity was significantly decreased, suggesting that *ODC* gene expression interference may upregulate *BAX*, *CASPASE 8* and *CASPASE 9* at the transcriptional level and promote cell apoptosis. He et al. [39] found that interfering with *ODC* gene expression in esophageal phosphorus cells reduced the levels of *PCNA* and *CCNB1*, thereby inhibiting the proliferation of cells, significantly increasing the level of activated *CASPASE 3* protein, and promoting the apoptosis of esophageal phosphorus cells. In HL-60 cell lines, the overexpression of *ODC* can inhibit the apoptosis induced by toxic carotene and maintain *BCL-2* expression [40]. *CCND1* is the core component of cell cycle regulation and can promote the cell proliferation cycle. The results of this experiment showed that *CCND1* expression was significantly decreased after interfering with *ODC* gene expression, and the expression levels of *CCND1* were significantly increased after transfection with the *ODC* overexpression vector, which indicated that the upregulation of *ODC* promoted the differentiation and proliferation of GCs. When *ODC* was downregulated, granulosa cell activity was significantly reduced, downregulating the *BCL-2*/*BAX* protein ratio ($P < 0.05$); when *ODC* was overexpressed, the activity of GCs was increased greatly, and the *BCL-2* / *BAX* protein ratio was upregulated ($P < 0.05$). Overexpression of *ODC* can block the apoptosis induced by dibenzoylmethane (DBM) in HL-60 cell lines, mainly by blocking the activation of *CASPASE 9*, reducing *BAX* expression, and upregulating the *BCL-2* / *BAX* ratio [41]. Studies have shown that the *BAX* protein cannot stimulate the release of cytochrome C. However, the oligomers formed by the transfer of *BAX* from the cytoplasm to the mitochondrial membrane form multimers with *BCL-2* to enhance mitochondrial permeability, contributing to the release of cytochrome C and activating *CASPASE 9*. The enzymolysis cascade of the Caspase protease family is activated, which eventually leads to cell apoptosis. Overexpression of *ODC* may affect the cell cycle, and *ODC* activity increases in many cell types during the G1 phase [42, 43]. Inhibition of *ODC* activity by polyamine inhibitors or analogs produces different effects at different stages of the cell cycle [44, 45]. Upregulation of *ODC* in the skin of transgenic mice can stimulate cell proliferation and enhance the activity of *CCNE* / *CDK2*- and *CCNA* / *CDK2*-associated kinases [42]. Moreover, the flow cytometry results of this work illustrated that the apoptosis rate of GCs was markedly increased after interfering with *ODC* ($P < 0.05$), and when *ODC* was overexpressed, the apoptosis rate was significantly reduced ($P < 0.05$). The above results suggested that *ODC* accelerated the proliferation of goose ovarian GCs and suppressed cell apoptosis.

Conclusions

Our study successfully achieved *ODC* interference and overexpression in goose ovarian GCs, and *ODC* mainly regulated the putrescine content in GCs and had little influence on spermidine and spermine. Furthermore, *ODC* participated in the adjustment of *P4* levels in GCs, promoted GC proliferation and inhibited cell apoptosis.

Abbreviations

GCs

Granulosa cells; ODC:Ornithine decarboxylase; OAZ:Ornithine decarboxylase antizyme; SSAT:Spermidine / spermine-N1-acetyltransferase; SPDS:Spermidine synthase; P4:Progesterone; AZIN1:Antizyme inhibitor; APAO:Acetyl-polyamine oxidase; SMO:Spermine oxidase; SAMDC:S-adenosylmethionine decarboxylase; SPMS:Spermine synthase; DFMO:Difluoromethylornithine; HPLC:High performance liquid chromatography

Declarations

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Not applicable.

Authors' contributions

BK and DMJ designed the study and supervised the project. SJZ, GLM and YLJ performed mainly the experiments. LL, HYX and CCH performed data analysis. BK, CYN, HZ and YHY contributed to writing the paper and modifying the manuscript. SQH and JWH assisted in animal handling. All authors submitted comments on drafts, and read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The animal study was approved by the Animal Ethics Committee of the College of Animal Science and Technology, Sichuan Agricultural University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Levels of ODC mRNA and protein in GCs after transfection with ODC siRNA. a Expression of ODC mRNA after transfection of three si-ODC plasmids at different times, b Expression of ODC protein in GCs at different times. * $P < 0.05$, ** $P < 0.01$.

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Figure 2

Expression levels of ODC mRNA and protein in GCs after transfection with the ODC plasmid. a Expression of ODC mRNA after transfection of the overexpression plasmid at different times, b Expression of ODC protein in GCs. * $P < 0.05$, ** $P < 0.01$.

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Figure 3

Effect of ODC on polyamine content (a) (b) and the expression of polyamine metabolism-related genes in GCs (c) (d). (a) (c) ODC interference, (b) (d) ODC overexpression. * $P < 0.05$, ** $P < 0.01$.

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Figure 4

Effect of ODC on the expression of reproductive hormone receptor genes (a) (b) in GCs and on the levels of P4 (c) (e) and E2 (d) (f) in culture medium. (a) (c) (d) ODC interference, (b) (e) (f) ODC overexpression. * $P < 0.05$, ** $P < 0.01$.

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Figure 5

Effects of ODC on the morphology (a) (c) and viability of goose GCs (b) (d) and (e) (f) cell proliferation-related genes. (a) (b) (e) ODC interference, (c) (d) (f) ODC overexpression. * P < 0.05, ** P < 0.01.

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Figure 6

Effect of ODC on the apoptosis of GCs. (a) (b) The expression of cell apoptosis-related genes, (c) (d) and proteins in GCs, and (e) (f) analysis of the cell apoptosis rate by flow cytometry. (a) (c) (e) ODC interference, (b) (d) (f) ODC overexpression. * $P < 0.05$, ** $P < 0.01$.

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