Adiponectin modulates steroid hormone secretion, granulosa cell proliferation and apoptosis via binding its receptors during hens' high laying period

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ABSTRACT Adiponectin is an important adipocytokine and plays the roles in multiple metabolic processes via binding its receptors - AdipoR1 and AdipoR2, which has also been found to participate in the regulation of the reproductive system of animals, in particular by influencing the secretion of ovarian steroid hormones. To further investigate the expression of adiponectin and its receptors in follicles after in vitro incubation, and their role in the steroid synthesis of laying hens' ovaries, we performed qRT-PCR and ELISA to detect the expressions of *AdipoQ, AdipoR1*, and *AidpoR2*, and determined the key genes involved in steroidogenesis and the secretion of estradiol (**E2**) and progesterone (**P4**) through the in vitro activation of adiponectin (**AipoRon**) and overexpression or knockdown of AdipoR1 and AdipoR2. Our results revealed that adiponectin and its receptors wildly exist in follicles and granulosa cells, and AdipoRon (5 and 10 μg/mL) had no effect on granulosa cell proliferation and apoptosis but significantly stimulated the secretion of adiponectin and its receptors in granulosa cells after incubation for 24 h. Furthermore, AdipoRon could significantly stimulate the secretion of P4 and inhibit E2 level compared to those of the control group through modulating the key genes expression of steroidogenesis (*CYP19A1, StAR, CYP11A1, FSHR*, and *LHR*). The secretion of E2 was also decreased in granulosa cells by the treatments of overexpression and knockdown of AdipoR1/2, however, there was no difference in terms of the level of P4 and *StAR* expression between them if there was overexpression or knockdown of AdipoR1/2. In addition, it was shown that the secretion of E2 only exhibits a marked drop if co-processing 10 μg/mL AdipoRon and pGMLV AdipoR2 compared to single treatments. Taken together, the study highlighted the role of adiponectin and its receptors in the regulation of steroid synthesis and secretion in ovarian granulosa cells in laying hens.

Key words: adiponectin, steroid hormones, AdipoR1/2, reproduction, laying hen

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INTRODUCTION

Adiponectin is well-known as an important adipocyte-derived protein hormone encoded by the AdipoQ gene. It participates in energy and lipid metabolism by binding to its two receptors- adiponectin receptor type 1 (**AdipoR1**) and adiponectin receptor type 2 (**AdipoR2**), which are strongly associated with metabolic syndrome, such as obesity-related disorders and type 2 diabetes (Kadowaki and Yamauchi, 2005; Kadowaki et al., 2006; Okamoto et al., 2006; Peters et al., 2013). The stimulation of AdipoR1 or AdipoR2 has been found to activate the adiponectin system through participating in different signaling pathways (Pierre et al., 2009). It was also discovered that Adiponectin receptor agonists (AdipoRon) could activate the expression of adiponectin and then limit the occurrence of these metabolic diseases and inflammatory responses via regulating AMPK and PPARα signaling pathways (Yamauchi et al., 2003; Kita et al., 2019; Adiyaman et al., 2020). In recent years, a number of studies have reported that adiponectin and its receptors are widely present in various tissues and cell types, including the reproductive tissues of human and animals (Lord et al., 2005; Nigro et al., 2013; Smolinska et al., 2014; Zhang et al., 2017), which indicated that it may be involved in multiple physiological processes, especially

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the reproductive process (Chabrolle et al., 2007; Maillard et al., 2010; Kamil et al., 2018). For example, it has been proved that AdipoRon fed mice could experience cardiac hypertrophy and fibrosis caused by overpressure in remission, which implied to affect by the activation of the AMPK signaling pathway (Ning et al., 2018). Furthermore, a number of researches reported that adiponectin and its receptors play a crucial role in the regulation of the hypothalamic-pituitary-gonadal axis and be involved in the reproductive process (Aristea Psilopanagioti et al., 2009; Kaminska et al., 2020). In humans, AdipoRon reduced the aromatase expression and estradiol (**E2**) production in luteinized granulosa cells through the activation of AMPK and PPAR signaling pathways (Grandhaye et al., 2021). In contrast, Chabrolle et al (2009) indicated that adiponectin could faciliate the secretion of progesterone (**P4**) and E2 in primary human granulosa cells via the stimulation of the MAPK signaling pathway (Chabrolle et al., 2009), and similar results were found in Cynopterus sphinx (Anuradha and Krishna, 2014). However, in cows, adiponectin can decrease the secretions of P4 and androstenedione in bovine theca cells via regulating LH and IGF-I (Lagaly et al., 2008). In geese, it was demonstrated that adiponectin increased the secretion of P4 and weakly inhibited E2 production via modulating the expression of steroidogenesis genes in geese follicular granulosa cells (Bo et al., 2019). Therefore, although the regulation pathways and conclusions are inconsistent, all these studies have proven that adiponectin is involved in the reproductive process via modulating steroid hormones in the ovaries. However, there remains little knowledge about the role of adiponectin and its receptors in the modulation of the reproductive system of chickens.

Our previous study showed that adiponectin was widely expressed in the tissues of hypothalamus-pituitary-ovarian (**HPO**) axis through qRT-PCR and Western blotting and was localized in follicular cells and basal cell in hens' ovaries, which indicated that it may be involved in the regulation of the reproductive process of hens (Li et al., 2021). Therefore, in the present study, our aims were directly to investigate the expression of adiponectin and its receptors in laying hens' ovarian follicles after in vitro incubation, and to evaluate the dose-dependent effects of AdipoRon on cell proliferation and apoptosis and the role of adiponectin and its receptors in modulating the steroid synthesis of granulosa cells.

MATERIALS AND METHODS

Ethics Statement

All experimental animals were treated in accordance with the guidelines for the administration of affairs concerning experimental animals. Animal care measures were approved by the Henan Agricultural University Institutional Animal Care and Use Committee (Permit Number: 19-0068).

Sample Collecting

A total of 45 healthy Hy-Line Brown laying hens aged 30 weeks old were chosen approximately 22 h after oviposition and anesthetized with 3% pentobarbital sodium (30 mg/kg body weight) through intravenous injection in a wing. Then, all of the individuals were euthanized by jugular vein bleeding while under deep anesthesia, after which the whole ovaries of 12 hens were removed and separated the growing follicles into small white follicle (**SWF**) in <4 mm diameter, large white follicle (**LWF**) in 4 to 6 mm diameter, small yellow follicle (**SYF**) in 6 to 8 mm diameter, and preovulatory follicles (F6-F1) in 9 to 12 mm or >12 mm diameters according to the different stages (Lovell and T., 2003). We mixed the different growing follicles of each pair of hens (i.e., 6 replicates per stage), then sterilized them in 75% ethanol and stored in a PBS buffer, including 3% double antibody, immediately for the genes' expression.

Granulosa Cell Culture

Numbers of small yellow follicles (6−8 mm in diameter) were separated from the whole ovaries of the 33 hens as mentioned above, and divided into the theca and granulosa layers; we collected the granulosa cells and washed them with in a PBS buffer before the following experiment. Some of them were sheared into smaller pieces and digested for 10 mins at 37°C with 0.25% trypsin. The digested solution was filtered through a 200 μm screen and extracted the precipitate by centrifugation. Then we added the complete medium with 2.5% bovine serum and 1% double antibody to suspend the precipitate and spread the granulosa cell solution into five six-well plates and placed in an incubator (set to 37°C and 5% CO_2). After 6 h, the adherent cells were treated with a medium with 0, 5, 10, 20, 40 μg/mL AdipoRon in each well plate, respectively.

Cell Proliferation and Apoptosis Assay

The CCK-8 assay was used to measure the granulosa cell proliferation. The cultured cells were spread into a 96-well plate and taken out of the incubator 12 h, 24 h, 48 h, or 72 h after the treatment, respectively. The cells at each time point continued to be cultured for an additional 2 h after the supplement of 100 μL medium, including 10% CCK-8 solution. We then measured the optical density (**OD**) value at 450 nm using a microplate reader. The cell vitality was calculated as follows:

Cell vitality (%) = [(OD value in treatment well – OD value in control well)/(OD value in control well – OD value in blank well)] x 100.

Granulosa cell apoptosis was assayed using a flow cytometer. The cultured cells were spread into a six-well plate and washed with a prewarmed PBS solution after 24 h of treatment. The cell solution was digested by trypsin with 0.25% EDTA for 1 to 2 min, then we terminated the digestion with complete medium, collected the precipitate by centrifugation and added 500 μL binding

buffer, 5 μL Annexin V-FITC and 5 μL propidium iodide to mix with the cells and reacted the mixture for 15 mins to determine the population of cells in the flow cytometer.

Cell Transfection

Two pairs of oligonucleotide sequences of *AipoR1* and *AdipoR2* genes were synthesized by Sangon Biotech (Shanghai, China) and cloned into the pGMLV-CMV-gga_ADIPOR1/2-EF1-ZsGreen1-T2A-Puro. The interference fragment-AdipoRs si-RNA was purchased from RiboBio (Guangzhou, China). We designed 15 treatments to transfect granulosa cells (e.g., control group, pGMLV-AdipoR1, pGMLV-AdipoR2, si-AdipoR1, si-AdipoR2, 5 μg/mL AdipoRon, 10 μg/mL AdipoRon, 5 μg/mL AdipoRon plus pGMLV-AdipoR1/2 or si-AdipoR1/2, and 10 μg/mL AdipoRon plus pGMLV-AdipoR1/2 or si-AdipoR1/2). First, the cultured granulosa cells were transfected with pGMLV-AdipoR1/2, pGMLV NC, si-AdipoR1/2 and siRNA NC using the transfection reagent-LTX (RiboBio), as well as 5 μg/mL and 10 μg/mL AdipoRon, respectively. All the experiments were followed the instructions of the manufacturer. Then some of the granulosa cells treated with pGMLV-AdipoR1/2 and si-AdipoR1/2 were given a new complete medium and added 5 or 10 μg/mL AdipoRon for the co-processing after transfection for 6 h. All the granulosa cells were cultured with complete medium in the incubator for 24 h after treatment for RNA extraction and ELISA assay.

ELISA Assay for Hormones

The treated cell solution was extracted and centrifuged at 3,000 r/min for 30 min. The supernatant was separated and used to prepare the antibody-antigen-enzyme-antibody complex using purified chicken AdipoQ, AdipoR1, AdipoR2, estrogen, and progesterone, with HRP labeled antibodies. After washing completely, we added TMB substrate solution and enzyme-catalyzed HRP, then reacted the mixture in a sulfuric acid solution and measured the OD value at 450 nm using a microplate reader, in accordance with the instructions of the Chicken ADP, ADIPOR1, ADIPOR2, E2, and P4 ELISA Kit provided by Jiangsu Meimian Industrial Co., Ltd (Yancheng, China). The samples in each treatment were done in 6 replicates.

The Expression of Gene and Protein Analysis

Total RNA was extracted using Trizol reagent after extracting different sizes of follicles (SWF, LWF, SYF and F6-F1) from hens' ovaries, culturing the granulosa cells, and then frozen at −80°C until use. A reverse transcription (PrimeScriptTM RT reagent Kit with gDNA Eraser; Takara; Beijing, China) was used to extract cDNA and quantitative real time PCR (**qRT-PCR**) was performed to determine mRNA expression following the manufacture's protocols. The cycling process of qRT-PCR was as follows: 95°C for 5 min, 35 cycles of denaturing at 95°C for 10 s, annealing at 60°C for 10 s, elongation at 72°C for 10 s, and the final elongation at 72°C for 1 min. The observed data were calculated using the $2-\Delta\Delta Ct$ method. All the primer sequences of genes and β-actin (as a house-keeping gene) were synthesized by Shangya (Table 1).

Western blotting analysis was performed to determine the protein concentration of AdipoR1 and AdipoR2. The total protein in granulosa cells was collected using the Whole Cell Extraction Kit (Solarbio, Beijing, China) after washing with a PBS solution, and the protein concentration was analyzed using Pierce Rapid Gold BCA Protein Assay kit (Elabscience; Wuhan, China). Then the protein samples were centrifuged at 12,000 r/min for 5 min, extracted the supernatant and the following process adhered to the method of a previous study (Li, 2020), which was also strictly in accordance with the manufacturer's recommendations. The primary and secondary antibodies of goat AdipoR1/2, rabbit GAPDH and HRP-goat/rabbit anti rabbit/goat were purchased from Abcam and Google, respectively. The OD value of target band was obtained using an AlphaEaseFC software system.

Data Analysis

All the experimental data are expressed as means ± SD and subjected to statistical analysis by one-way ANOVA of IBM SPSS Statistics V22.0. The comparison of the mean in each group was performed by multiple comparison and Duncan's analysis. A value of P below 0.05 was considered a statistically significant difference.

Table 1. The primers of genes presented in the study.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
AdipoQ	GCCAGGTCTACAAGGTGTCA	CCATGTGTCCTGGAAATCCT
AdipoR1	CCAGGAGAAGGTTGTGTTTG	TGATCAGCAGTGCAATTCCT
AdipoR2	CTGCAACAACACAGACAGCC	GGGCTTGTAGAAGGGGTGAC
StAR	CGCTGCCATCTCCTACCAACAC	GGACATCTCCATCTCGCTGAAGG
CYP11A1	CTGCTCCGCCACCTCAAC	CCCGGTAAACGTTTTGGATACA
CYP19A1	CTCGGGGCTGTGTAGGAAAG	TGTCTGTACTCTGCACCGTC
LHR	GGGCTTTCCCAAGCCTACAT	TGGTGTCTTTATTGGCGGCT
FSHR	TCCTGTGCTAACCCTTTCCTCTA	AACCAGTGAATAAATAGTCCCATC

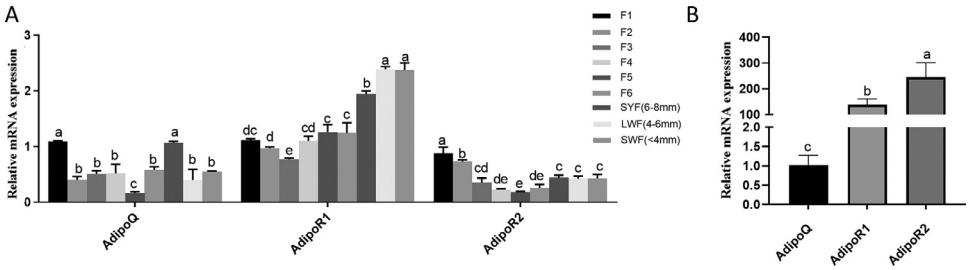

Figure 1. The mRNA expression levels of *AdipoQ, AdipoR1* and *AdipoR2* in follicles of different sizes (A) and granulosa cell (B). Different letters above each bar represent significant difference ($P < 0.05$) and all the values in the bars with the vertical lines represent mean ± SD, which are the same as the following figures.

RESULTS

The Expression of Adiponectin and Its Receptors in Follicles and Granulosa Cells

To validate the presence of adiponectin and its receptors in different follicles and granulosa cells of hens' ovaries, the gene expressions of *AdipoQ* and its receptors were analyzed and observed (Figure 1A, B). The qRT-PCR results showed that adiponectin, AdipoR1 and AdipoR2 were prevalent in all the growing follicles with different sizes and granulosa cells, of but higher expression levels of adiponectin existed in the larger prehierarchical and preovulatory follicles. Furthermore, the highest expression of *AdipoQ* and *AdipoR2* were found in F1 and the lowest expression levels of them were found in F5 follicle (Figure 1A; $P < 0.05$). However, higher expression levels of *AdipoR1* were obtained in prehierarchical follicles (SWF, SYF, LWF) than in preovulatory follicles (F6-F1; $P < 0.05$). In granulosa cells, the expression levels of *AdipoR1* and *AdipoR2* were significantly higher than those of *AdipoQ* (Figure 1B; $P < 0.05$).

The Effect of AdipoRon on the Proliferation and Apoptosis of Granulosa Cell

AdipoRon served as an adiponectin receptor agonist to activate the adiponectin system, as we mentioned before; thus, we designed 5 dose levels of AdipoRon (0, 5, 10, 20, and 40 μg/mL) to investigate the effect of adiponectin on the proliferation and apoptosis of granulosa cells. The results showed that all the AdipoRon treatments at doses of 20 μg/mL or 40 μg/mL markedly decreased the cell vitalities after incubation for 12 h, 24 h, 48 h, or 72 h. In addition, the cell vitalities following AdipoRon treatment at the doses of 5 μg/mL and 10 μg/mL were no different from those in the control group after incubation for 12 h or 24 h (Figure 2A). However, the cell vitalities for 5 μg/mL and 10 μg/mL of AdipoRon also decreased after treatment for 48 h or 72 h. Moreover, the proportions of apoptotic cells were determined by a flow cytometer and there was no significant difference among the groups with 0, 5, or 10 μg/mL of AdipoRon (Figure 2B, C). Therefore, the dose levels of AdipoRon (5 μg/mL and 10 μg/mL) for 24 h incubation after the treatment were selected to treat the granulosa cells for further study.

The Regulation of AdipoRon in the Secretion of Steroid Hormones

As described above, we suggested adding AdipoRon (5 or 10 μg/mL) for 24 h to detect the levels of adiponectin, AdipoR1/2, and steroid hormones in granulosa cells by ELISA after adding. In the results, the supplement of AdipoRon significantly promoted the secretion of adiponectin, AdipoR1, and AdipoR2 compared to the control group ($P < 0.05$; Figure 3 A−C). The progesterone level in AdiopRon group was much higher than in that of control ($P < 0.05$; Figure 3 D). However, the concentration of estrogen decreased drastically when adding AdipoRon to granulosa cells ($P < 0.05$; Figure 3 E). The higher the dosage of AdipoRon, the lower the level of estrogen. These results indicated that AdipoRon altered the

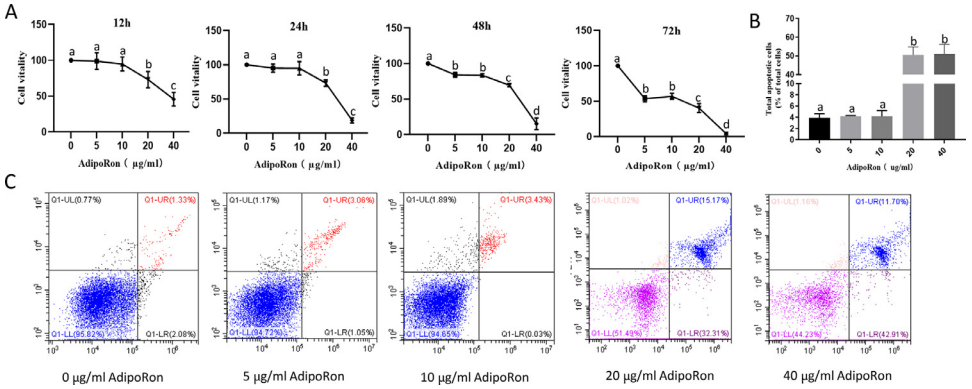

Figure 2. Effect on granulosa cell proliferation and apoptosis of the supplement of AdipoRon. (A) The granulosa cell vitality of the supplements of AdipoRon at different levels and incubation times. (B) The proportion of apoptotic cells in the total granulosa cells. (C) Annexin-V/PI analysis of granulosa cells treated with 0, 5, 10, 20, 40 μg/mL AdipoRon, respectively.

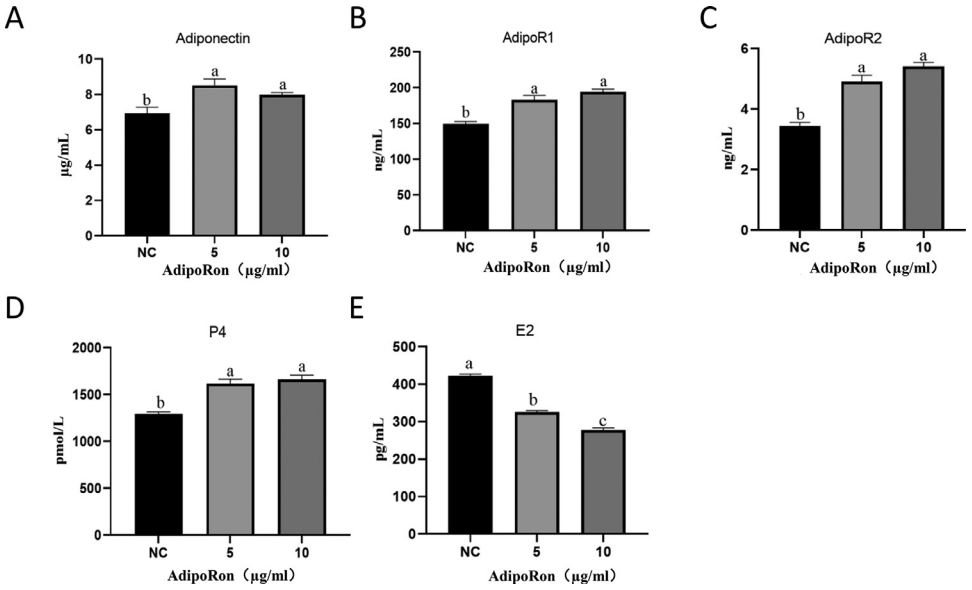

Figure 3. The secretion of Adiponectin (A), AdipoR1 (B), AdipoR2 (C), P4 (D) and E2 (E) in granulosa cells treated with 0, 5, 10 μg/mL AdipoRon, respectively.

secretion of steroid hormones via enhancing the secretion of adiponectin and its receptors.

The Gene and Protein Expression of AdipoR1/2 is Changed by Overexpression or Knockdown of AdipoR1/2

To confirm the effect of the overexpression and inhibition of AdipoR1/2, we determined the gene and protein expression of AdipoR1/2 using qRT-PCR and Western blotting. The results showed that the treatment for the over-expression of AdipoR1 and AdipoR2 significantly upregulated the gene and protein expressions of AdipoR1 and AdipoR2 compared to that in the pGMLV negative control (NC) group ($P < 0.01$; Figure 4A−F). Furthermore, the gene and protein expression of these two receptors in the knockdown of AdipoR1/2 groups were significantly lower than those in the siRNA NC groups as we expected ($P < 0.01$). Thus, these overexpression and interference vectors of AdipoR1/2 were used for the following study.

The Expression of Steroidogenesis Related Genes Following Treatment With AdipoRon and Overexpression or Knockdown of AdipoR1/2

To further investigate the role of adiponectin and its receptors in the regulation of steroid synthesis, we

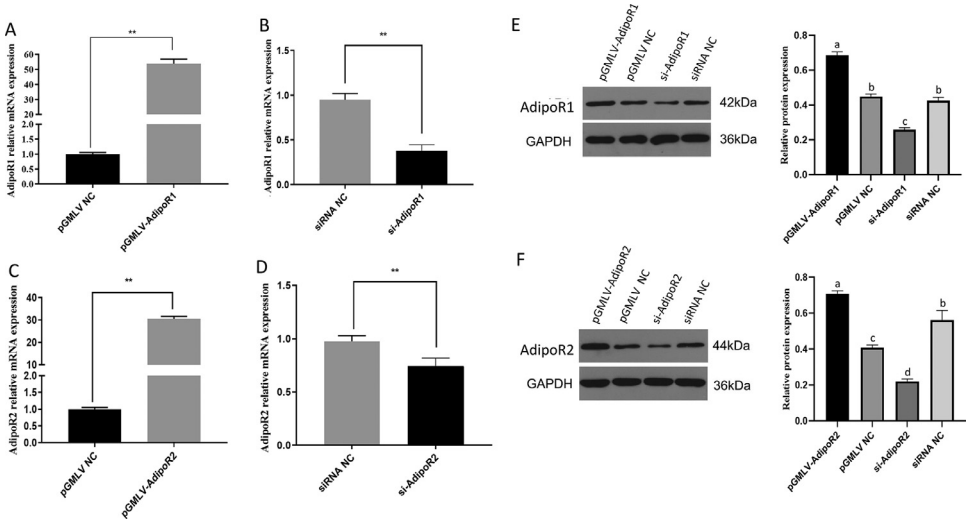

Figure 4. Effect of overexpression or knockdown of AdipoR1 and AdipoR2 on the expressions of AdipoR1 and AdipoR2. AdipoR1 overexpression (A) and knockdown (B) in granulosa cells enhances or suppresses the mRNA expression levels of AdipoR1. AdipoR2 overexpression (C) and knockdown (D) in granulosa cells enhances or suppresses the mRNA expression levels of AdipoR2. AdipoR1 (E) and AdipoR2 (F) overexpression and knockdown in granulosa cells led to a higher or lower protein expression of AdipoR1 or AdipoR2. * means $P < 0.05$, ** means $P < 0.01$ and different letters above each bar means $P < 0.05$.

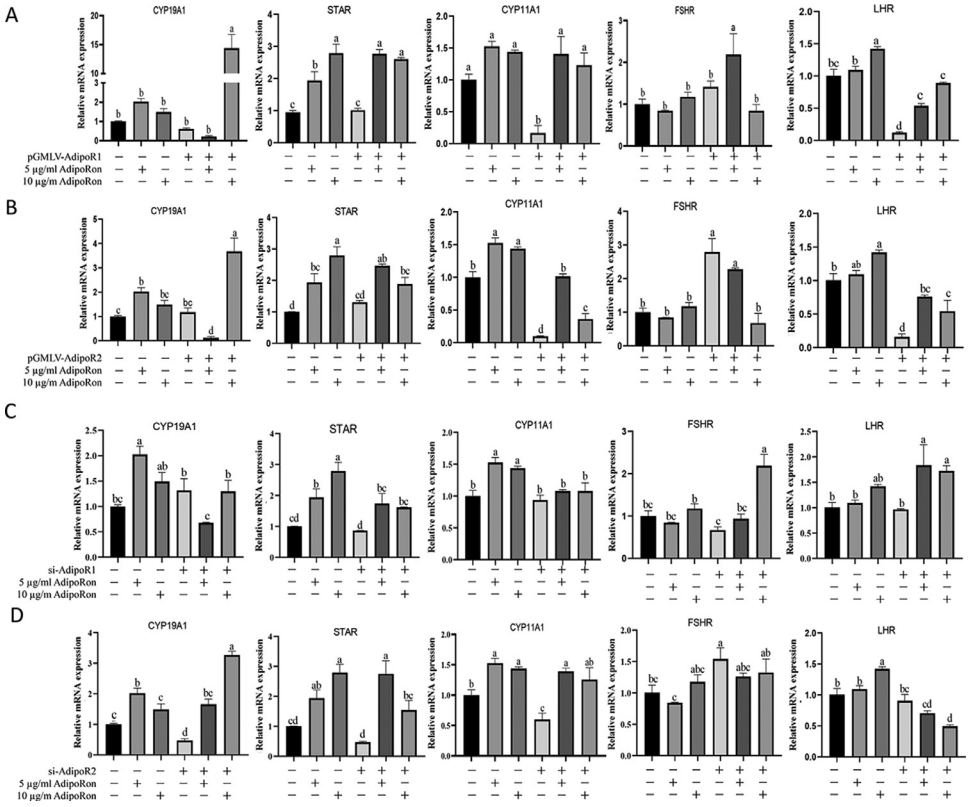

Figure 5. Effect of AdipoRon and overexpression or knockdown of AdipoR1/2 on the mRNA expressions of steroidogenesis related genes in granulosa cells. The mRNA expressions of *CYP19A1, StAR, CYP11A1, FSHR*, and *LHR* in granulosa cells treated with overexpression AdipoR1 (A), overexpression AdipoR2 (B), knockdown AdipoR1(C) and knockdown AdipoR2(D) individual or co-processing with AdipoRon at different concentrations, respectively.

determined the mRNA expression levels of the key related genes including Cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*), steroid acute regulatory protein (*StAR)*, P450 sidechain cleavage enzyme (*CYP11A1)*, FSH receptor (*FSHR)*, and LH receptor (*LHR*; Figure 5A−D). The results showed that, after treating the granulosa cells with the overexpression or knockdown of AdipoR1/2, the gene expression levels of *CYP19A1* and *STAR* were no different compared to those in the control groups, except that much lower expression levels of *CYP19A1* were obtained in the si-AdipoR2 group ($P < 0.05$). Moreover, the expression levels of *CYP11A1* and *LHR* were significantly downregulated in the pGMLV AdipoR1/2 groups compared to those in the control group. The expression levels of *StAR, CYP11A1*, and *LHR* were obviously upregulated when 10 μg/mL adipoRon was added to the granulosa cells ($P < 0.05$); however, there was no difference in the expression level of FSHR under the same treatment. Furthermore, after co-processing with pGMLV AdipoR1/2 and AdipoRon (10 μg/mL), the expression levels of *StAR* and *CYP19A1* were significantly increased compared to those in the control groups ($P < 0.05$). It was also found that the expression levels of *FSHR* and *LHR* were significantly upregulated if co-processed with si AdipoR1 and AdipoRon (10 μg/mL) ($P < 0.05$), while only the expression levels of *StAR* and *FSHR* were enhanced in the co-processing of the pGMLV AdipoR1/2 and 5 μg/mL AdipoRon group. Taken together, these results demonstrated that the expression levels of steroidogenesis related genes could be partly varied by individual or co-processing of AdipoRon and overexpression or knockdown of AdipoR1/2.

The Secretion of Steroid Hormones in the Co-processing of AdipoRon and Overexpression or Knockdown of AdipoR1/2

To valid the effect of adiponectin and its receptors in the secretion of steroid hormones, we detected the concentration of estradiol (E2) and progesterone (P4). The levels of E2 were significantly decreased in all the treatment groups compared with those in the control group ($P < 0.05$; Figure 6A−D). This indicated that, regardless of the overexpression or knockdown of AdipoR1/2 in granulosa cells, the E2 level would be impacted, and if co-processing with AdipoRon and overexpression of AdipoR1, stronger suppression of E2 would be obtained. However, the level of P4 was not changed in the pGMLV AdipoR1/2 or si AdipoR1/2 groups compared to that in the control group (Figure 6A−D), which indicated that the receptors could directly act on the secretion of P4. Furthermore, P4 secretion was significantly enhanced in the groups with AdipoRon supplement ($P < 0.05$), except for the group with co-processing of pGMLV-AdipoR1 and 10 μg/mL AdipoRon, which also showed an upward trend but no statistically significant difference (Figure 6A). Thus, the results verified that adiponectin

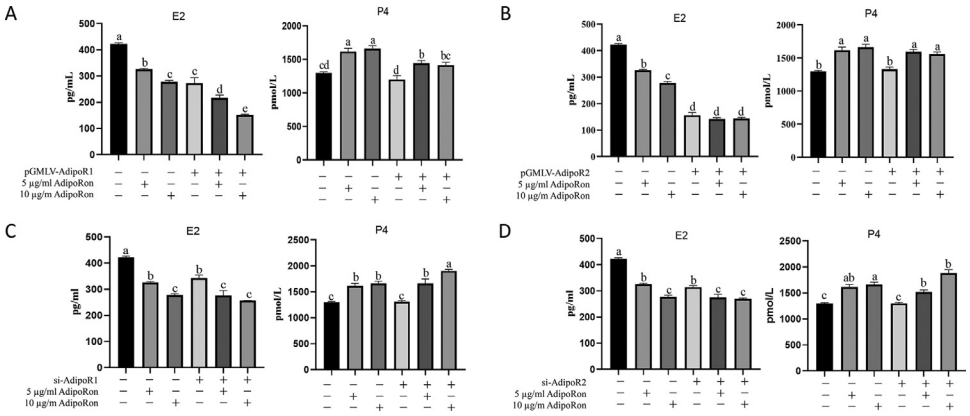

Figure 6. Effect of AdipoRon and overexpression or knockdown AdipoR1/2 on the secretion of steroid hormones in granulosa cells. The secretions of E2 and P4 in granulosa cells treated with overexpression AdipoR1 (A), overexpression AdipoR2 (B), knockdown AdipoR1 (C) and knockdown AdipoR2 (D) individual or co-processing with AdipoRon at different concentrations, respectively.

and its receptors could be involved in the regulation of steroid hormones.

DISCUSSION

Adiponectin has been reported as a member of the adipokine family that plays a crucial role in a variety of metabolic processes, especially in glucose and lipid metabolism (Scherer et al., 1995; Ghadge et al., 2018). However, a number of studies have also proven that adiponectin is involved in the modulation of the reproductive system via inducing ovarian steroid hormone secretion (Comim et al., 2016; Szeszko et al., 2016; Grandhaye et al., 2019). In the present study, we found that adiponectin and its receptors are wildly present in ovarian follicles, and there are higher expression levels of adiponectin in the F1 follicle and SYF, which may be related to higher steroid hormone secretion during the periods of follicular growth and maturation. However, in vitro study, the expression of adiponectin in granulosa cell was much lower than that of the two receptors, which was similar to the findings of a previous study (Chabrolle et al., 2007). By contrast (Hadley et al., 2020) reported that adiponectin was only expressed in the theca layer, not in the granulosa layer of chicken ovary and found that this impacted cell proliferation and steroid synthesis by increasing pERK and pACC abundance and inhibiting *StAR* expression (Hadley et al., 2020). Furthermore, high levels of AdipoRon (an activator of adiponectin; 20 μg/mL or 40 μg/mL) and incubation for 48 h or 72 h could impact the cell proliferation and apoptosis of granulosa cells, however, there was no influence on cell vitality if adding small amounts (5 μg/mL or 10 μg/mL) of AdipoRon for 12 h and 24 h, consistent with previous studies (Chabrolle et al., 2007; Maillard et al., 2010), which indicated that the effect of AdipoRon on cell proliferation and apoptosis of granulosa cells depended on the concentration and treatment time.

In mammals, adiponectin has been evidenced to influence steroidogenesis in the ovaries, which indicates that adiponectin can trigger E2 and P4 secretion via promoting the expressions of *LHR, StAR*, and *3β-HSD* or via increasing the expression of *IGF-I* (Chabrolle et al., 2009; Anuradha and Krishna, 2014). However, unlike mammals, poultry have a unique reproduction system with numbers of various sizes of follicles in the ovary to maintain continuous ovulation during the high laying period. A hen's follicle consists of oocytes surrounded by granulosa and theca layers that store theca cells and granulosa cells individually and produce different steroid hormones that mainly participate in the regulation of follicular growth, maturation, and atresia (Johnson, 2015; Li et al., 2020). Thus, to investigate the effect of adiponectin on steroid synthesis and secretion in hens' ovaries, we detected the levels of E2 and P4 in the granulosa cells via adding 5 μg/mL or 10 μg/mL AdipoRon and found that the activation of adiponectin could significantly enhance the secretion of P4 and suppress E2 in granulosa cells to further affect ovarian function, which was consistent with the findings in bat and geese (Singh and Krishna, 2012; Bo et al., 2019). Furthermore, we determined the expression of key related genes - *CYP11A1, CYP19A1, StAR, FSHR*, and *LHR*, which have been proven to be involved in steroid hormones' synthesis of follicles. *CYP11A1, CYP19A*1, and *StAR* are well-known steroidogenic genes that directly affect the synthesis of E2 and P4; FSHR and LHR are receptors of FSH and LH, respectively, to induce the actions of FSH and LH for accelerating the production of ovarian steroid hormones via the HPO axis; Vikkels et al., 2012; Kempisty et al., 2014; Kaewlert et al., 2018). In our study, adiponectin (5 or 10 μg/mL AdipoRon) was found to increase the expression levels of *StAR* and *CYP11A1* in varying degrees, as well as to influence the expression of *LHR* if adding 10 μg/mL AdipoRon to granulosa cells. That may be due to the response of increased secretion of P4 to induce follicle development, which was consistent with a study of pigs (Smolinska et al., 2016). However, the expression of FSHR did not cause the activation of adiponectin, which indicates adiponectin impacts the secretion of ovarian steroid hormones not based on the induction of FSH

(Chabrolle et al., 2007). Interestingly, the expression of *CYP19A1* was upregulated in the above treatment, but there was not a higher E2 level, and in Huoyan geese, it was shown that adiponectin could slightly downregulate the expression of *CYP19A1* (Bo et al., 2019). Furthermore, it was found that the expression of *CYP19A1* was no different after the treatment with pGMLV AdipoR1/2 and si-AdipoR2 compared with that in the control group, but the level of E2 following these treatments was significantly lower than that in the control group. Thus, it might be inferred that the stimulation of adiponectin and its receptors might inhibit E2 secretion via modulating other key related genes.

In addition, after transfection with the overexpression of AdipoR1/2 in the granulosa cells, as previously described, the expressions of *CYP11A1* and *LHR* was much lower than in the control group but there was no difference in the si-AdipoR1/2 groups (except for a reduction of *CYP11A1* in si-AdipoR2). However, the levels of P4 were not affected by the overexpression or knockdown of AdipoR1/2. This may be due to the stable *StAR* gene expression in the above groups, which was considered as a prerequisite of progesterone biosynthesis in granulosa cells transferring cholesterol to the inner mitochondrial membrane (Nobuhiro et al., 2007; Johnson et al., 2002). Despite the partially inconsistent trend between the expression of key genes involved in steroid biosynthesis and hormone secretion in the treatments of AdipoR1/2, it seems clear that adiponectin receptors can modulate the secretion of E2, but not P4 production. Therefore, it is necessary to conduct further research to confirm the role of adiponectin receptors-AdipoR1 and AdipoR2 in the regulation of steroid synthesis and secretion of granulosa cells in hens' ovaries.

CONCLUSION

In the present study, we systematically elucidated the expression of adiponectin and its receptors in ovarian follicles and determined the effect of different doses of AdipoRon on granulosa cell proliferation and apoptosis, as well as their role in steroid synthesis and hormone secretion in hens' ovarian granulosa cells. The results highlighted that adiponectin and its receptors were universally expressed in follicles with different sizes and high levels of AdipoRon (20 μg/mL and 40 μg/mL) could inhibit cell proliferation and promote apoptosis of granulosa cell. Furthermore, the activation of adiponectin facilitated the secretion of progesterone and inhibited the secretion of estradiol, and its receptors could also decrease the secretion of estradiol. However, there was no further impact on progesterone and estradiol secretion if co-processing with adiponectin and its receptors compared to the treatment group of AdipoRon or AdipoR1/2. A marked drop in the E2 level was only obtained if co-processing with 10 μg/mL AdipoRon and pGMLV AdipoR1. In summary, our study highlighted that adiponectin and its receptors modulated the secretion of E2 and P4 in granulosa cells, probably via the regulation of the expression of key genes related to steroid synthesis.

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DISCLOSURES

All the authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Yadong Tian, Xiangtao Kang and Xiaojun Liu acquired the funding for the project and conceived the study; Jing Li and Xuejie Ma performed the experimental process and wrote the manuscript; Guoxi Li and Pengkun Yang were involved in the study design; Xing Wu, Sujin Si and Chong Li conducted all the data analysis and prepared the experimental materials. All authors approved the final manuscript.

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