Expression pattern and developmental behaviour of cellular nucleic acid-binding protein (CNBP) during folliculogeneseis and oogenesis in fish

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Abstract

In vertebrates, folliculogenesis establishes an intricate system for somatic cell–oocyte interaction, and ultimately leads to the acquisition of their respective competences. Although the formation process and corresponding interactions are strikingly similar in diverse organisms, knowledge of genes and signaling pathways involved in follicle formation is very incomplete and the underlying molecular mechanisms remain enigmatic. CNBP has been identified for more than ten years, and the highest level of CNBP transcripts has been observed in adult zebrafish ovary, but little is known about its functional significance during folliculogenesis and oogenesis. In this study, we clone CNBP cDNA from gibel carp (Carassius auratus gibelio), and demonstrate its predominant expression in gibel carp ovary and testis not only by RT-PCR but also by Western blot. Its full-length cDNA is 1402 bp, and has an ORF of 489 nt for encoding a peptide of 163 aa. And its complete amino acid sequence shared 68.5%–96.8% identity with CNBPs from other vertebrates. Based on the expression characterization, we further analyze its expression pattern and developmental behaviour during folliculogenesis and oogenesis. Following these studies, we reveal an unexpected discovery that the CagCNBP is associated with follicular cells and oocytes, and significant distribution changes have occurred in degenerating and regenerating follicles. More interestingly, the CagCNBP is more highly expressed in some clusters of interconnected cells within ovarian cysts, no matter whether the cell clusters are formed from the original primordial germ cells or from the newly formed cells from follicular cells that invaded into the atretic oocytes. It is the first time to reveal CNBP relevance to folliculogenesis and oogenesis. Moreover, a similar stage-specific and cell-specific expression pattern has also been observed in the gibel carp testis. Therefore, further studies on CNBP expression pattern and developmental behaviour will be of significance for understanding functional roles of CNBP during gametogenesis.

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Keywords: Folliculogenesis; Oogenesis; Cyst formation; Granulosa cell; Gibel carp; Cellular Nucleic acid-binding protein; Immunofluorescence

Abbreviations: CNBP, cellular nucleic acid-binding protein; CagCNBP, Carassius auratus gibelio cellular nucleic acid-binding protein; CagVasa, Carassius auratus gibelio Vasa; ZP3, Zona Pellucide typeIII; SNX, sorting nexin; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; nt, nucleotide; ORF, open reading frame; UTR, untranslated region; SMART, switch mechanism at the 5’ end of RNA templates; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate saline buffer; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; TBS, Tris-buffered saline; PI, propidium iodide; TRITC, tetramethyl-rhodamine isothiocyanate; FITC, fluorescein isothiocyanate.

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The nucleotide sequence data reported in this paper have been submitted to the GenBank under accession number: CagCNBP (AY773183).
1. Introduction

Ovarian follicles are special compartments for oocyte differentiation, proliferation, growth and maturation (Matova and Cooley, 2001). In ovary, a single layer or multiple layers of follicular cells, such as granulosa cells and theca cells, generally surround a single oocyte to establish a follicle. Extensive communications and continuous interactions have been observed between the oocyte and the surrounding follicular cells (Matova and Cooley, 2001; Amlich and Dean, 2002). Although the assembly processes and corresponding interactions are strikingly similar in diverse organisms, knowledge of genes and signaling pathways involved in follicle formation is very incomplete and the underlying molecular mechanisms remain enigmatic as commented by Matova and Cooley (2001).

Cellular nucleic acid-binding protein (CNBP) is a zinc finger protein that binds DNA or RNA in a sequence-specific manner (Klug and Rhodes, 1987; Saotome et al., 1995). It contains seven tandem zinc finger repeats that are composed of 14 same amino acid arrangement of Cys–X2–Cys–X4–His–X4–Cys (Armas et al., 2001). So far, CNBP cDNAs have been cloned from human (Rajavashisth et al., 1989), mouse (Warden et al., 1994), rat (Yasuda et al., 1998), Xenopus (Flink et al., 1998), Bufo arenarum (Armas et al., 2001), and Danio rerio (Armas et al., 2004), and all of them have been demonstrated to be highly conserved at the amino acid and nucleotide levels.

During embryogenesis, the CNBP expression patterns have been analyzed in Xenopus leavis (Flink et al., 1998), Bufo arenarum (Armas et al., 2001), mouse (Chen et al., 2003; Shimizu et al., 2003), and Danio rerio (Armas et al., 2004). In Xenopus leavis, CNBP mRNA could be detected in the unfertilized eggs and in the embryonic cells derived from early ectoderm, endoderm, and mesoderm (Flink et al., 1998). In Bufo arenarum, Northern blot analysis revealed two transcripts with different expressions of pattern behaviour, and subcellular localization of CNBP was reported during oogenesis and early embryogenesis (Armas et al., 2001). During mouse embryogenesis, CNBP plays important roles in cell proliferation and tissue patterning during anterior–posterior axis, craniofacial and limb development by targeting c-Myc (Shimizu et al., 2003), and it is essential for the forebrain induction and specification (Chen et al., 2003). In fish, the highest level of CNBP transcripts has been observed in adult zebrafish ovary (Armas et al., 2004), but its distribution and functional roles during oogenesis is unclear up to the present.

Gibel carp (Carassius auratus gibelio), because of its unique triploid level and two different reproduction modes (Zhou et al., 2000; Zhou and Gui, 2002), has been used as a promising study model for developmental biology and evolutionary genetics (Dong et al., 2004; Yang and Gui, 2004). Recently, a systematic study has been initiated to screen differentially expressed genes during oogenesis and early embryogenesis. Some important genes involved in oocyte maturation, egg fertilization and early embryogenesis, such as cyclin A1, cyclin B, cyclin A2 (Xie et al., 2001, 2003), SNX (Wen et al., 2003), hatching enzymes (Liu et al., 2003), C-type lectin (Dong et al., 2004), and C1q-like factor (Chen and Gui, 2004), have been identified in the model system. In the present study, we clone CNBP cDNA from the gibel carp. Based on the characterization analysis and antibody preparation, we find that the CagCNBP is highly expressed in ovary and testis. Following this finding, we analyze its expression pattern during oogenesis, and observe its distribution in different stage oocytes and follicles. An unexpected discovery is revealed that the CagCNBP is intensively associated with follicular cells, and significant distribution changes have occurred in degenerating and regenerating follicles.

2. Materials and methods

2.1. RNA extraction and SMART cDNA synthesis

The gibel carp were bred in Guanqiao Experimental Station of the Institute of Hydrobiology, Chinese Academy of Sciences. The mature eggs and gastrula embryos were sampled in the spawning season. Total RNAs were isolated with SV total RNA Isolation System (Promega), and SMART cDNA was synthesized using a Clontech SMART PCR cDNA Synthesis Kit as described previously (Xie et al., 2003). PCR product was ligated to TOPO-XLPCR vector by T4 DNA ligase and then the ligated products were electrotransformed into TOPO10 cell by Eppendorf Electroporator 2510. White clones were selected and amplified by LD-PCR to detect the insertion fragments size and the PCR products were used for dot-blotting.

2.2. Dot-blot hybridization

The dot-blot hybridization was carried out as described previously (Xie et al., 2001). The gastrula and egg first-strand cDNA were respectively labeled with digoxigenin using a DIG High Prime system (Boehringer Mannheim) as positive and negative probes for the differential screening (Kajiwara et al., 1996). The blots of PCR products from gastrula SMART cDNA library were neutralized for 2 min in 0.5 M Tris–HCl (pH 7.5) and baked for 2 h at 75–80 °C. The membrane was pre-hybridized for 1.5 h and hybridized for 16 h in 5 × SSC, 0.1% N-lauroylsarcosine, 0.2% SDS, 1 × blocking solution (Boehringer Mannheim) and 100 mg/ml denatured salmon sperm DNA at 68 °C. Blots were washed in 2 × SSC and 0.1% SDS two times at room temperature, followed by two washes in 0.1 × SSC and 0.1% SDS at 68 °C under constant agitation for 15 min each. Immunological detection was performed...
using anti-DIG-AP and BCIP/NBT as described in the user manual.

2.3. Sequence analysis

DNA sequencing of the positive clones were performed in Biosasia sequencing company. Homology searches were performed by BLAST at web servers of USA National Center for Biotechnology Information. Homology comparison was performed using the ClustalW1.8 program. The prediction of glycosylation site, and phosphorylation site was done using software at the ExPASy Molecular Biology Server (http://expasy.pku.edu.cn).

2.4. RT-PCR analysis

Total RNAs were isolated by SV total RNA Isolation System (Promega) from brain, heart, kidney, liver, ovary, spleen, muscle and testis of 1-year-old gibel carp, and from different embryos at developmental stages including mature eggs, 5 min after fertilization, multi-cell stage, blastula, gastrula, neurula, tail bud, cardiopalmsus, hatching larvae and larvae. Aliquots were subjected to 1% agarose gel electrophoresis and stained by ethidium bromide to verify the quantity and quality of RNA. For RT-PCR detection, about 1 µg of the different RNAs were reverse-transcribed with MMLV (Gibco) at 37 °C with oligo(dT) primer, and α-tubulin was used to adjust the concentration of the reverse-transcribed first stand cDNAs. Then the well adjusted first strand cDNAs were used as templates to amplify the specific fragment by PCR with the gene-specific primers. The condition of enzymatic amplification was established for 35 cycles involving an initial denaturation step at 94 °C for 3 min, then each cycle performed at 94 °C 30 s, 58 °C 45 s and 72 °C for 1 min. As a negative control, water was used instead of cDNAs for the RT-PCR to exclude any contamination from buffers and tubes. In order to exclude the contamination of genomic DNA, the genomic DNA was used as template for the control because there were different sizes of the amplified DNA fragments from cDNA and genomic DNA. Aliquots (5 ul) of the PCR reaction products were separated by electrophoresis on 1% agarose gel containing ethidium bromide.

2.5. Expression of CNBP protein and preparation of polyclonal antibody

The cDNA fragment coding for CNBP protein (163 aa) was subcloned between the EcoR I and Xho I sites of pET-32α expression vector. A about 28 KD soluble His fusion protein was expressed in BL21 (DE3) and purified by affinity chromatography (Novogene) following the manufacturer’s instructions. The purified protein was used to prepare poly-antiserum by immunizing white rabbit described as previously (Dong et al., 2004).

2.6. Western blot analysis

Western blot analysis was performed as described previously (Dong et al., 2004). In brief, tissue extracts were respectively prepared from equal amount tissues (50 mg each tissue in 3 ml extraction buffer) of liver, spleen, kidney, heart, brain, muscle, ovary and testis. Egg and embryo extracts were prepared from equal amount (100 embryos/1 ml extraction buffer) of eggs and embryos at different developmental stages including mature eggs, 5 min after fertilization, multi-cell stage, blastula, gastrula, neurula, tail bud, cardiopalmsus, hatching larvae and larvae. For Western blot detection, the equal amount (10 µl/each sample) of extracts were loaded from each sample and separated on 15% SDS-PAGE gel. To further adjust the equal amount of proteins from the samples of oocytes, eggs and embryos, the running gels were generally stained with Coomassie brilliant blue R-250 before using for Western blot detection. Then the well adjusted extracts were separated on the SDS-PAGE gels, and then were electrophoretically blotted to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TTBS buffer (100 mM NaCl, 100 mM Tris–Cl, 0.05% Triton, pH 7.5). Blocked membrane was incubated with the rabbit antiserum at a dilution of 1:200 in TTBS buffer containing 1.0% milk at room temperature for 1 h. The membrane was washed 5 times for 10 min each in TBS buffer and then incubated with 1:2000 diluted alkaline phosphatase conjugated goat anti-rabbit IgG (Sino–American). After four washes of 10 min each in TBS buffer, detection was performed using BCIP/NBT staining.

2.7. Immunofluorescence localization

The ovaries at different stages, including vitellogenic stage ovaries in November and post-ovulation ovaries from June to August, and the testes in February, were freshly fixed by 4% paraformaldehyde in PBS (pH 7.0) at 4 °C overnight. After washing with PBS (pH 7.0) three times, the samples were immersed in 30% saccharose-PBS buffer overnight at 4 °C. They were then embedded in O.C.T. (Optimal Cutting Temperature, Germany), and sectioned at 7 µm in thickness with frozen microtomy (Leica). The cryostat sections were rehydrated in PBS for 30 min, and incubated for 1 h with 5% dry milk in PBS at room temperature to prevent non-specific binding of antibodies. The sections were then incubated with the rabbit antiserum (1:100 dilution) for about 20 h at 4 °C, washed five times with PBST (10 min each), subsequently incubated for 1 h with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (goat anti-rabbit IgG, 1:100 dilution, Zhongshan) in the dark and washed five times with PBS (10 min each). Then, the sections were stained by PI for 5 min and washed for four times (5 min each). Finally, the sections were observed with a Leica confocal fluorescence micro-
scope. Control sections were treated with pre-immune serum as primary antibody.

To distinguish different cells within the ovarian cysts simultaneously, primordial germ cells were visualized using the CagVasa antibody prepared by our laboratory (Xu et al., 2005). Double immunolabeling was performed by the CagVasa antibody with red fluorescence (TRITC) to CagVasa in combination with the CagCNBP antibody to CagCNBP with green fluorescence (FITC) to show localization of CagVasa and CagCNBP at the same time described as above.

3. Results and discussion

3.1. Cloning and characterization of Carassius auratus gibelio CNBP cDNA

To reveal differentially expressed genes at gastrula stage relative to mature eggs, a total of 1500 clones from the gastrula Smart cDNA library were screened with two probes respectively prepared from gastrula first-strand cDNAs and egg first-strand cDNAs. As shown previously, 130 positive clones specific to gastrula embryos were obtained (Liu et al.,

Fig. 1. Amino acid alignment of 8 complete CNBP polypeptides from different vertebrates, such as Carassius auratus gibelio (Cag) (GenBank accession AY773183), Danio rerio (Dan) (GenBank accession AY228240.1), Bufo arenarum (Buf) (GenBank accession AF144698), Rattus norvegicus (Rat) (GenBank accession AF242550), Mus musculus (Mus) (GenBank accession AK075760.1), Xenopus laevis (Xen) (GenBank accession XLU20977), Gallus gallus (Gal) (GenBank accession AF004942), and Homo sapiens (Hom) (GenBank accession NM003418.1). Seven repetitive 14-amino acid sequences of the zinc finger motif are underlined. Potential serine and threonine phosphorylation sites (italic) and O-GlcNAc sites (shadow) in CagCNBP are indicated.
Sequencing analysis and database searches revealed a positive clone of the cellular nucleic acid-binding protein (CNBP) gene. The CagCNBP full-length cDNA is 1402 bp, and has an ORF of 489 bp for encoding a peptide of 163 aa. It has a 100 bp of 5′-untranslated region (UTR) and a 813 bp of 3′UTR including a typical polyadenylation signal sequence AATAAA and one 23 bp of poly(A)+tail. N-linked glycosylation site analysis by NetNGlyc 1.0 didn’t find any potential N-glycosylation site, but YinOYang 1.2 showed three potential O-glycosylation sites (Ser 4, Ser 137, and Thr 158). NetPhos 2.0 analysis revealed five potential phosphorylation sites (Ser6, Ser70, Ser83, Thr56 and Thr158). Seven repetitive zinc finger motives of 14-amino acids were observed in the deduced polypeptide sequence (Fig. 1).

Homology searches revealed highly evolutionary conservation among the vertebrate CNBP homologues. As shown in Fig. 1, the amino acid identities between the CagCNBP and others range from 68.5 to 96.8%. The highest identity exists between the CagCNBP and zebrafish.

Fig. 2. Expression characterization of CagCNBP during embryogenesis revealed by RT-PCR (A) and Western Blot (B). Ubiquitous expression of β-actin was used as the RT-PCR control (A, top). E1 to E10 respectively indicate 10 samples from mature eggs (E1), fertilized eggs (E2), multi-cell stage embryos (E3), blastula embryos (E4), gastrula embryos (E5), neurula embryos (E6), tail bud embryos (E7), heartbeat embryos (E8), hatching embryos (E9), and hatched larvae. The amplified fragment of CagCNBP and the western-blot band of CagCNBP protein are indicated by arrows. The equal amount of proteins loaded in each lane for Western blot detection (bottom) were adjusted by the Coomassie brilliant blue R-250 staining (B, top).

Fig. 3. RT-PCR detection of CagCNBP transcripts (A) and Western blot detection of CagCNBP expression (B) in different adult tissues, including Ov—ovary, Te—testis, Br—brain, Mu—muscle, Ki—kidney, Sp—spleen, Li—liver, and He—heart. The genomic DNA (G) was used to exclude the contamination of genomic DNA because there were different sizes of the amplified DNA fragments from the cDNA and genomic DNA. Ubiquitous expression of β-actin was used as the control (A, top). CagCNBP transcripts (A) and CagCNBP (B) are indicated by arrows. The protein samples were adjusted to equal amount (data not shown) before using for Western blot detection as described in Materials and methods.
Danio rerio) CNBP, and the lowest identity is with that of Gallus gallus.

3.2. Predominant expression of CagCNBP in ovaries

CagCNBP is ubiquitously distributed during embryogenesis, as shown in Fig. 2. Before fertilization, maternal CagCNBP mRNA and protein existed in the mature eggs. When the embryos developed to gastrula stage, new zygotic CagCNBP mRNA began to transcribe in the embryos, and to keep the high level from gastrula to the hatched larvae stage (Fig. 2A). This may be the reason why the CagCNBP cDNA was screened from the gastrula Smart cDNA library. Furthermore, Western blot analysis showed similar expression content changes during embryogenesis. Obvious differences were that the CagCNBP content decreased to the lowest level at gastrula and neurula stages, and the zygotic CagCNBP protein was expressed from tail bud stage (Fig. 2B).

Tissue distribution of CagCNBP transcripts and CagCNBP protein was further examined by RT-PCR and Western blot analysis. RT-PCR analysis was carried out using total RNAs from adult tissues including brain, heart, kidney, liver, ovary, spleen, muscle and testis. As shown in Fig. 3A, the CagCNBP is predominantly expressed in ovary,
testis and spleen, and lower levels of transcription are also detected in heart, kidney, liver, brain and muscle. Following this observation, the same tissues were also subjected to Western blot detection. As shown in Fig. 3B, one positive protein band of about 25 KD was predominantly detected in ovary, testis and spleen, whereas the CagCNBP protein was hardly detected in other tissues, including heart, kidney, liver, brain and muscle. The data are consistent with previous observation in zebrafish that CNBP is predominantly expressed in ovary (Armas et al., 2004), and predominant expression is also observed for the testis.

3.3. Differential expression of CagCNBP at different stage ovaries and follicles

In the above results, we have observed predominant expression of CagCNBP in adult fish ovary, but its expression characterization and cellular distribution during oogenesis have not been uncovered. At first, different stage ovaries were further subjected to Western blot analysis, and an unexpected phenomenon was revealed that the CagCNBP protein is highly expressed not only in vitellogenic stage ovaries but also in post-ovulation ovaries. As shown in Fig. 4A, the CagCNBP content in the post-ovulation ovaries is higher than that in the ovaries from mature fish in the April spawning season. In the growing season from August to September, the protein content decreases in ovaries. However, when gibel carp enter the vitellogenic stage in November no matter whether they are adult individuals after spawning or young fish propagated from this year, the protein content increases again. Moreover, four kinds of follicles containing different oocytes, such as vitellogenic oocytes, fully grown oocytes, degenerating oocytes, and atretic oocytes, were isolated from the ovaries after spawning in June, and were also subjected to Western blot analysis. As shown in Fig. 4B, the CNBP protein band about 25 kDa is detected in the all oocyte extracts, but its content in degenerating oocytes and atretic oocytes is obviously higher than that in vitellogenic oocytes and fully grown oocytes. This suggests that CagCNBP expression should increase during oocyte degeneration after spawning.

3.4. Differential distribution of CagCNBP in different stage follicles and oocytes

The CagCNBP antibody prepared from the expressed protein was able to trace localization and distribution of CagCNBP in gibel carp ovaries. Firstly, the gibel carp ovaries in November were used for immunofluorescence observation, because vitellogenesis had started, and different stage oocytes and ovarian follicles existed simultaneously in the ovaries. Strongly immunoreactive signals were revealed in different stage follicles and oocytes. As shown in Fig. 5A, the positive CagCNBP signals are widely distributed over cytoplasm, nucleus and follicular epithelium in previtellogenic and vitellogenic oocytes, whereas they are mainly distributed over follicular epithelium in fully grown oocytes. Interestingly, strong immunoreactive signals were also observed in some cell clusters.

Double staining of PI for nucleus and immunofluorescence for CagCNBP further revealed the differential distribution and developmental behavior of CagCNBP during oogenesis. As shown in Fig. 5C, the CagCNBP is mainly localized within the nucleus in previtellogenic oocytes. When vitellogenesis initiates, the CagCNBP signals are widely distributed over cytoplasm, nucleus and follicular epithelium, but some stronger signals still appear at nucleolus-like granules, and distribute at the nuclear periphery. In fully grown oocytes, intensive immunofluorescence rings were observed around them, because the CagCNBP signals strongly appeared on follicular epithelium.

Fig. 6. Distribution changes of CagCNBP in degenerating and atretic follicles in post-ovulation ovaries. (A) shows a degenerating follicle with intensive immunofluorescence ring between oocyte zona radiata and follicular epithelium stained by CagCNBP antibody. (B) shows the atretic follicle in which most of the CagCNBP fluorescence particles invade into atretic oocyte. And, some cell clusters extended from the follicular epithelium are indicated by white arrows.
3.5. Intensive expression and developmental behaviour of CagCNBP on follicular epithelium in degenerating and atretic follicles

In post-ovulation ovaries, significant distribution changes of CagCNBP occur in degenerating and atretic follicles. As shown in Fig. 6, the CagCNBP signals on follicular epithelium become stronger and stronger in some degenerating follicles, and form an intensive immunofluorescence ring around the degenerating oocyte (Fig. 6A). As the oocyte degeneration progresses, the fluorescence ring gradually shrinks, and become a lot of particles. Then, most of the fluorescence particles invade into atretic oocytes, and some form cell clusters with strong immunofluorescence in the atretic oocytes (Fig. 6B).

Fig. 7. Detail cell structure and change processes of the CagCNBP immunofluorescence ring and particles on follicular epithelium detected by double staining of PI for nucleus and immunofluorescence for CagCNBP in some typical atretic follicles and oocytes. A1, B1, and C1 respectively show the green fluorescence immunostained by CagCNBP antibody. A2, B2, and C2 respectively show the corresponding results stained by PI. A3, B3, and C3 show the corresponding overlaps of the CagCNBP green fluorescence and nucleus red fluorescence. Fe—follicle epithelium, indicated by yellow arrowheads, IC—invaded cells, indicated by white arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Double staining of PI and the CagCNBP antibody revealed detail change process and developmental behaviour in the CagCNBP fluorescence ring and particles on follicular epithelium. As shown in Fig. 7, the follicular epithelium is firstly destroyed in the degenerating follicle. Some granulosa cells invade into the oocyte following Zona radiata breakdown even if the CagCNBP fluorescence ring exists. And the invaded cells still produced strong CagCNBP fluorescence despite some has broken (Fig. 7A). In some atretic oocytes, the invaded cells become more and extensive, and most of them become cellular debris (Fig. 7B). Recently, Kamo et al. (2004) characterized invasive granulosa cells found between zona pellucida and oocyte during follicular atresia in mice, and suggested that the invasive granulosa cells might have a macrophage-like cell function for the elimination of oocytes from atretic follicles. The intensive expression of CagCNBP on follicular epithelium in degenerating follicles implicates that CagCNBP might be involved in follicle and oocyte degeneration, and related to ovarian cell apoptosis.

More interestingly, even though most of the invaded cells die in the atretic oocytes, some of them survive and proliferate. And, new cell clusters and ovarian cysts are produced from the proliferated cells, and high amount of CagCNBP still associates with the regenerating cell clusters (Fig. 7C).

3.6. CagCNBP distribution within ovarian cyst and association with cyst and follicle formation

Follicle formation and oogenesis initiate in ovarian cyst that is composed of primordial germ cell and pre-follicular somatic cell clusters (Matova and Cooley, 2001). One of the important findings in this study is that the CagCNBP is intensively expressed in cell clusters within ovarian cysts no matter whether the ovarian cysts produce from original gonad cell clusters or from the regenerating cell clusters. As shown in Fig. 8, numerous cells in primitive ovarian cyst appear to be formed by incomplete cytokinesis, and seem to be interconnected by intercellular bridges that may be mainly composed of the CagCNBP because they are intensively immunostained by the CagCNBP antibody (Fig. 8A). As the ovarian cysts develop, the cells within the cysts seem to differentiate into two kinds of cells. CagCNBP expresses in the major kind of cells, because most of cells could be immunostained by the CagCNBP antibody, and co-localized with PI staining. The minor kind of cells was not recognized by the CagCNBP antibody (Fig. 8B). The data implicate that CagCNBP might be also involved ovarian cyst formation.

To further distinguish the two kinds of cells within the ovarian cysts, double immunolabeling was performed using the CagVasa antibody (as primordial germ cell marker, Xu

![Fig. 8. Distribution and cell localization of CagCNBP in primitive ovarian cyst (A) and in the cyst that are composed of major somatic cells and minor germ cells (B). A1 and B1 respectively show the green fluorescence immunostained by CagCNBP antibody. A2 and B2 respectively show the corresponding results stained by PI. A3 and B3 show the corresponding overlaps of the CagCNBP green fluorescence and nucleus red fluorescence. Cc—cell cluster, Fe—follicular epithelium. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image)
et al., 2005) with red fluorescence (TRITC) in combination with the CagCNBP antibody to CagCNBP with green fluorescence (FITC) to show localization of CagVasa and CagCNBP at the same time. As shown in Fig. 9, the CagVasa antibody is able to recognize the minor kind of cells, and the recognized cells are larger in size than that immunostained by the CagCNBP antibody. Vasa, as a molecular marker of promordial germ cells, has been found conservatively in the germline of a wide variety of animals from invertebrates to mammals, and has been identified as a regulator during germ cell specification, germ cell specificity maintenance and gamete formation (Tanaka et al., 2000; Raz, 2003). Its RNA and protein have been always used to reveal the germ line cells, such as oogonia. Obviously, the positive cells recognized by CagVasa antibody in this study should be primordial germ cells distributed in the ovarian cysts, and will form oogonia and oocytes, whereas the major kind of cells immunostained by the CagCNBP antibody should be pre-follicular somatic cells, and they will develop into granulosa cells and theca cells.

3.7. Differential expression and developmental behaviour of CagCNBP during oogenesis

In vertebrates, cyst and follicle formation are complicated somatic cell-oocyte interaction event. Through cyst and follicle formation, germ cells and their surrounding somatic cells establish an intricate system of mutual interactions that ultimately lead to the acquisition of their respective competences (Cecconi et al., 2004). Previous studies have demonstrated that granulosa cells allow the transfer of about 85% of the oocyte metabolic needs and modulate oocyte transcriptional activity (De La Fuente and Eppig, 2001). In this study, we confirmed a predominant expression of CNBP gene and protein in fish ovary, and observed the cell-specific expression during oogenesis. As summarized in Table 1, the precursors of follicular somatic cells in ovarian cysts and granulosa and theca cells in fully grown follicles and regenerating ovarian cysts are characterised by stronger CNBP expression than other stage somatic and germ cells. However, strong CNBP expression is also observed in oocytes of previtellogenic and vitellogenic follicles, but the CNBP positive signals is hardly observed within the fully grown oocytes and mature eggs. The expression pattern might be related to follicle formation and oocyte growth, because in early stages of follicle formation, numerous cells are produced in ovarian cysts by incomplete cytokinesis, while in the stages of previtellogenic and vitellogenic follicles, intensive RNA transcription and protein synthesis activities occur in the growing oocytes. CNBP has been demonstrated to have high affinity for single stranded nucleic acids and binding to mRNA regulatory regions, and therefore, the expression difference in somatic and germ cells of different development stages is associated with the transcriptional and

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translational activity discrepancy. Additionally, the reason why CagCNBP signals is hardly observed within the fully grown oocytes and mature eggs may be because its signals in follicular cells are so intensively that the weak CNBP signals within the germ cells could not be observed by the immunofluorescence microscope, although Western blot detection has indicated that it is a maternal transfer protein.

Gibel carp CNBP is highly conserved through the animal kingdom, with particular sequence similarity to zebrafish. The high level of conservation implicates that CNBP may play an essential biological roles across different species throughout evolution (Shimizua et al., 2003). Recently, Armas et al. (2004) have observed the highest level of CNBP transcripts in adult zebrafish ovary, but little is known about its functional significance during oogenesis. In the current study, we demonstrate its predominant expression in gibel carp ovary not only by RT-PCR but also by Western blot. Based on the expression characterization, we further analyze its expression patterns and developmental behaviour during oogenesis. Following these studies, we reveal an unexpected discovery that the CagCNBP is associated with follicular cells and oocytes, and significant distribution changes have occurred in degenerating and regenerating follicles. More interestingly, the CagCNBP is more highly expressed in some clusters of interconnected cells within ovarian cysts, no matter whether the cell clusters are formed from the original primordial germ cells or from the newly formed cells from follicular cells that invaded into the atretic oocytes. All of the results suggest that CNBP should play crucial roles during oogenesis because its high expression is associated with high transcriptional and translational activities in follicular cells and oocytes.

In this study, the predominant CNBP expression is found not only in ovary but also in testis. In order to understand the significance, we have observed the expression and developmental behaviour in testis. Preliminary data indicate that the CagCNBP signals distribute mainly on somatic cells and some stage-specific germ cells, such as spermatogonia, primary spermatocytes and secondary spermatocytes, whereas no signals are observed on spermatids and sperms (data not shown). This implicates that the stage-specific and cell-specific expression pattern in testis is similar to that in ovary. Therefore, further studies on CNBP expression pattern and developmental behaviour will be of particular interests for understanding functional roles of CNBP during oogenesis and spermatogenesis.

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