Expression pattern and regulation of head-to-head genes \textit{Vps36} and \textit{Ckap2} during chicken follicle development

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Abstract Vacuolar protein sorting 36 (VPS36), a protein primarily known for its role in the Endosomal Sorting Complex Required for Transport pathway, has recently been shown to be linked to chicken reproduction. Previous research showed that \textit{Vps36} is significantly downregulated in sexually mature chicken ovaries compared to immature ones. In this study, using real-time quantitative RT-PCR, we investigated the expression pattern of \textit{Vps36} and its head-to-head gene \textit{Ckap2} mRNA in chicken follicles. Small white follicles were found to have significantly higher expression of \textit{Vps36} and \textit{Ckap2} mRNA than any other sized follicles (\( P < 0.05 \)). The expression of \textit{Vps36} and \textit{Ckap2} mRNA were detected in both granulosa and theca layers of pre-ovulatory follicles, the expression of \textit{Ckap2} in theca layers was slightly higher than in granulosa cells. Treatment of small yellow follicles with follicle-stimulating hormone and estradiol resulted in a marked decrease of both \textit{Vps36} and \textit{Ckap2} mRNA (\( P < 0.05 \)); however, progesterone, transforming growth factor-\( \beta \) 1 and luteinating hormone induced no significant changes in \textit{Vps36} and \textit{Ckap2} mRNA expression in these follicles. These results indicate that the head-to-head genes of \textit{Vps36} and \textit{Ckap2} exhibit similar expression in chicken follicles and are involved in chicken follicle development.

Keywords chicken, \textit{Vps36}, \textit{Ckap2}, mRNA, follicle

1 Introduction

Vacuolar protein sorting 36 (VPS36) is important in the ESCRT (Endosomal Sorting Complex Required for Transport) pathway. Fifteen class E vps (vacuolar protein sorting) genes encoding the components of four ESCRT protein complexes have been identified, including ESCRT 0 (Vps27, Hse1), I (Vps23, Vps28, Vps37, Mvb12), II (Vps22, Vps25, Vps36) and III (Vps2, Vps20, Vps24, Snf7/Vps32) [1]. Loss of class E vps function in yeast leads to accumulation of ubiquitinated proteins on the limiting membrane of enlarged endosomes [2]. Biochemical studies in mammalian cells also revealed a similar function for endosomal protein sorting [3,4]. The VPS36 protein contains Npl4 zinc finger (NZF) and GLUE (GRAM-like Ub binding) domains, a ‘hub’ that mediates interaction with ESCRT-I (Vps28) [5] and binds to PI(3)P (phosphatidylinositol-3-phosphate) on endosomes and to ubiquitinated cargo [6–9]. Genetic analysis of class E vps genes has mainly been performed in yeast [2,10]. In \textit{Drosophila}, the \textit{Vps36} mutant increases apoptotic resistance and shows neoplastic characteristics [11]. Mutants in all subunits of the ESCRT-II complex (Vps22, Vps25 and Vps36) abolish the final Staufen-dependent step in bcd RNA localization [12]. \textit{Ckap2} (cytoskeleton associated protein 2) is the head-to-head gene of \textit{Vps36} and shares a 247 bp promoter with the latter on chicken chromosome 1. \textit{Ckap2} is an important mitotic regulator [13–15], and has been shown to be upregulated in various human malignancies [16–18].

It is widely accepted that follicle growth and differentiation are mediated by endocrine, paracrine and autocrine factors, among which the most important are the gonadotropins and growth factors [19]. Follicle-stimulating hormone (FSH) is responsible for follicular recruitment and growth of the smaller follicles. Subsequent to selection, a follicle undergoes a transition from largely FSH-dependence to luteinizing hormone (LH)-dependence [20–22]. LH is the primary gonadotropin responsible for promoting progesterone production in pre-ovulatory follicles [19,23]. Our previous study showed that \textit{Vps36} is significantly downregulated in sexually mature ovaries compared to immature ones in chicken ovary [24],
suggested that \( Vps36 \) is likely to be involved in the process of ovarian follicular development. The objective of this study is to investigate the mRNA expression pattern of \( Vps36 \) and \( Ckap2 \) genes and the effect of gonadotrophins, steroid hormones and transforming growth factor-\( \beta \) (TGF-\( \beta \)) on their expression in chicken follicles. We demonstrated that the expression of the head-to-head genes \( Vps36 \) and \( Ckap2 \) is regulated by FSH and estradiol in chicken follicles.

## 2 Materials and methods

### 2.1 Birds, follicle collection, and separation of granulosa and theca layer cells

The Hy-Line Brown laying hens were housed under standard conditions with food and water. Sexually mature hens (23 weeks old, \( n = 4 \)) were slaughtered to collect follicles of various sizes, including pre-ovulatory follicles (F1, F2, F3 and F5) and pre-hierarchical follicles (small white follicles, SWF, 2–4 mm; and small yellow follicles, SYF, 4–8 mm). Then the yolk in follicles was removed and the separated follicles were immediately frozen in liquid nitrogen. All the tissues were stored at \(-80^\circ\text{C}\) until processed. The granulosa and theca cell layers from pre-ovulatory follicle (F1, F3 and F6) were separated using a dissection microscope following the method described previously [25].

### 2.2 Follicle culture and treatment

The SYF of the ovaries of egg-laying hens (35–48 weeks old) were collected, then individual follicles were washed with three dishes of \( 1 \times \text{ PBS (pH 7.2) } \) and placed in M199 medium (1 mL) (HyClone, Logan, UT, USA) with 1% ITS (Sigma, St. Louis, MO, USA) and seeded in 24-well culture plates at a density of one per well according to the protocol. The cultured SYF were divided into four groups: one control group and three treatment groups. The cultured SYF in treatment groups were treated with 10, 50 and 100 ng mL\(^{-1}\) of porcine FSH (Sioux Biochemical, Sisoux, Israel) and equine LH (Sigma), and the SYF that were treated with human TGF-\( \beta \)1 (Prospectbio, Rehovot, Israel) were divided into three groups (control, 2.5 and 5 ng mL\(^{-1}\)). Each group had four repeats. Then all SYF were cultured at 39°C in a water-saturated atmosphere of 95% air and 5% \( \text{ CO}_2 \) for 16 h [27]. After 16 h, the yolk in SYF was removed and the remaining SYF were immediately frozen in liquid nitrogen for RNA isolation. Each treatment was repeated at least three times.

### 2.3 Total RNA isolation and cDNA synthesis

Total RNA from follicles of different levels was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions and treated with DNase I (Qiagen, Beijing, China) to remove DNA contamination. Total RNA from SYF cultured and treated as above was isolated using MicroElute\textsuperscript{TM} Total RNA Kit (OmegaBiotek, Norcross, GA, USA) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed using oligo-d(T) primer (10 \( \mu \text{mol L}^{-1} \) ) and 2 \( \text{ U reverse transcriptase (Roche Applied Science, Mannheim, Germany) according to manufacturer’s protocol. The amount and integrity of isolated total RNA were measured using a spectrophotometer (Eppendorf, Hamburg, Germany) and checked by loading total RNA onto a 1% agarose gel that was stained with ethidium bromide.

### 2.4 Real-time quantitative RT-PCR

Chicken \( Vps36 \) and \( Ckap2 \) mRNA were quantified using 3 \( \mu \text{L} \) of the reverse transcription reaction product (equivalent of 150 ng of single-stranded cDNA) as template in real-time quantitative RT-PCR (qRT-PCR) with \( \beta\text{-actin} \) as internal control. The qRT-PCR was performed in a 20 \( \mu \text{L} \) reaction volume consisting of SYBR\textsuperscript{®} Premix Ex Taq\textsuperscript{TM} (Takara Biotechnology, Dalian, China) and 0.2 \( \mu \text{mol L}^{-1} \) of forward and reverse primers (Table 1) on Mx3000 Real Time PCR-Cycler (Agilent Technologies, Reno, NV, USA) with the settings: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 30 s, and 72°C for 25 s. At the end of amplification, a melting curve analysis was performed to ensure the specificity of the amplification.

| Table 1 | Oligonucleotide primer sequences for real-time quantitative PCR |
|---------|---------------------------------------------------------------|
| Primer  | GenBank accession number | Sequence | Product size/bp |
| \( Vps36 \) Sense | XM_417077 | 5’-TTGTAAGATGCTGGAGCCCG-3’ | 187 |
| \( Vps36 \) Antisense | | 5’-TTAGAGAAACAGACATTCCCACC-3’ | |
| \( Ckap2 \) Sense | NM_001006274 | 5’-CTGGATGCGCTTGAAACAGA-3’ | 168 |
| \( Ckap2 \) Antisense | | 5’-TCTATCACAGCTCCCCCATC-3’ | |
| \( \beta\text{-actin} \) Sense | NM_205518 | 5’-TGGATGATGATATTGCTGC-3’ | 253 |
| \( \beta\text{-actin} \) Antisense | | 5’-ATCTTTCCTCATATCATCCC-3’ | |
confirm the presence of a single amplification product. Each sample was run in duplicate to obtain average log-linear threshold (CT) values for *Vps36* and *Ckap2* mRNA and β-actin mRNA. The efficiencies were close to 100%, allowing the use of the 2-ΔΔCT method for calculation of relative gene expression [28]. All qRT-PCR were performed at least in triplicate and with a negative control. The expression of chicken *Vps36* and *Ckap2* mRNA in different sized follicles and in cultured SYF was examined individually, except that for SWF, due to their small sizes, three to four were pooled for analysis.

2.5 Statistical analysis

For the quantitative measurement of the mRNA levels of *Vps36* and *Ckap2* genes in follicles, values are expressed as the means±SEM. Data from the experiments were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan’s multiple range test. When *P* < 0.05, the difference was considered as significant.

3 Results

3.1 Expression of *Vps36* and *Ckap2* mRNA in follicles

The mRNA expression pattern of chicken *Vps36* and *Ckap2* genes were investigated in chicken follicles of various sizes, i.e. SWF, SYF and from F5 to F1. The mRNA expression of chicken *Vps36* gene was markedly reduced from SWF to SYF (*P* < 0.05), then remains stable from F5 to F1 follicles (Fig. 1a). The expression of chicken *Ckap2* mRNA was progressively reduced from SWF to F2 follicles (*P* < 0.05) and then slightly increased in F1 follicles (Fig. 1b). Both *Vps36* and *Ckap2* exhibited the highest mRNA expression levels in chicken SWF. The expression of *Vps36* and *Ckap2* mRNA were detected in both granulosa and theca layers of chicken pre-ovulatory follicles, and the expression of *Ckap2* in theca layers was slightly higher than in granulosa cells (Fig. 2).

3.2 Effect of FSH and LH on the expression of *Vps36* and *Ckap2* mRNA in SYF

The expression of chicken *Vps36* and *Ckap2* mRNA in SYF was significantly decreased in response to FSH treatment (*P* < 0.05, Fig. 3a). The effect of FSH on chicken *Vps36* and *Ckap2* mRNA expression was not significant by different concentrations (10, 50 and 100 ng·mL⁻¹). LH treatment in chicken SYF produced no significant effect on mRNA expression of *Vps36* and *Ckap2* genes (Fig. 3b).

3.3 Effect of E2 and P4 on *Vps36* and *Ckap2* mRNA expression in SYF

In SYF, E2 treatment for 16 h resulted in a significant decrease in the mRNA expression of chicken *Vps36* and *Ckap2* genes (*P* < 0.05, Fig. 4a). With the increasing concentration of E2, a gradual decrease in mRNA expression level of chicken *Vps36* was observed. Treatment of SYF with 10 ng·mL⁻¹ E2 did not affect chicken *Ckap2* mRNA expression, however, when the concentration of E2 was increased to 50 and 100 ng·mL⁻¹, *Ckap2* mRNA expression was remarkably decreased (*P* < 0.05, Fig. 4a). By contrast, P4 treatment produced no significant effect on chicken *Vps36* mRNA expression (*P* > 0.05, Fig. 4b). The same situation occurred with chicken *Ckap2* (Fig. 4b) except that *Ckap2* mRNA expression was significantly decreased after treatment with higher concentration of P4 (100 ng·mL⁻¹) compared to any other concentrations (Fig. 4b).

![Fig. 1](image_url) *Vps36* (a) and *Ckap2* (b) mRNA abundance in different sized follicles as measured by real-time quantitative RCR. The different letters above each bar indicate significant difference at *P* < 0.05. Data are means±standard error of the mean (*n* = 4). SWF (small white follicles, 2–4 mm); SYF (small yellow follicles, 4–8 mm); F5 (12–14 mm); F3 (22–24 mm); F2 (the second largest follicle); F1 (the largest follicle).
3.4 Effect of TGFβ1 on the expression of \(Vps36\) and \(Ckap2\) mRNA in SYF

In chicken SYF, the expression of \(Vps36\) mRNA levels was not significantly affected by TGFβ1 at the concentrations of 2.5 and 5.0 ng\(\cdot\)mL\(^{-1}\) (Fig. 5). When the concentration of TGFβ1 was increased, the chicken \(Ckap2\) mRNA expression level was gradually decreased, but the effect was not significant (Fig. 5).

4 Discussion

The process of ovarian follicle development in vertebrates is closely associated with the functional differentiation of granulosa cells. In the process of chicken follicular development, through selection from the pool of SYF, follicles successively become dominant. The functional differentiation of granulosa cell regulated by hormone and many paracrine/autocrine factors is coupled with changes in the expression level of many related genes. By cDNA-
investigated.

different sized follicles, as well as in cultured SYF, were expression pattern and regulation of these two genes in ovulation remains unknown. Therefore, in this study, the more active in SWF than any other sized follicles. be postulated that the cellular process, mentioned above, is involved in follicle recruitment, which is likely to be a previous study that the regulatory mechanism on the transcription of head-to-head genes is similar [30].

Follicle-stimulating hormone promotes the development of granulosa cells, and causes them to proliferate, subsequently differentiate, and finally to become steroidogenic via de novo synthesis of steroidogenic factors, steroidogenic enzymes, and transcription factors [31–35]. Transcription factors like AP-1, CBP, Egr-1, SF-1, and SP1 were suggested to be regulated through the ERK signaling pathway that is stimulated by FSH [36]. It is also reported that FSH promotes rapid activation of protein kinase A (PKA) [37]. The cAMP-response-element binding-protein (CREB) is the best-known transcription factor regulated by PKA [38,39] and was initially predicted to regulate expression of most, if not all, PKA-regulated target genes in granulosa cells. In this study, bioinformatics analysis revealed two CREB binding sites in Vps36 gene (data not shown), indicating a PKA-CREB pathway that FSH regulates Vps36 transcription. The biological actions of estrogens are mediated by estrogen binding to one of two specific estrogen receptors, ERα and ERβ, which belong to the nuclear receptor superfamily [40]. Bioinformatics analysis shows that both Vps36 and Ckap2 genes contain ERα binding sites. We postulate β-estradiol inhibits the mRNA expression of Vps36 and Ckap2 mainly through the ERα pathway, but this idea requires investigation.

5 Conclusions

In this study, we found that Vps36 and Ckap2 were highly expressed in pre-hierarchical follicles. The mRNA expression of Vps36 and Ckap2 in chicken SYF was strongly inhibited by FSH and estrogen treatment. In addition, the head-to-head genes of Vps36/Ckap2 exhibit similar expression and regulatory modes in chicken follicles. These results indicate that Vps36 and Ckap2 are important in follicle development.

Acknowledgements This work was funded by the National Natural Science Foundation of China (30871777), Platform Construction of Genetic Resources of Livestock and Poultry Breeds in China (2005DKA21101) and Agricultural Elite Breeds (Poultry) Project of Shandong Province (2009LZ09-03).

Compliance with ethics guidelines Xinxing Cui, Chunhong Yang, Li Kang, Guiyu Zhu, Qingqing Wei and Yunliang Jiang declare that they have no conflict of interest or financial conflicts to disclose.
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