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The role of AdipoQ on proliferation, apoptosis, and hormone Secretion in chicken primary adenohypophysis cells

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ABSTRACT Adiponectin (**AdipoQ**), an adipokine secreted by adipocytes, has been reported to exist widely in various cell types and tissues, including the adenohypophysis of chickens. However, the molecular mechanism by which AdipoQ regulates the function of chicken adenohypophysis remains elusive. In this study, we investigated the effects of AdipoQ on proliferation, apoptosis, secretion of related hormones (FSH, LH, TSH, GH, PRL and ACTH) and expression of related genes (*FSHβ, LHβ, GnRHR, TSHβ, GH, PRL* and *ACTH*) in primary adenohypophysis cells of chickens by using real-time fluorescent quantitative PCR (**RT-qPCR**), cell counting kit-8 (**CCK-8**), flow cytometry, enzyme-linked immunosorbent assay (**ELISA**) and Western blot (**WB**) assays. Our results showed that AdipoQ promoted the proliferation of chicken primary adenohypophysis cells, up-regulated the mRNA expression of proliferation-related genes *CDK1, PCNA, CCND1* and *P21* ($P < 0.05$), as well as the increased protein expression of CDK1 and PCNA ($P < 0.05$). Furthermore, AdipoQ inhibited apoptosis of chicken primary adenohypophysis cells, resulting in down-regulation of pro-apoptotic genes *Caspase3, Fas*, and *FasL* mRNA expression, and decreased Caspase3 protein expression ($P < 0.05$). Moreover, there was an up-regulation of anti-apoptotic gene Bcl2 mRNA and protein expression ($P < 0.05$). Additionally, AdipoQ suppressed the secretion of FSH, LH, TSH, GH, PRL, and ACTH ($P < 0.05$), as well as the mRNA expression levels of related genes ($P < 0.05$). Treatment with AdipoRon (a synthetic substitute for AdipoQ) and co-treatment with RNA interference targeting AdipoQ receptors 1/2 (***AdipoR1/2***) had no effect on the secretion of FSH, LH, TSH, GH, PRL, and ACTH, as well as the mRNA expression levels of the related genes. This suggests that AdipoQ's regulation of hormone secretion and related gene expression is mediated by the AdipoR1/2 signaling axis. Importantly, we further demonstrated that the mechanism of AdipoQ on FSH, LH, TSH and GH secretion is realized through AMPK signaling pathway. In conclusion, we have revealed, for the first time the molecular mechanism by which AdipoQ regulates hormone secretion in chicken primary adenohypophysis cells.

Key words: adiponectin, primary adenohypophysis cell, AdipoR1/2, AMPK, chicken

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INTRODUCTION

Adiponectin (**AdipoQ**), also known as Acrp30, GBP28 or apM1, is a pivotal protein hormone secreted by adipose tissue subsequent to leptin. It plays a crucial role in various physiological processes, including glucose and lipid metabolism, growth and reproduction (Scherer et al., 1995; Kurose et al., 2021; Chen and Whiting 2022; Onodera et al., 2023; Bernardi et al., 2024; Zhou et al., 2024). Extensive research has provided evidence of its ability to enhance insulin sensitivity, ameliorate glucose and lipid metabolism, and reduce body weight in obese individuals (Berg et al., 2002; Straub and Scherer 2019; Tadiotto et al., 2023). Moreover, AdipoQ has been found to inhibit the growth of pancreatic cancer cells and nasopharyngeal carcinoma cells through the β-Catenin and AMPK signaling pathways (Jiang et al., 2019; Zhang et al., 2022). Furthermore, maternal AdipoQ levels have been shown to affect fetal growth (Duval et al.,

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2016; Qiao et al., 2016). Lastly, AdipoQ has been reported to potentially affect the secretion of reproductive-related hormones such as E2, P4, and GnRH (Grandhaye et al., 2021; Li et al., 2021b; Li et al., 2023).

The physiological effects of AdipoQ are primarily regulated through its interaction with AdipoQ receptors 1 (**AdipoR1**) and AdipoQ receptors 2 (**AdipoR2**). Initial studies have demonstrated that AdipoR1 is predominantly expressed in skeletal muscle in various mammals, including humans, mice, and pigs, while AdipoR2 is primarily expressed in the liver (Yamauchi et al., 2003; Lord et al., 2005). Additionally, the expression of AdipoR1 and AdipoR2 has been confirmed in various cell types and tissues, such as cardiomyocytes, pancreatic β cells (Kharroubi et al., 2003), endothelial cells (Motoshima et al., 2004), bone-forming cells (Berner et al., 2004), placenta (Chen et al., 2006). Interestingly, several studies have identified the presence of AdipoQ and its receptors in the adenohypophysis of vertebrates, including chickens (Maddineni et al., 2005; Ramachandran et al., 2007; Kiezun et al., 2013; Kaminska et al., 2020; Li, et al., 2021a). Psilopanagioti et al. (2009) reported the localization of AdipoQ and its receptors, within a specific subpopulation of pituitary cells responsible for the secretion of FSH, LH, GH, and TSH, suggesting a potential role in regulating endocrine function within the pituitary gland.

The upstream pituitary gland is under direct control of the central nervous system and regulated by hypothalamic releasing hormones, including follicle stimulating hormone (**FSH**), luteinizing hormone (**LH**), thyroid stimulating hormone (**TSH**), growth hormone (**GH**), prolactin (**PRL**) and adrenocorticotropic hormone (**ACTH**) (Marshall, 1952; Nagra et al., 1963; Proudman et al., 1999). hese hormones play crucial roles in regulating fundamental physiological functions such as reproduction, growth, development, and metabolism. They are transported to target organs and tissues throughout the body via abundant lymphatic vessels and capillaries surrounding the pituitary gland. This transportation occurs through systemic lymphatic and blood circulation, thereby influencing the activity of downstream endocrine glands. Research has provided evidence that AdipoQ exerts regulatory effects on the pituitary gland in mammals. For instance, studies have shown that AdipoQ can stimulate FSH release from porcine pituitary cells and enhance GnRH/insulin-induced FSH and LH release (Kiezun et al., 2014). A study conducted by Rodriguez-Pacheco et al. (2007) on rat pituitary cells demonstrated the inhibition of GH and LH release following a short-term (4h) treatment with AdipoQ. Another study by Sarmento-Cabral et al. (2017) on baboon pituitary cells revealed that AdipoQ inhibited ACTH release and promoted PRL release. These findings further support the regulatory role of AdipoQ in the mammalian pituitary endocrine system.

However, the molecular mechanisms by which AdipoQ regulates the physiological functions of the poultry adenohypophysis remain unknown. Therefore, the purpose of this study was to investigate the effects of AdipoQ on the proliferation, apoptosis, hormone secretion (FSH, LH, TSH, GH, PRL and ACTH) and expression of related genes (FSHβ, LHβ, GnRHR, TSHβ, GH, PRL and ACTH) in chicken primary adenohypophysis cells, and to further reveal its potential signaling pathways.

MATERIALS AND METHODS

Ethics Approval

All experimental protocols in this study strictly followed the guidelines of the Institutional Animal Care and Use Committee (**IACUC**) of Henan Agricultural University, with approval number 19-0068.

Isolation and Culture of Chicken Primary Adenohypophysis Cells

Intact adenohypophysis tissue were isolated from 16-embryo-old Hy-Line brown embryos and to washed three times in PBS containing 10% bull serum albumin (**BSA**) (Solarbio, Beijing, China) and 1% double antibody (Solarbio, Beijing, China). Neuropituitaries were carefully discarded and the remaining adenopituitaries were transferred to 1.5 mL EP tubes. The adenopituitaries were aseptically cut into pieces and digested with 0.25% trypsin (Solarbio, Beijing, China) at 37 °C for 10 min. Afterwards, the digested tissue were immediately transferred to a DEME (Gibco, Grand Island, USA) containing 10% BSA, 5% fetal bovine serum (**FBS**) (Solarbio, Beijing, China), 5 μg/mL bovine insulin (Solarbio, Beijing, China), 5 μg/mL transferrin (Solarbio, Beijing, China), and 1% double antibody to halt the digestion process. The cell suspension was filtered through a 200-mesh cell sieve to remove undigested tissues and cell aggregates, followed by centrifugation at 1,500 rpm/min for 10 min. The resulting cell precipitate was then diluted to a concentration of 5×10^6 cells/mL in DEME supplemented with 10% BSA, 5% FBS, 5 μg/mL bovine insulin, 5 μg/mL transferrin, and 1% double antibody. Finally, the cell suspension was seeded into poly-L-lysine-coated (Sigma, St Louis, MO) 12-well plates and incubated at 37 °C in a 5% CO_2 incubator.

Plasmid Construction and Cell Transfection

The *AdipoQ* gene of chicken was isolated by PCR amplification using gene-specific primers. The resulting PCR products were digested with EcoR I and HindIII, and then cloned into pcDNA3.1 vector after digestion. The modified vector was designated as pcDNA3.1-AdipoQ, while the empty vector pcDNA3.1 served as a negative control. All constructs were verified by sequencing, and plasmids were purified using an endotoxin-free plasmid extraction kit (Tiangen, Beijing, China). Small interfering RNAs (**siRNAs**) targeting AdipoQ (si-AdipoQ), AdipoR1 (si-AdipoR1), AdipoR2 (si-AdipoR2),

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

Gene	Sequence of nucleotide	Gene ID	Product length (bp)
Adiponectin	F 5′- GCCAGGTCTACAAGGTGTCA -3′ R 5′- CCATGTGTCCTGGAAATCCT -3′	NM_20691.1	86
AdipoR1	F 5′- CCAGGAGAAGGTTGTGTTTG-3′ R 5′- TGATCAGCAGTGCAATTCCT-3′	NM_001031027.1	149
AdipoR2	F 5′- CTGCAACAACACAGACAGCC -3′ R 5′- GGGCTTGTAGAAGGGGTGAC-3′	NM_001007854.1	171
P21	F 5′- GAAGAGTTGTCCACGATAAGC -3′ R 5′- TTCCAGTCCTCCTCAGTCC -3′	NM_001396336.1	247
CDK1	F 5′- TAATAGATGACAAAGGGGT -3′ R 5′- GAGTGGAATACAGAGCAGA -3′	NM_205314.2	146
CCND1	F 5′- CAGAAGTGCGAAGAGGAAGT -3′ R 5′- CTGATGGAGTTGTCGGTGTA -3′	XM_046941491.1	188
PCNA	F 5′- AGCACCAAATCAGGAAAAG -3′ R 5′- GCACAGGAGATGACAACAG -3′	NM_204170.3	177
Bcl2	F 5′- CAACGGAGGATGGGATGCC -3′ R 5′- CTTATGTCCAAGATAAGCG -3′	NM_205339.3	145
Caspase3	F 5′- GGCTACTACTCCTGGAGGA -3′ R 5′- ACACAATGCATGGAATCTG -3′	NM_204725.2	193
Fas	F 5′- TAACACAGCTGCAGCAGACA -3′ R 5′- TCACAATGTCAGGGACGTGG -3′	NM_001199487.2	101
FasL	F 5′- CTGGAGAAGCTCATAGGGCAG -3′ R 5′- ACCAGAGACAGGTTCCCACT -3′	NM_001031559.2	120
FSHβ	F 5′- CTGCGGTGACCATCCTGAAT -3′ R 5′- CTCACAGTGCAGTCAGTGCT -3′	NM_204257.2	96
LHβ	F 5′- GTGTTGGTGCTGATGACCCT -3′ R 5′- ACCGCCACCGTTACGTTTAT -3′	HQ872606.1	155
GnRHR	F 5′- GAGCTTCTCCTGCCTCTTCC -3′ R 5′- TAGTAGGGGGTCCAGCAGAG -3′	NM_001012609.1	219
TSHβ	F 5′- GCCTGGAGTGTATAAAGCACA -3′ R 5′- AGGGACTCATGCTCTTGTCAG -3′	NM_205063.4	115
GH	F 5′- ACATGGAGCTGCTTCGGTTT -3′ R 5′- AACACTCTGTCTGAGGTGCC -3′	NM_204359.2	115
PRL	F 5′- GAGTGACCTCCCTGCCAATC -3′ R 5′- AATGAAACCCCGACCCTGAG -3′	NM_205466.3	164
ACTH	F 5′- GCAGCTCCTCCGCAGTT -3′ R 5′- ACTTGCTGTTCTCCCAGCAT -3′	NM_001398117.1	139
GAPDH	F 5′- GAACATCATCCCAGCGTCCA-3′ R 5′- CGGCAGGTCAGGTCAACAAC-3′	NM_204305	132

and a negative control (**NC**) were obtained from Gene Pharma (Shanghai, China).

The cells were transfected with LipofectamineTM 3000 Transfection Reagent (Invitrogen, Carlsbad, CA). After seeding, the cells were grown to 70 to 80% confluence before transfection with 1,000 ng of plasmid and 50 nM siRNA. All procedures strictly followed the manufacturer's instructions. At 36 h post-transfection, cells were harvested for RNA and protein extraction, while cell supernatants were collected for ELISA analysis.

RNA Extraction and Real-Time PCR

Total RNA was extracted from chicken primary adenohypophysis cells using TRIzol reagent (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. RT-PCR was performed using 500 ng of total RNA and the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) at 37 °C for 15 min, followed by 85 °C for 5 s. The qRT-PCR was conducted using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) with gene-specific primers. The analysis was performed on the Light Cycler 96 qRT-PCR system (Roche, Basel, Switzerland) with cycling conditions of 95 °C for 5 min, followed by 30 cycles of 95 °C for 10 s and 60 °C for 30 s. The primers used for qRT-PCR are listed in Table 1. The relative expression of each gene was calculated according to the 2-(delta delta Ct) method, and the results were normalized by GAPDH levels (Livak and Schmittgen, 2001; Wang et al., 2011).

Cell Viability Assay

Cell viability was assessed using the cell counting kit (**CCK**)-8 (Dojindo, Japan) according to the manufacturer's protocol. Chicken primary adenohypophysis cells were seeded in 96-well plates and incubated at 37°C with 5% CO_2 until transfection completion. At 24 h, 36 h, and 48 h post-transfection, the medium was replaced with fresh medium supplemented with 10% CCK-8 reagent. After an additional 2 h incubation at 37°C, the absorbance was measured at 450 nm.

Apoptosis Assay

Cell apoptosis was detected using the Annexin V-633/PI Apoptosis Detection Kit (Dojindo, Japan). Chicken primary adenohypophysis cells were harvested 36 h post-transfection and labeled with 5 μL Annexin V-633

and 5 μL PI for 15 min at room temperature in the dark. Subsequently, flow cytometry analysis was performed on the labeled cells using a BD Biosciences flow cytometer (BD Biosciences, San Jose, CA).

Cell Cycle Assay

Cells were fixed in 75% cold ethanol and incubated overnight at 4°C. Subsequently, DNA was stained with propidium iodide (**PI**) (Solarbio, Beijing, China) for 1 h at room temperature in the absence of light. The stained cells were then analyzed using a flow cytometer (BD Biosciences, San Jose, CA).

Western Blot Analysis

After 36 h of transfection, the culture medium was discarded, and the chicken primary adenohypophysis cells were washed with PBS. Total proteins were extracted from the cells using RIPA lysate (Shanghai, China). The protein samples were separated using 10% SDS-PAGE based on their molecular weight and transferred onto a PVDF membrane. To prevent nonspecific binding, the PVDF membrane was blocked with 5% defatted milk powder. Primary antibodies against AdipoQ (1:1,000, Bioss, Beijing, China), p-AMPK (1:1,000, Cell Signaling Technology, Boston, MA), CDK1 (1:1,000, proteintech, Wuhan, China), PCNA (1:2000, abcom, Cambridge, UK), Bcl2 (1:2,000, abcom, Cambridge, UK), Caspase3 (1:2,000, abcom, Cambridge, UK) and GAPDH (1:5,000, Boster, Wuhan, China) were incubated together with the membrane overnight at 4°C. Subsequently, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:3,000, Elabscience, Wuhan, China) for 50 min. Finally, the bands were quantified by measuring the optical density using the AlphaEaseFC program.

Enzyme Linked Immunosorbent Assay

After collecting the cell supernatant, the cells were centrifuged at 3,000 rpm/min for 20 min. The levels of FSH, LH, TSH, GH, PRL and ACTH in the supernatant were measured using a commercial ELISA kit (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China). Briefly, 40 μL of cell supernatant, 10 μL of antibody, and 100 μL of enzyme reagent were added to each well. The plate was then sealed with plate sealing film and incubated at 37°C for 1 h. After incubation, the plates were washed five times with washing solution, and 50 μL of color development solutions A and B were added to each well. The plates were further incubated for 15 min at 37°C in the dark. Subsequently, 50 μL of termination solution was added to each well, and the plates were immediately measured at 450 nm OD using a microreader (Rayto, Shenzhen, China).

Statistical Analysis

All experiments underwent statistical analysis using SPSS version 22.0 (IBM SPSS, Armonk, NY). The data are presented as mean ± standard deviation. Student's *t*-test was employed for comparisons between two groups, while one-way ANOVA followed by post hoc Duncan's test was used for comparisons involving more than two groups. A *p*-value of less than 0.05 was considered indicative of a statistically significant difference.

RESULTS

AdipoQ Promoted the Proliferation and Inhibited the Apoptosis of Chicken Primary Adenohypophysis Cells

To investigate the impact of *AdiopoQ* overexpression and interference on the proliferation and apoptosis of chicken primary adenohypophysis cells, a series of experiments were conducted. The cells were cultured with PCDNA3.1, PCDNA3.1-AdipoQ, NC, and si-AdipoQ for a duration of 36 h. First, qRT-PCR was utilized to detect *AdiopoQ* expression and confirm the successful overexpression and interference of *AdiopoQ* (Figures 1A and 1B). Then, the impact of AdipoQ manipulation on cell proliferation and apoptosis in chicken primary adenohypophysis cells was evaluated using various assays, including the CCK-8 assay, flow cytometry, qRT-PCR and WB assay. The results showed that overexpression of *AdipoQ* significantly increased cell viability in chicken primary adenohypophysis cells ($P < 0.01$) while interference with *AdipoQ* significantly decreased cell viability ($P < 0.01$) at 36 h (Figures 1C and 1D).The cell cycle analysis revealed that overexpression of *AdipoQ* markedly reduced the number of G0/G1 cells, with a higher number of S-phase cells compared to the control group ($P < 0.05$) (Figure 1E). Conversely, interference with *AdiopoQ* increased the number of G0/G1 cells and decreased the number of S-phase cells compared to controls ($P < 0.01$) (Figure 1F). Moreover, we found that overexpression of *AdipoQ* markedly upregulated the mRNA expression of proliferation-related genes, including *CDK1*, PCNA, *CCND1* and *P21*, along with the protein levels of CDK1 and PCNA ($P < 0.05$) (Figures 1G and 1M). In contrast, interference with *AdipoQ* resulted in the opposite effect (P<0.05) (Figures 1H and 1N). Additionally, the overexpression of *AdiopoQ* resulted in a significantly lower percentage of apoptotic cells, whereas interference with *AdiopoQ* led to a significantly higher percentage when compared to the control group ($P < 0.01$) (Figures 1I−1J). Similarly, overexpression of *AdipoQ* significantly up-regulated the mRNA and protein expression of the anti-apoptotic gene Bcl2, while significantly down-regulated the mRNA expression of the pro-apoptosis genes *Caspase3, Fas*, and *FasL* as well as the protein expression of the Caspase3 ($P < 0.05$) (Figures 1K and 1M). Conversely, interference with *AdipoQ* yielded contrasting results ($P < 0.05$) (Figures 1L and 1N). Based on these collective findings, it can be

concluded that AdipoQ promotes cell proliferation and inhibits apoptosis in chicken primary adenohypophysis cells.

AdipoQ Inhibits the Expression of Hormone Secretion and Relevant Genes in Chicken Primary Adenohypophysis Cells

To unveil the role of AdipoQ in the regulation of gene expression and hormone secretion associated with chicken primary adenohypophysis cells, we examined the expression of *FSHβ, LHβ, GnRHR, TSHβ, GH, PRL* and *ACTH* genes as well as hormone secretion after overexpression or interference with *AdipoQ* by RT-qPCR and ELISA analysis. The results demonstrated that overexpression of the AdipoQ gene in chicken primary adenohypophysis cells led to the downregulation of *FSHβ, LHβ, GnRHR, TSHβ, GH, PRL* and *ACTH* mRNA levels ($P < 0.05$) (Figure 2A). Similarly, hormone secretion of FSH, LH, TSH, GH, PRL, and ACTH was significantly inhibited ($P < 0.05$) (Figures 2B−2G). Conversely, interference with *AdipoQ* resulted in the opposite expression pattern of the aforementioned mRNAs and hormones ($P < 0.05$) (Figures 2I−2N). In short, AdipoQ inhibits hormone secretion and related gene expression in chicken primary pituitary cells.

Hormone Secretion and Related Gene Expression Following AdipoRon Treatment and AdipoR1/2 Interference

To investigate the influence of AdipoQ on hormone secretion and the mRNA expression of related genes in chicken primary adenohypophysis cells, we employed an AdipoQ analogue called AdipoRon. Co-treatment of AdipoRon with interference of *AdipoR1/2* was conducted to determine the receptor through which AdipoQ exerts its effects. Prior to the experiments, we employed the CCK-8 method to determine the safe concentration of AdipoRon that would not significantly affect cell

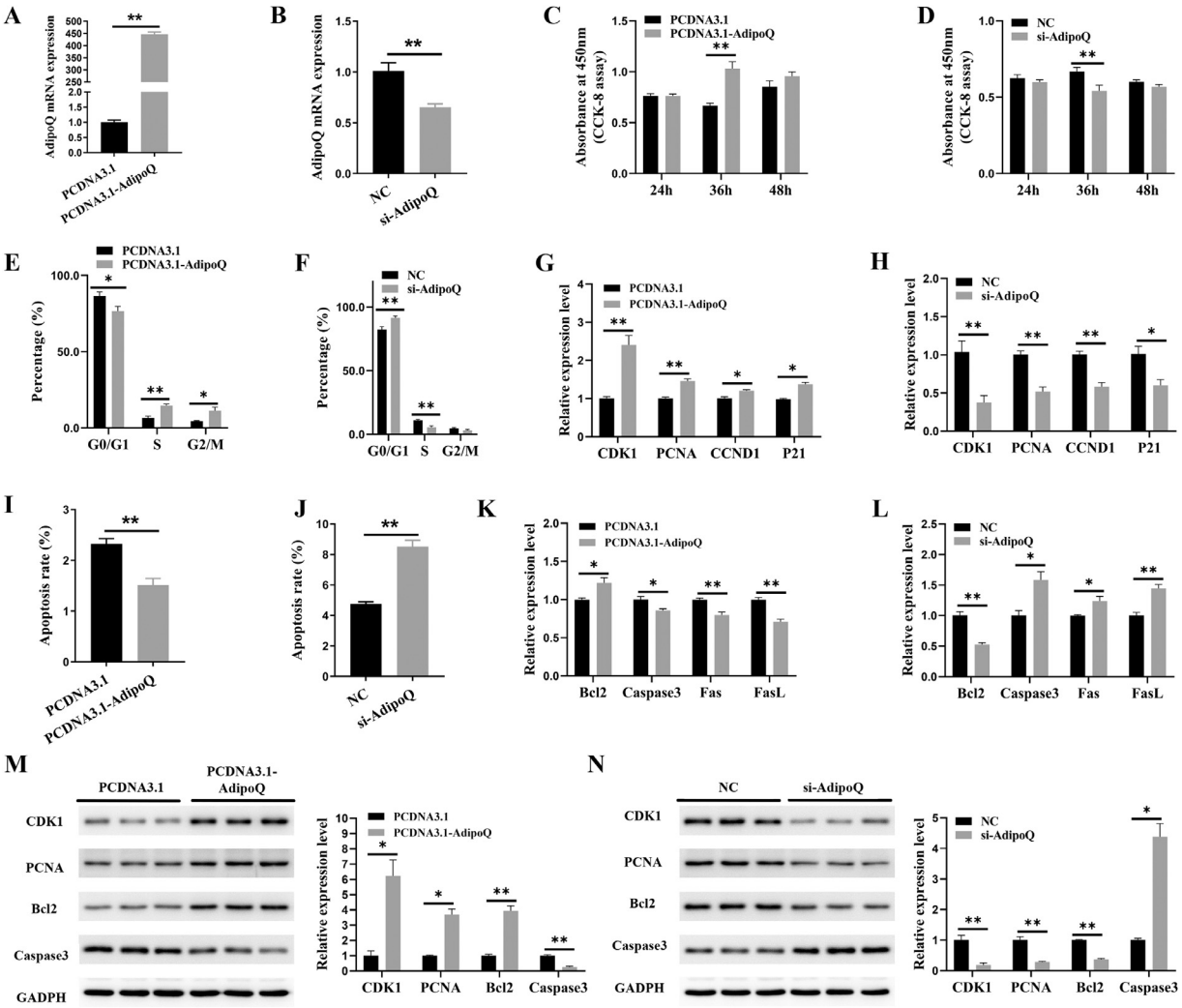

Figure 1. Effects of overexpression and interference of AdipoQ on proliferation and apoptosis of chicken primary adenohypophysis cells. (A−B) The overexpression or interference efficiency of AdipoQ was examined by RT−PCR; (B−C) Cell growth curves determined by the CCK-8 assay following transfection with AdipoQ overexpression or interference in chicken primary adenohypophysis cells; (E−F) Flow cytometry to determine proliferation of treated cells; (G−H) The mRNA expression of key genes related to proliferation following cell treatment; (I−J) Flow cytometry for the apoptotic rate of treated cells; (K−L) The mRNA expression of key genes related to apoptosis following cell treatment.

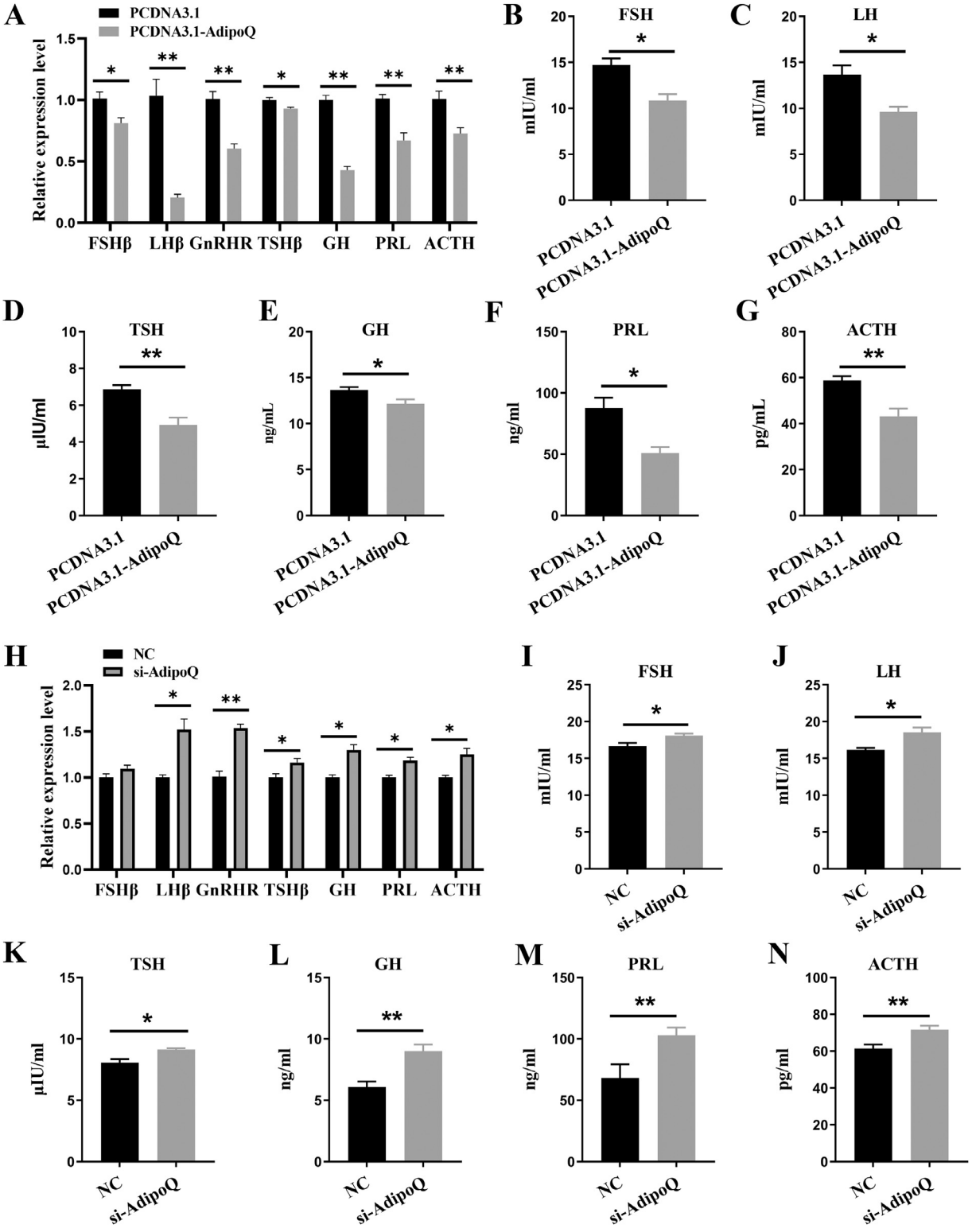

Figure 2. Effects of overexpression and interference of AdipoQ on hormone secretion and related gene expression of chicken primary adenohypophysis cells. (A) Effect of overexpression of AdipoQ on mRNA expression of related genes; (B−G) Effects on hormone secretion after overexpression of AdipoQ; (C) Effect of interference of AdipoQ on mRNA expression of related genes; (I−N) Effects on hormone secretion after interference of AdipoQ.

viability in chicken primary adenohypophysis cells. The results indicated that treatment with 5 μg/mL of AdipoRon for 12 h did not cause a significant difference in cell viability compared to the control group. However, cell viability significantly decreased after 24 or 36 h of treatment with 5 μg/mL of AdipoRon ($P < 0.05$). Furthermore, concentrations of 10 μg/mL and 20 μg/mL of AdipoRon resulted in a significant reduction in cell activity at 12, 24, and 36 h ($P < 0.05$) (Figure 3A). Based on these findings, we selected the concentration of 5 μg/mL AdipoRon for 12 h for subsequent experiments.

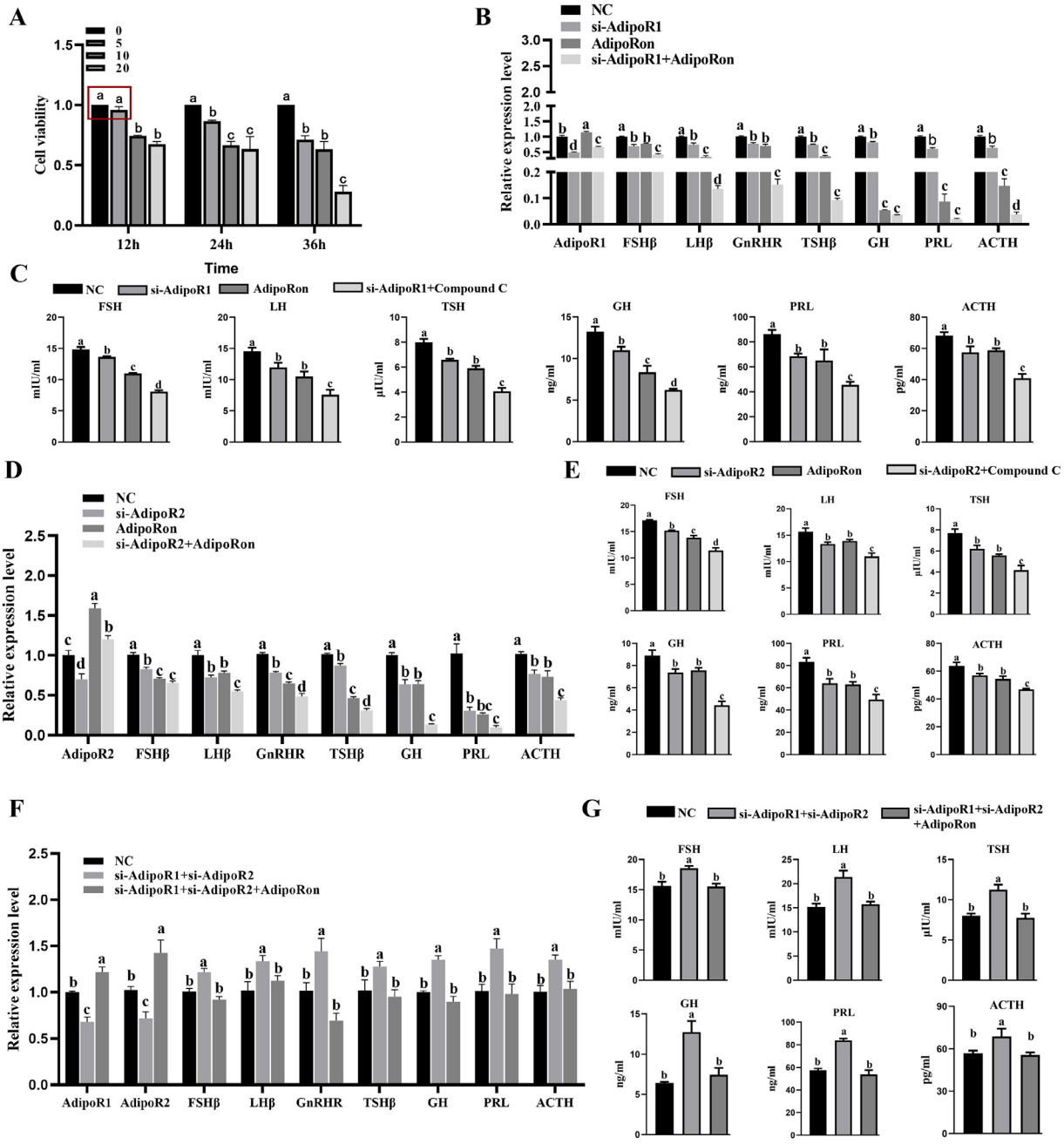

Figure 3. Cotreatment of AdipoRon and Interfering AdipoR1/2 on hormone secretion and related gene expression. (A) Effect of different concentrations of AdipoRon on the viability of chicken primary adenohypophysis cells; (B) The effect of AdipoRon cotreatment with interfering AdipoR1 on mRNA expression of related genes; (C) The effect of AdipoRon cotreatment with interfering AdipoR1 on hormone secretion; (D) The effect of AdipoRon cotreatment with interfering AdipoR2 on mRNA expression of related genes; (E) The effect of AdipoRon cotreatment with interfering AdipoR2 on hormone secretion; (F) Effect of cotreatment of AdipoRon with interfering AdipoR1 and interfering AdipoR2 on the mRNA expression of related genes; (G) Effect of cotreatment of AdipoRon with interfering AdipoR1 and interfering AdipoR2 on hormone secretion.

Following interfering with *AdipoR1* or *AdipoR2*, the mRNA expression of *AdipoR1* or *AdipoR2* was significantly reduced ($P < 0.05$) (Figures 3B and 3D). Moreover, the mRNA expression of *FSHβ, LHβ, GnRHR, TSHβ, GH, PRL* and *ACTH* as well as the secretion of FSH, LH, TSH, GH, PRL and ACTH hormones were significantly decreased in si-AdipoR1 or AdipoR2 group ($P < 0.05$) (Figure 3B–E). Similarly, in the AdipoRon group, there was a significant decrease in the mRNA expression of *FSHβ, LHβ, GnRHR, TSHβ, GH, PRL* and *ACTH* as well as the secretion of FSH, LH, TSH, GH, PRL and ACTH hormones ($P < 0.05$) (Figure 3B–E). Compared with the NC group, si-AdipoR1 or si-AdipoR2 plus AdipoRon resulted in a decrease in the mRNA expression of *FSHβ, LHβ, GnRHR, TSHβ, GH, PRL* and *ACTH* as well as the secretion of FSH, LH, TSH, GH, PRL and ACTH hormones ($P < 0.05$) (Figure 3B–E). Our results showed that when *AdipoR1* was interfered, AdipoQ inhibited the secretion of FSH, LH, TSH, GH, PRL and ACTH by AdipoR2, and vice versa.

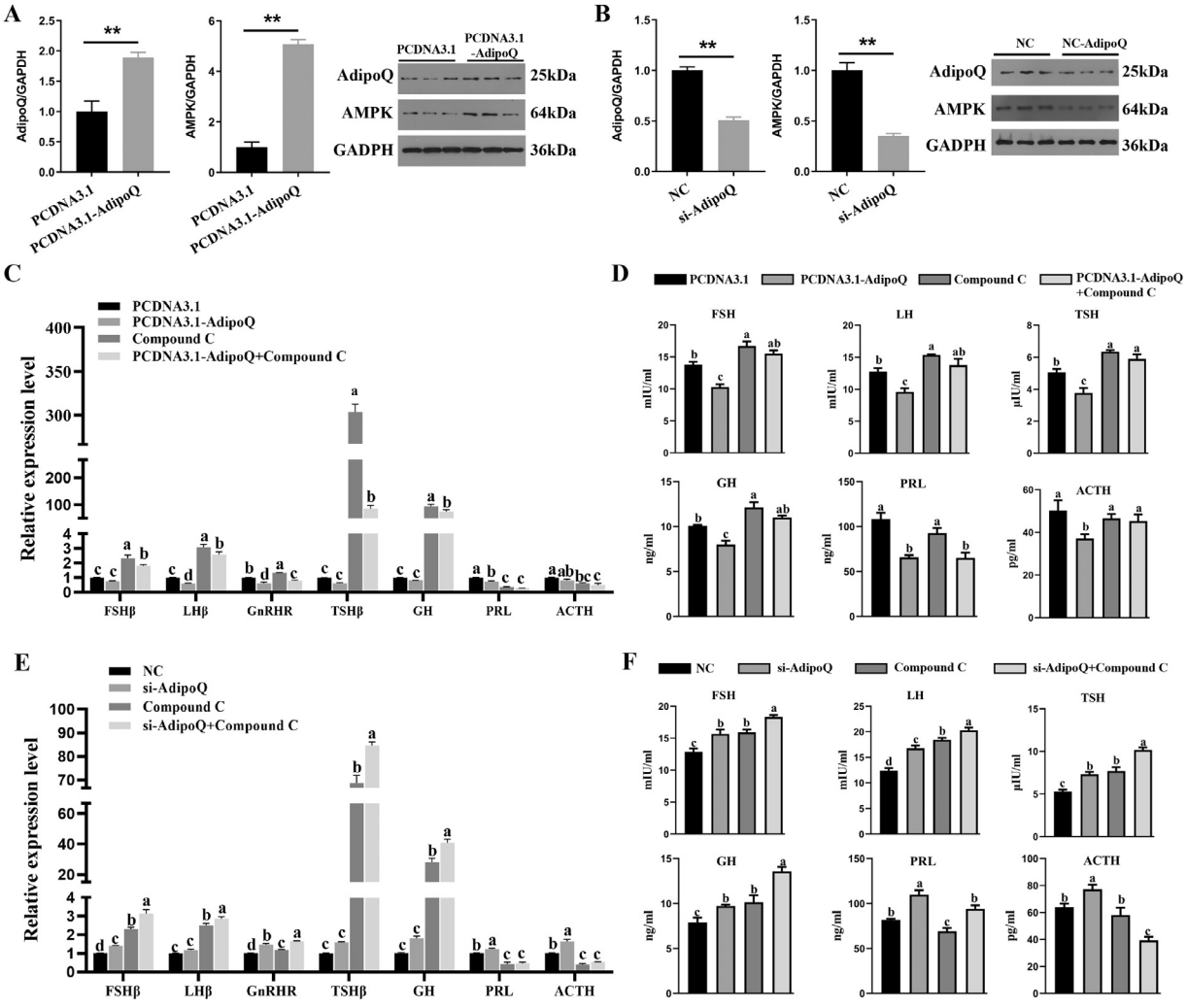

Figure 4. AdipoQ regulates hormone secretion and related gene expression in chicken primary adenohypophysis cells through the AMPK signaling pathway. (A−B) Effects of AdipoQ overexpression or interference on AdipoQ and AMPK protein expression; (C) Effect of cotreatment of Compound C with overexpressed AdipoQ on mRNA expression of related genes; (D) Effect of cotreatment of Compound C with overexpressed AdipoQ on hormone secretion; (E) The effect of Compound C cotreatment with interfering AdipoQ on mRNA expression of related genes; (F) The effect of Compound C cotreatment with interfering AdipoQ on hormone secretion.

Additionally, we simultaneously interfered with *AdipoR1* and *AdipoR2* with the addition of AdipoRon. The results showed that in si-AdipoR1 plus si-AdipoR2 group, the mRNA expression of *AdipoR1* and *AdipoR2* significantly decreased, while the mRNA expression of *FSHβ, LHβ, GnRHR, TSHβ, GH, PRL* and *ACTH* as well as the secretion of FSH, LH, TSH, GH, PRL and ACTH were significantly increased ($P < 0.05$) (3F−G). Interestingly, compared to the NC group, the mRNA expression of *AdipoR1* and *AdipoR2* significantly increased in the si-AdipoR1 plus si-AdipoR2 plus AdipoRon group ($P < 0.05$). However, there were no significant differences in the mRNA expression of *FSHβ, LHβ, GnRHR, TSHβ, GH, PRL* and *ACTH* as well as the secretion of FSH, LH, TSH, GH, PRL and ACTH (Figures 3F−3G).These results suggested that AdipoQ analogue, AdipoRon, inhibits the secretion of FSH, LH, TSH, GH, PRL and ACTH hormones as well as the expression of related genes, which was mediated by activating AdipoR1 and AdipoR2.

AdipoQ Regulates Hormone Secretion and Related Gene Expression in Chicken Adenohypophysis Cells Via the AMPK Signaling Pathway

To further explore the regulatory role of AdipoQ in hormone secretion and gene expression in chicken primary adenohypophysis cells through the AMPK signaling pathway, we initially examined the effects of overexpression or interference of *AdipoQ* on the phosphorylation level of AMPK in these cells. Our results showed a significant up-regulation of AdipoQ protein levels and AMPK phosphorylation in the AdipoQ overexpression group, while a down-regulation was observed in the AdipoQ interference group compared to the control group ($P < 0.01$) (Figure 4A and B). Subsequently, we employed AMPK pathway inhibitors (Compound C), PCDNA3.1, PCDNA3.1-AdiopoQ, NC and si-AdiopoQ for the next studies. The findings revealed noticeable increases in the mRNA levels of *FSHβ, LHβ,*

GnRHR, TSHβ, and *GH* in the Compound C group compared to the PCDNA3.1 group or the NC group (P<0.05). accompanied by an elevation in the secretion of FSH, LH, TSH, and GH hormones ($P < 0.05$). However, the mRNA levels of *PRL* and *ACTH* were significantly decreased ($P < 0.05$), while their hormone levels remained unaffected or down-regulated (Figure 4C−F). PCDNA3.1-AdiopoQ or si-AdiopoQ led to a decrease or increase in the mRNA levels of *FSHβ, LHβ, GnRHR, TSHβ, GH, PRL* and *ACTH*, respectively. Additionally, these interventions significantly inhibited or promoted the secretion of FSH, LH, TSH, GH, PRL and ACTH hormones ($P < 0.05$) (Figure 4C−F). In addition, in the PCDNA3.1-AdipoQ plus Compound C group, the expression of *FSHβ, LHβ, GnRHR, TSHβ, GH* mRNA as well as the secretion of FSH, LH, TSH, GH hormone was lower than that in the Compound C group but higher than that in the PCDNA3.1-AdiopoQ group (Figure 4C and D). However, in the si-AdipoQ plus Compound C group, there was a significant increase in the expression of *FSHβ, LHβ, GnRHR, TSHβ, GH* mRNA, and the secretion of FSH, LH, TSH, and GH hormones compared to the Compound C group and the si-AdipoQ group ($P < 0.05$) (Figure 4E and F). In summary, these data indicated that AdipoQ regulates the secretion of FSH, LH, TSH and GH hormones and the expression of *FSHβ, LHβ, TSHβ* and *GH* genes in chicken adenohypophysis cells through the AMPK signaling pathway.

DISCUSSION

AdipoQ, a protein secreted by adipose tissue, exhibits various effects such as antioxidant stress regulation, glucose and lipid metabolism regulation, and anti-inflammatory properties (Brochu-Gaudreau et al., 2010; Schindler et al., 2017; Singh et al., 2018). Recent studies have shed light on the role of AdipoQ in cell proliferation and apoptosis regulation. For instance, Zhang et al. (2015) found that AdipoQ inhibits the proliferation and promotes apoptosis of endometrial cancer cells by activating AMPK pathway. Similarly, Feng et al. (2018) discovered that AdipoQ promoted the proliferation of ovarian cancer cells through PI3K/Akt and Raf/MEK/ERK pathways. In another study, Zhang et al. (2011) found that AdipoQ promotes the proliferation of hippocampal neural stem cells by activating the p38MAPK/GSK-3β/β-catenin signaling pathway, without affecting apoptosis. These studies collectively indicate that AdipoQ has a wide range of regulatory effects on the process of proliferation and apoptosis of different cell lines, exhibiting cell-specific differences. To investigate the impact of AdipoQ on the proliferation of chicken adenohypophysis cells, we examined cell viability, cell cycle progression, and the expression of *P21, CDK1, CCND1*, and *PCNA* genes associated with cell proliferation following *AdipoQ* overexpression or interference. Cyclin-dependent kinases (**CDKs**), a vital family of protein kinases, play a critical role in regulating the cell cycle, activating checkpoints, and repairing DNA damage, with CDK1 particularly essential for controlling the entry of cells into mitosis (Nasa et al., 2020). The CCND1 gene encodes a highly conserved cell cycle protein that forms a complex with CDK4 and CDK6, critically influencing the G1/S transition (Liu et al., 2008). Our findings revealed that AdipoQ promotes the proliferation of chicken primary adenohypophysis cells. To explore the effect of AdipoQ on apoptosis of chicken primary adenohypophysis cells, we assessed apoptosis levels and the expression of apoptosis-related genes *Bcl2, Fas, FasL* and *Caspase3*, upon *AdipoQ* overexpression or interference. Fas, a death receptor molecule and member of the tumor necrosis factor (**TNF**) family, induces apoptosis when bound to FasL, a type II membrane protein (Vickers et al., 2000). The caspase family plays a pivotal role in the morphological and biochemical changes associated with apoptosis, with Caspase3 being a key player. Activated Caspase3 cleaves various intracellular target proteins, triggering nuclear and cytoplasmic morphological alterations that ultimately lead to cell death (Kurokawa and Kornbluth, 2009). Bcl2 protects cells from apoptotic signaling by preventing the release of cytochrome C from mitochondria (an important event in apoptosis) thus inhibiting it (Flaws et al., 2001). Our results demonstrated that AdipoQ inhibits apoptosis in chicken primary adenohypophysis cells. Although the signaling pathway underlying AdipoQ's effects on proliferation and apoptosis in chicken primary adenohypophysis cells remains unclear and requires further investigation, the wealth of research on AdipoQ's effects on proliferation and apoptosis across diverse cell lines provides valuable insights and direction. Notably, AdipoQ has been observed to inhibit the expression of the interleukin-6 (***IL-6***) gene in porcine adenohypophysis cells (Szeszko et al., 2019). IL-6 is a multifunctional cytokine that plays a crucial role in cell growth regulation. Autocrine IL-6 has been associated with cell senescence, as reported by Sapochnik et al. (2017). This discovery opens a new avenue for investigating the regulatory mechanisms by which AdipoQ influences the proliferation and apoptosis of chicken primary adenohypophysis cells. That is, AdipoQ may promote cell proliferation and inhibit apoptosis in chicken primary adenohypophysis cells by suppressing the expression of the *IL-6* gene.

In chicken primary adenohypophysis cells, various hormones are secreted, including FSH, LH, TSH, GH, ACTH, and PRL, which play significant physiological roles in poultry organisms. Among these hormones, FSH, LH and PRL specifically contribute to reproduction. FSH primarily promotes follicular maturation, while LH promotes both follicular maturation and ovulation. Additionally, PRL stimulates the production of follicular LH receptors. TSH and ACTH are primarily involved in body development. TSH supports the growth and function of the thyroid, whereas ACTH maintains the normal morphology and function of the adrenal gland. GH is closely associated with growth and metabolism, promoting bone and organ growth, protein

synthesis, and influencing fat and mineral metabolism. It plays a crucial role in overall body growth and development. Our findings demonstrate that AdipoQ indirectly participates in the regulation of reproduction, development, growth, and metabolism by inhibiting the secretion of FSH, LH, TSH, GH, PRL and ACTH by the pituitary gland. Further results obtained from co-treatment with AdipoRon (a synthetic analogue of AdipoQ) and interference of *AdipoR1/2* suggest that AdipoQ's regulatory effect on adenohypophysis hormone secretion is mediated through the activation of AdipoR1 and AdipoR2. This view is supported by previous studies. For example, Rodriguez-Pacheco et al. (2007) demonstrated that in short-term (4 h) cultured rat pituitary cells, AdipoQ inhibited the release of GH and LH as well as the secretion of GH and LH induced by ghrelin and GnRH stimulation. This is consistent with the results of our experiment. Other studies have also shown the specific effects of AdipoQ on pituitary endocrine function across different species and physiological stages. In vitro studies conducted by Kiezun et al. (2014) showed that AdipoQ regulated FSH secretion without affecting LH secretion from porcine pituitary cells during estrus, but exhibited different effects at different stages of the estrous cycle. Punyadeera et al. (2005) demonstrated that intravenous injection of adenovirus expressing AdipoQ into male mice reduced serum LH levels while leaving FSH levels unchanged. Moreover, some studies suggest that AdipoQ may influence the secretory function of adenohypophysis cells by down-regulating the expression of *IL-6* and interleukin-1α (***IL-1α***) genes. Szeszko et al. (2019) observed that AdipoQ down-regulated the expression of *IL-6* and *IL-1α* genes in porcine pituitary cells, which are associated with positive regulation of pituitary cell secretion. Fukata et al. (1989) and Spangelo et al. (1989) confirmed that IL-6 induced the secretion of ACTH, GH, and PRL in rat pituitary cells in vivo or in vitro. Braden et al. (1998) found that IL-1α stimulated the release of LH in sheep pituitary cells in vitro. Based on these studies, it can be speculated that AdipoQ may inhibit the secretory function of chicken primary adenohypophysis cells by down-regulating the expression of the *IL-6* or *IL-1α* gene.

Previous studies have widely acknowledged the role of AMPK in maintaining energy homeostasis and facilitating metabolic adaptations. However, recent research has revealed that AMPK may also have significant involvement in other physiological processes, including its participation in the signaling pathway of AdipoQ (Wen et al., 2010; Hu et al., 2022). Lu et al. (2008) showed that AdipoQ increased the phosphorylation level of AMPK and significantly suppressed LH secretion, as well as GnRH-stimulated LH fraction. This finding is partially consistent with our findings that AdipoQ may regulate

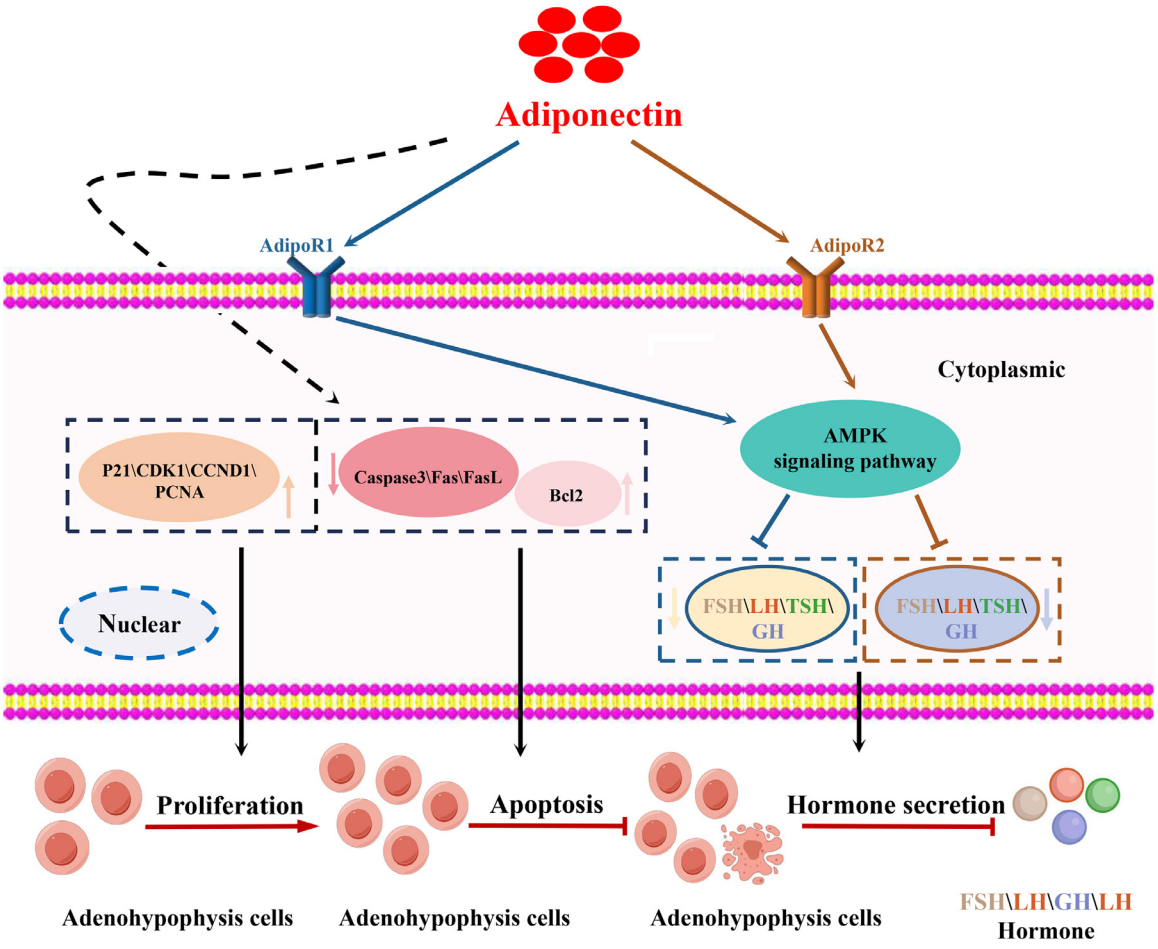

Figure 5. A schematic diagram illustrating the role of AdipoQ in the proliferation, apoptosis and hormone secretion function of chicken primary adenohypophysis cells.

the secretion of FSH, LH, TSH and GH in chicken adenohypophysis through AMPK signaling pathway. We hypothesize that AMPK, acting as a signal transducer, functions by sensing the body's nutritional status through monitoring ATP concentration and cellular energy levels. Subsequently, it transmits secretory signals to regulate the release of FSH, LH, TSH, and GH, indirectly influencing processes related to reproduction, growth, and development. Notably, an investigation into the impact of AdipoQ on the transcriptional profile of porcine anterior pituitary cells revealed differential expression of genes associated with the AMPK signaling pathway. Specifically, AdipoQ up-regulated the expression of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (**PIK3CG**), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (**PFKFB1**) and glycogen synthase 2 (**GYS2**) (Szeszko et al., 2019). This suggests that AdipoQ may indirectly affect AMPK signaling pathway by regulating the expression of these three genes.

CONCLUSIONS

The objective of this study was to investigate the potential role of AdipoQ in regulating the proliferation, apoptosis, hormone secretion (FSH, LH, TSH, GH, PRL, and ACTH) and related gene expression of chicken primary adenohypophysis cells. This study revealed that AdipoQ promotes the proliferation and inhibits apoptosis of chicken primary adenohypophysis cells. Furthermore, AdipoQ regulates the hormone secretion (FSH, LH, TSH, GH, PRL, and ACTH) and mRNA expression (*FSHβ, LHβ, GnRHR, TSHβ, GH, PRL*, and *ACTH*) in chicken primary adenohypophysis cells by binding to AdipoR1/2. Additionally,the regulatory effect of AdipoQ on hormone secretion (FSH, LH, TSH, and GH) may be mediated through the AMPK signaling pathway (Figure 5). These findings provide a groundbreaking insight into the molecular mechanism underlying AdipoQ's regulation of secretory function in chicken adenohypophysis.

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DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.104137.

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