RESEARCH ARTICLE

Expression pattern and regulation of head-to-head genes *Vps36* and *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 *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 *Vps36* and its head-to-head gene *Ckap2* mRNA in chicken follicles. Small white follicles were found to have significantly higher expression of *Vps36* and *Ckap2* mRNA than any other sized follicles ($P < 0.05$). The expression of *Vps36* and *Ckap2* mRNA were detected in both granulosa and theca layers of pre-ovulatory follicles, the expression of *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 *Vps36* and *Ckap2* mRNA ($P < 0.05$); however, progesterone, transforming growth factor-β 1 and luteinizing hormone induced no significant changes in *Vps36* and *Ckap2* mRNA expression in these follicles. These results indicate that the head-to-head genes of *Vps36* and *Ckap2* exhibit similar expression in chicken follicles and are involved in chicken follicle development.

Keywords   chicken, *Vps36*, *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

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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 (phosphatidyl-inositol-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 *Drosophila*, the *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]. *Ckap2* (cytoskeleton associated protein 2) is the head-to-head gene of *Vps36* and shares a 247 bp promoter with the latter on chicken chromosome 1. 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 *Vps36* is significantly downregulated in sexually mature ovaries compared to immature ones in chicken ovary [24],

suggesting 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-β1 (TGF-β1) 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 $-80°C$ 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 $1 \times$ 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 reference [26]. The 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 center, IA, USA), β-estradiol (E2; Sigma), progesterone (P4; Sigma) and equine LH (Sigma), and the SYF that were treated with human TGF-β1 (Prospecbio, 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% 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™ 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 µmol·L^{-1}) and 2 U reverse transcriptase (Roche Applied Science, Mannheim, Germany) according to manufacture's protocol. The amount and integrity of isolated total RNA were measured using a spectro-photometer (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 µL 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 *β-actin* as internal control. The qRT-PCR was performed in a 20 µL reaction volume consisting of SYBR® Premix Ex Taq™ (Takara Biotechnology, Dalian, China) and 0.2 µmol·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

Table 1 Oligonucleotide primer sequences for real-time quantitative PCR

Primer	GenBank accession number	Sequence	Product size/bp
Vps36	XM_417077		187
Sense		5′-TTGTAAGATGCTGGAGCCG-3′	
Antisense		5′-TAAGAGAACAGACATTCCCACC-3′	
Ckap2	NM_001006274		168
Sense		5′-CTGGATGCGTCTTGAACAGA-3′	
Antisense		5′-TCTATCACAGCTCCCCCATC-3′	
β-actin	NM_205518		253
Sense		5′-TGGATGATGATATTGCTGC-3′	
Antisense		5′-ATCTTCTCCATATCATCCC-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 $2^{-\Delta\Delta C_T}$ 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).
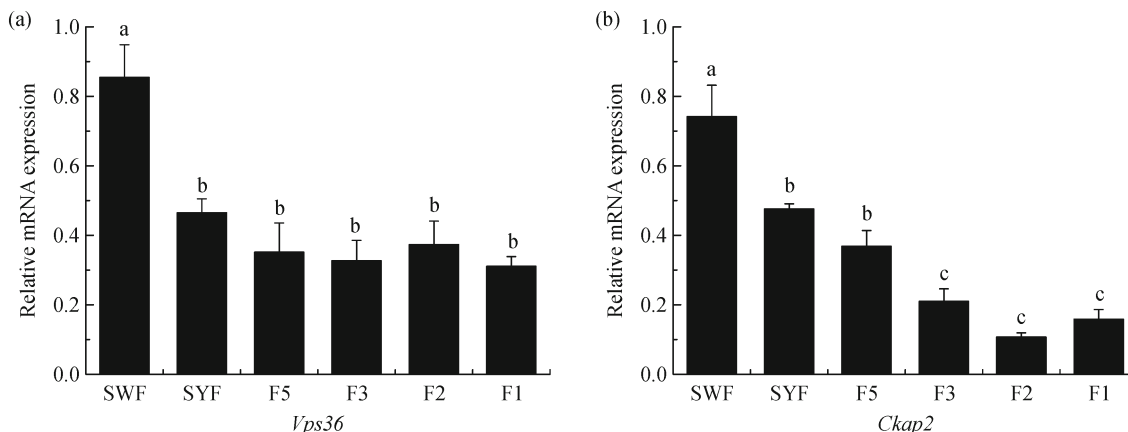

Fig. 1 *Vps36* (a) and *Ckap2* (b) mRNA abundance in different sized follicles as measured by real-time quantitative RCR. The different letters above each bar indicate significant difference at $P < 0.05$. Data are means±standard error of the mean ($n = 4$). SWF (small white follicles, 2–4 mm); SYF (small yellow follicles, 4–8 mm); F5 (12–14 mm); F3 (22–24 mm); F2 (the second largest follicle); F1 (the largest follicle).
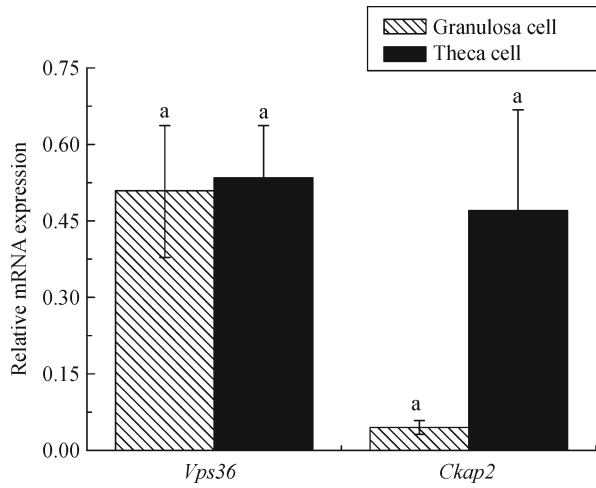
Fig. 2 Real-time quantitative PCR analysis of *Vps36* and *Ckap2* gene expression in granulosa and thecal layer cells of pre-ovulatory (F1, F3 and F6) follicles. Values marked with different letters differ significantly ($P < 0.05$). Data are means±SEM ($n = 5$).
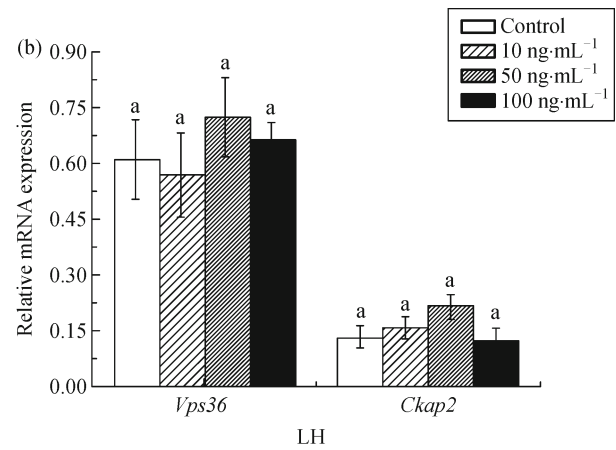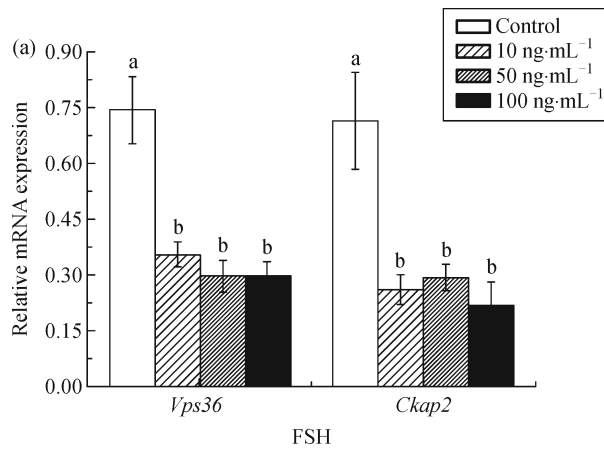
3.4 Effect of TGFβ1 on the expression of *Vps36* and *Ckap2* mRNA in SYF

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·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-

Fig. 3 Effect of FSH (a) and LH (b) on mRNA expression of *Vps36* and *Ckap2* in chicken small yellow follicles. Values marked with different letters differ significantly ($P < 0.05$). Data are means±SEM ($n = 4$).
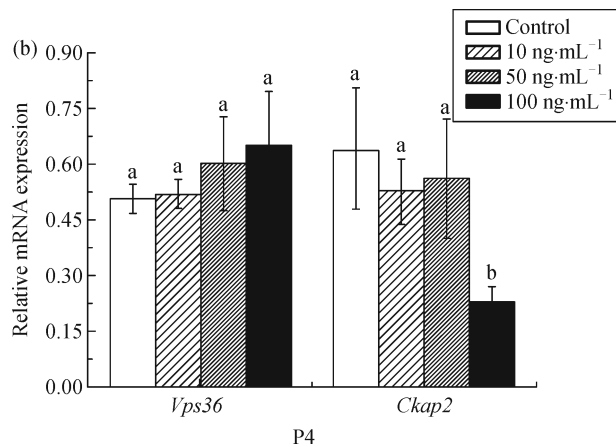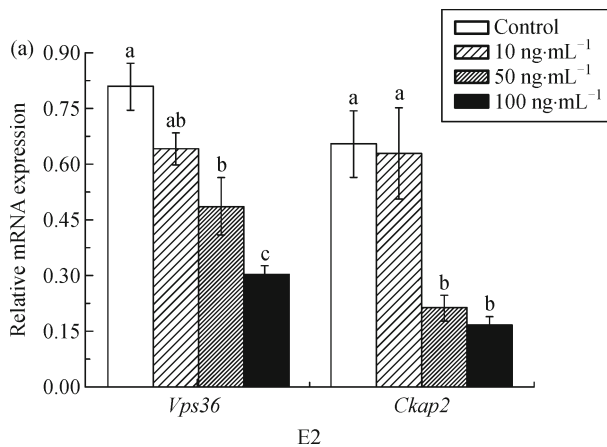

Fig. 4 Effects of E2 (a) and P4 (b) on mRNA expression of *Vps36* and *Ckap2* in chicken small yellow follicles. Values marked with different letters differ significantly ($P < 0.05$). Data are means±SEM ($n = 4$).
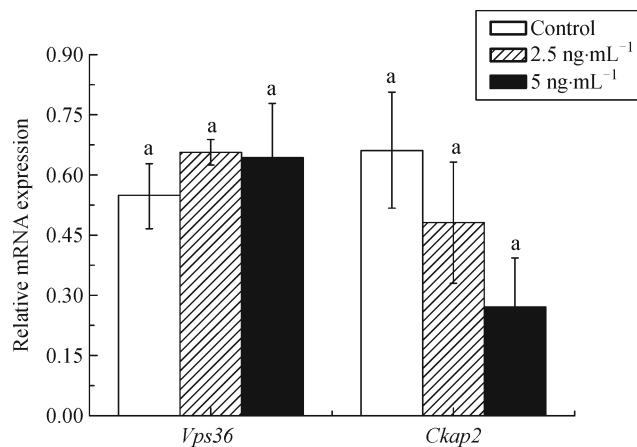
Fig. 5 Effect of TGFβ1 on mRNA expression of Vps36 and Ckap2 in chicken small yellow follicles. Values marked with different letters differ significantly ($P < 0.05$). Data are means±SEM ($n = 4$).

AFLP, *Vps36* was indentified to be related to sexual maturity in chicken and its expression was related to the ovary function [24]. *Ckap2* is the head-to-head gene to *Vps36* on the same chicken chromosome. However, the role of *Vps36* and *Ckap2* in animal follicle growth and ovulation remains unknown. Therefore, in this study, the expression pattern and regulation of these two genes in different sized follicles, as well as in cultured SYF, were investigated.

The result indicated that *Vps36* was expressed in chicken pre-ovulatory and pre-hierarchical follicles, and was much higher in SWF than any others. In addition, both granulosa and theca layers of chicken pre-ovulatory follicles expressed *Vps36* mRNA. These results indicate that *Vps36* is involved in follicle development. It is known that activated growth factors, hormones and cytokine receptors are brought inside the cell by endocytosis and delivered to lysosomes by multivesicular endosomes of which *Vps36* is one important component [29]. Thus it can be postulated that the cellular process, mentioned above, is more active in SWF than any other sized follicles.

To investigate whether the head-to-head gene of *Vps36* is also closely associated with the functional differentiation of follicles, the mRNA expression of *Ckap2* was analyzed in follicles of different sizes. The result showed that there was a similar expression pattern between *Vps36* and *Ckap2* mRNA in follicles sized from SWF to F1, but there were also slight differences in which the mRNA expression of *Ckap2* gradually reduced from SWF to F2, implying that, like *Vps36*, *Ckap2* is also involved in follicle development and the related cellular process.

The decrease in *Vps36* and *Ckap2* mRNA levels in SYF compared to SWY follicles indicates their possible involvement in follicle recruitment, which is likely to be also regulated by sex hormones and gonadotropins. Therefore, the regulatory mechanism of *Vps36* and

Ckap2 in the process of follicle recruitment was investigated by treating cultured chicken SYF with FSH, β-estradiol, progesterone, TGF-β1 and LH. The results indicated that FSH and β-estradiol inhibited the mRNA expression of both *Vps36* and *Ckap2*, but progesterone, TGF-β1 and LH produced no effect on the mRNA expression of *Vps36* and *Ckap2*. These results are consistent with 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.

All applicable institutional and national guidelines for the care and use of animals were followed.

References

1. Henne W M, Buchkovich N J, Emr S D. The ESCRT pathway. *Developmental Cell*, 2011, 21(1): 77–91

2. Katzmann D J, Odorizzi G, Emr S D. Receptor downregulation and multivesicular-body sorting. *Nature Reviews-Molecular Cell Biology*, 2002, 3(12): 893–905

3. Williams R L, Urbé S. The emerging shape of the ESCRT machinery. *Nature Reviews-Molecular Cell Biology*, 2007, 8(5): 355–368

4. Babst M. A protein's final ESCRT. *Traffic*, 2005, 6(1): 2–9

5. Teo H, Gill D J, Sun J, Perisic O, Veprintsev D B, Vallis Y, Emr S D, Williams R L. ESCRT-I core and ESCRT-II GLUE domain structures reveal role for GLUE in linking to ESCRT-I and membranes. *Cell*, 2006, 125(1): 99–111

6. Alam S L, Sun J, Payne M, Welch B D, Blake B K, Davis D R, Meyer H H, Emr S D, Sundquist W I. Ubiquitin interactions of NZF zinc fingers. *The EMBO Journal*, 2004, 23(7): 1411–1421

7. Alam S L, Langelier C, Whitby F G, Koirala S, Robinson H, Hill C P, Sundquist W I. Structural basis for ubiquitin recognition by the human ESCRT-II EAP45 GLUE domain. *Nature Structural & Molecular Biology*, 2006, 13(11): 1029–1030

8. Slagsvold T, Aasland R, Hirano S, Bache K G, Raiborg C, Trambaiolo D, Wakatsuki S, Stenmark H. Eap45 in mammalian ESCRT-II binds ubiquitin via a phosphoinositide-interacting GLUE domain. *The Journal of Biological Chemistry*, 2005, 280(20): 19600–19606

9. Hirano S, Suzuki N, Slagsvold T, Kawasaki M, Trambaiolo D, Kato R, Stenmark H, Wakatsuki S. Structural basis of ubiquitin recognition by mammalian Eap45 GLUE domain. *Nature Structural & Molecular Biology*, 2006, 13(11): 1031–1032

10. Raymond C K, Howald-Stevenson I, Vater C A, Stevens T H. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. *Molecular Biology of the Cell*, 1992, 3(12): 1389–1402

11. Herz H M, Woodfield S E, Chen Z, Bolduc C, Bergmann A. Common and distinct genetic properties of ESCRT-II components in Drosophila. *PLoS ONE*, 2009, 4(1): e4165

12. Irion U, St Johnston D. *bicoid* RNA localization requires specific binding of an endosomal sorting complex. *Nature*, 2007, 445(7127): 554–558

13. Whitfield M L, Sherlock G, Saldanha A J, Murray J I, Ball C A, Alexander K E, Matese J C, Perou C M, Hurt M M, Brown P O, Botstein D. Identification of genes periodically expressed in the human cell cycle and their expression in tumors. *Molecular Biology of the Cell*, 2002, 13(6): 1977–2000

14. Jeon S M, Choi B, Hong K U, Kim E, Seong Y S, Bae C D, Park J. A cytoskeleton-associated protein, TMAP/CKAP2, is involved in the proliferation of human foreskin fibroblasts. *Biochemical and Biophysical Research Communications*, 2006, 348(1): 222–228

15. Hong K U, Park Y S, Seong Y S, Kang D, Bae C D, Park J.

Functional importance of the anaphase-promoting complex-Cdh1-mediated degradation of TMAP/CKAP2 in regulation of spindle function and cytokinesis. *Molecular and Cellular Biology*, 2007, 27(10): 3667–3681

16. Eichmuller S, Usener D, Dummer R, Stein A, Thiel D, Schadendorf D. Serological detection of cutaneous T-cell lymphoma-associated antigens. *Proceedings of the National Academy of Sciences of the United States of America*, 2001, 98(2): 629–634

17. Bae C D, Sung Y S, Jeon S M, Suh Y, Yang H K, Kim Y I, Park K H, Choi J, Ahn G, Park J. Up-regulation of cytoskeletal-associated protein 2 in primary human gastric adenocarcinomas. *Journal of Cancer Research and Clinical Oncology*, 2003, 129(11): 621–630

18. Jin Y, Murakumo Y, Ueno K, Hashimoto M, Watanabe T, Shimoyama Y, Ichihara M, Takahashi M. Identification of a mouse cytoskeleton-associated protein, CKAP2, with microtubule-stabilizing properties. *Cancer Science*, 2004, 95(10): 815–821

19. Johnson A L. Regulation of follicle differentiation by gonadotropins and growth factors. *Poultry Science*, 1993, 72(5): 867–873

20. You S, Bridgham J T, Foster D N, Johnson A L. Characterization of the chicken follicle-stimulating hormone receptor (cFSH-R) complementary deoxyribonucleic acid, and expression of cFSH-R messenger ribonucleic acid in the ovary. *Biology of Reproduction*, 1996, 55(5): 1055–1062

21. Calvo F O, Bahr J M. Adenylyl cyclase system of the small preovulatory follicles of the domestic hen: responsiveness to follicle-stimulating hormone and luteinizing hormone. *Biology of Reproduction*, 1983, 29(3): 542–547

22. Johnson A L, Bridgham J T, Wagner B. Characterization of a chicken luteinizing hormone receptor (cLH-R) cDNA, and expression of cLH-R mRNA in the ovary. *Biology of Reproduction*, 1996, 55(2): 304–309

23. Johnson A L, Bridgham J T. Regulation of steroidogenic acute regulatory protein and luteinizing hormone receptor messenger ribonucleic acid in hen granulosa cells. *Endocrinology*, 2001, 142(7): 3116–3124

24. Kang L, Zhang Y, Zhang N, Zang L, Wang M, Cui X, Jiang Y. Identification of differentially expressed genes in ovaries of chicken attaining sexual maturity at different ages. *Molecular Biology Reports*, 2012, 39(3): 3037–3045

25. Gilbert A B, Evans A J, Perry M M, Davidson M H. A method for separating the granulosa cells, the basal lamina and the theca of the preovulatory ovarian follicle of the domestic fowl (*Gallus domesticus*). *Journal of Reproduction and Fertility*, 1977, 50(1): 179–181

26. Diaz F J, Anthony K, Halfhill A N. Early avian follicular development is characterized by changes in transcripts involved in steroidogenesis, paracrine signaling and transcription. *Molecular Reproduction and Development*, 2011, 78(3): 212–223

27. Jia Y D, Yan F F, Zeng W D, Zhang C Q. Promoting effect of IGF-I on prehierarchical follicle development in laying chickens. *Scientia Agriculture Sinica*, 2011, 44(20): 4295–4301 (in Chinese)

28. Livak K J, Schmittgen T D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. *Methods*, 2001, 25(4): 402–408

29. Raiborg C, Stenmark H. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature*, 2009,

458(7237): 445–452

30. Li Y Y, Yu H, Guo Z M, Guo T Q, Tu K, Li Y X. Systematic analysis of head-to-head gene organization: evolutionary conservation and potential biological relevance. *PLoS Computational Biology*, 2006, 2(7): e74

31. Amsterdam A, Selvaraj N. Control of differentiation, transformation, and apoptosis in granulosa cells by oncogenes, oncoviruses, and tumor suppressor genes. *Endocrine Reviews*, 1997, 18(4): 435–461

32. Richards J S. New signaling pathways for hormones and cyclic adenosine 3′,5′-monophosphate action in endocrine cells. *Molecular Endocrinology*, 2001, 15(2): 209–218

33. Morohashi K I, Omura T. Ad4BP/SF-1, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. *The FASEB Journal*, 1996, 10(14): 1569–1577

34. Omura T, Morohashi K. Gene regulation of steroidogenesis. *The Journal of Steroid Biochemistry and Molecular Biology*, 1995, 53 (1–6): 19–25

35. Amsterdam A, Rotmensch S. Structure-function relationships during granulosa cell differentiation. *Endocrine Reviews*, 1987,

8(3): 309–337

36. Hunzicker-Dunn M, Maizels E T. FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. *Cellular Signalling*, 2006, 18(9): 1351–1359

37. DeManno D A, Cottom J E, Kline M P, Peters C A, Maizels E T, Hunzicker-Dunn M. Follicle-stimulating hormone promotes histone H3 phosphorylation on serine-10. *Molecular Endocrinology*, 1999, 13(1): 91–105

38. Hagiwara M, Brindle P, Harootunian A, Armstrong R, Rivier J, Vale W, Tsien R, Montminy M R. Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. *Molecular and Cellular Biology*, 1993, 13(8): 4852–4859

39. Mukherjee A, Park-Sarge O K, Mayo K E. Gonadotropins induce rapid phosphorylation of the 3′,5′-cyclic adenosine monophosphate response element binding protein in ovarian granulosa cells. *Endocrinology*, 1996, 137(8): 3234–3245

40. Moggs J G, Orphanides G. Estrogen receptors: orchestrators of pleiotropic cellular responses. *EMBO Reports*, 2001, 2(9): 775–781