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Molecular characterization and expression pattern of AFPIV during embryogenesis in gibel carp(*Carassiu auratus gibelio*)

Jing-Xia Liu · Yan-Hua Zhai · Jian-Fang Gui

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Abstract As a new type of AFPs, *AFPIV* has been firstly identified in longhorn sculpin (*Myoxocephalus octodecimspinosus*), and in recent years, its cDNA and amino acid sequence have been reported, and its pancreatic synthesis has been firstly reported in polar fish. However, its expression patterns during fish embryogenesis have not been elucidated yet. By differential screening, we cloned the *CagAFPIV* in gibel carp, *Carassius auratus gibelio*, demonstrated its predominant expression during embryogenesis. RT-PCR detection revealed that *CagAFPIV* was first transcribed from blastula stage and kept a high level during embryogenesis and declined remarkably in hatched larva. In situ hybridization revealed that *CagAFPIV* transcripts were firstly distributed over the margin and marginal blastomere in blastula stage embryos, at the early-gastrula stage the positive signals distributed in the marginal cells and the internalization cells, and later restricted to the cells the yolk syncytial layer (YSL) from later gastrula stage to larva stage. Consistently, the *CagAFPIV* protein also kept a high level during embryogenesis, and the high protein level retained some days after the larva hatched. Our work, for the first time, revealed the dynamic expression and distribution of *CagAFPIV* during embryogenesis.

Keywords AFPIV · Embryogenesis · Gibel carp

Abbreviations

AFPIV	Antifreeze type IV protein
CagAFPIV	*Carassius auratus gibelio* antifreeze type IV protein
CagApo-14	*Carassius auratus gibelio Apo-14*
Cagfetuin-B	*Carassius auratus gibelio fetuin-B*
nt	Nucleotide
ORF	Open reading frame
UTR	Untranslated region
SMART	Switch mechanism at the 5′ end of RNA templates
PBS	Phosphate saline buffer
FITC	Fluorescein isothiocyanate
HE	Hematoxylin and Eosin

Introduction

Numerous species of marine polar fish synthesize antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs), as a defense to protect living organisms by freezing temperatures and protecting cell membranes from cold-induced damage. To data, four distinct classes of AFPs have been isolated and identified from fish, and were, respectively, named types I–IV based on their structural features. Type I AFPs have been isolated from righteye flounders (*Pleuronectidae*), sculpins (*Myoxocephalus scorpius*), winter flounder (*Pseudopleuronectes americanus)* and Atlantic (*Liparis atlanticus*) and dusky (*Liparis*

The nucleotide sequence data reported in this paper have been submitted to the GenBank under accession number: *CagAFPIV* (**AY365004**).

J.-X. Liu · Y.-H. Zhai · J.-F. Gui (✉)
State Key Laboratory of Freshwater Ecology and Biotechnology, Wuhan Center for Developmental Biology, Institute of Hydrobiology, Chinese Academy of Sciences, 430072 Wuhan, China
e-mail: jfgui@ihb.ac.cn

J.-X. Liu
Group of Molecular Toxicology, Institute of Hydrobiology, Chinese Academy of Sciences,
430072 Wuhan, China

gibbus) snailfish, characterized with α-helices and alanine-rich [1–4]; type II AFPs in sea raven, smelts and herring, characterized by disulfide bridges and an extensive β-structure [5, 6]; type III AFPs are compact β-stranded structure and globular, and isolated from eelpouts and wolffishes [5, 6]; and the type IV AFPs, a new kind of fish antifreeze identified from longhorn sculpin (*Myoxocephalus octodecimspinosus*), characterized by four-helix bundle [7–9].

In recently, the reports related to AFPs and AFGPs all focus on their antifreeze activity and lower solution freezing point characterization [10, 11], or reveal their synthesis sites, and at least three different tissues, including liver, skin and pancreatic, have been identified secreting AFPs or AFGPs [4, 11–13]. But no report has been found regarding the AFPs or AFGPs expression characterization during embryogenesis.

Gibel carp (*Carassius auratus gibelio*), an unique triploid species with dual reproductive modes of gynogenesis and bisexual reproduction [14, 15], has been used as a promising research model for developmental biology and evolutionary mechanisms [16–18]. And some important genes involved in embryogenesis, such as *CagCNBP*, *Cagfetuin-B*, *CagApo-14* and *CagMdkb* have been identified in the model system [19–22]. In this study, *CagAFPIV* was cloned from gibel carp. Based on the characterization analysis and antibody preparation, we demonstrated its predominant expression during embryogenesis by RT-PCR and western blot detection, and its transcripts and protein distribution during embryogenesis was also revealed in this paper.

Materials and methods

Full-length cDNA cloning and sequence analysis

The positive clones containing *AFPIV* full-length cDNA were screened from the gastrula SMART cDNA library as described previously [23]. Homology comparison was performed using the ClustalW1.8 program.

RT-PCR detection

Total RNAs were isolated by SV total RNA Isolation System (Promega) from embryos at different developmental stages. RT-PCR detection was performed as reported previously [19]. Briefly, aliquots of RNA were subjected to 1% agarose gel electrophoresis and stained by ethidium bromide to verify the quantity and quality of RNA firstly. For RT-PCR detection, about 1 μg of the different RNAs were reverse-transcribed with MMLV (Gibco) at 37°C with oligo(dT) primers, and α-tublin 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. Aliquots (5 μl) of the PCR reaction products were separated by electrophoresis on 1% agarose gel containing ethidum brimide.

Riboprobes synthesis and in situ hybridization

Embryos were fixed in 4%(w/v) paraformaldehyde according to a previous report [24]. Antisense or sense digoxigenin-UTP labeled RNA probes were synthesized using T7 or Sp6 polymerase by in vitro transcription (DIG RNA labeling kit; Roche Molecular Biochemicals). Briefly, the specific fragment of the gene was amplified by PCR and cloned to pEGM-T vector. After DNA sequencing, the vector with correct gene fragment was linearized with appropriate enzyme. The linearized DNA was then used for labeling the Antisense or sense RNA probes. The procedure of whole-mount in situ hybridization (WISH) was performed as described previously [24]. In brief, the embryos were fixed in 4%(w/v) paraformaldehyde overnight at 4°C, after rinsed with PBS for several times, the embryos were transferred into 100% Methanol and stored at −20°C until use. For in situ hybridization, the embryos were permeabilized with proteinase K firstly and then prehybridized in Hyb + (50% Formamide, 5× SSC, 0,1% Tween 20, 50 μg/ml Heparin, 500 μg/ml tRNA) for 3 h at 65°C. Then incubate with Hyb + containing ∼100 ng/ml of antisense DIG RNA probe overnight at 65°C. Washed the embryos for several times with different concentration SSC buffer after hybridization and then for detection with alkaline phosphatase conjugated antibody, and staining the embryos at RT in the dark with BCIP/NBT staining kit finally. Whole mounts were photographed and processed for paraffin section. And the paraffin sections were directly observed.

Western blot detection

GST-fetuin-B fusion protein was expressed in BL21 (DE3) and purified by a FPLC procedure using glutathione-Sepharose 4B columns (Amersham Biosciences) according to the manufacturer's instructions. The purified protein was used to prepare poly-antiserum by immunizing white rabbit as described previously [17]. Western blot detection

was performed as described previously [17, 19]. In brief, 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, fertilized eggs, multi-cell stage embryos, morula embryos, blastula embryos, gastrula embryos, neurula embryos, tail bud embryos, heartbeat embryos, hatching embryos and hatched larva. 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 by 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 1:2,000 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.

Immunofluorescence localization

Two day larva was fixed with 4% paraformaldehade in PBS at 4°C overnight. Immunofluorescence localization was performed according to previous report [19]. In briefly, the larva was freshly fixed by 4% paraformaldehyde in PBS (pH 7.0) at 4°C overnight. After washing with PBS (pH 7.0) 3 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 isothiolyanate (FITC)-conjugated secondary antibody (goat anti-rabbit IgG, 1:100 dilution, Zhongshan) in the dark and washed 5 times with PBS (10 min each). Finally, the sections were observed with a Leica confocal fluorescence microscope. Control sections were treated with pre-immune serum as primary antibody. After photographed, the sections were staining with HE and then were observed.

Results

Molecular characterization of *CagAFPIV*

Full-length cDNA of *CagAFPIV* is 641 bp, and has an ORF of 390 bp for encoding a peptide of 130aa with a 16 amino acid signal-peptide. It has a 64 bp of 5′-untranslated region (UTR) and a 184 bp 3′-untranslated region (UTR) including a typical polyadenylation signal sequence AATAAA and one 30 bp of poly(A) + tail(Fig. 1a).

Homology searches revealed evolutionary conservation among the three fish *AFPIV* homologues in Fig. 1b. The high identity exists between the *CagAFPIV* and zebrafish (*Danio rerio*) *AFPIV*, nearly reaches 83%, but low identity exists between the *CagAFPIV* and longhorn sculpin, only reaches 33%. Although gibel carp *AFPIV* with low identity compare to longhorn sculpin *AFPIV*, on the phylogenetic tree, gibel carp *AFPIV* is clustered together with *AFPIV* but not *AFP I- III* (Fig. 1c), suggesting it appears to be a gibel carp *AFPIV* homolog.

Expression pattern of *CagAFPIV* during embryogenesis

Mature eggs, fertilized eggs and embryos and larva at different developmental stages were subjected to RT-PCR and Western blot detection. As shown in Fig. 2a, the *CagAFPIV* began to transcribe in the embryos at blastula stage, and the transcription rapidly reached high level at neurula stage and kept the high level from neurula stage to the hatching stage, but declined remarkably in hatched larva. Consistently, western blot detection revealed that *CagAFPIV* protein could be firstly detected in blastula stage, appeared a weak full-length protein band with signal-peptide. In gastrula embryos, the protein firstly produced 2 weak protein bands, the secreted form which signal-peptide was removed had been detected. Following this stage, its content reached a high level and kept the high level to the hatched larva, and we still detected two protein bands in new-hatched larva. But in the 3 day larva, the protein only appeared the secreted form, and the secreted form retained in larva till 13 day and disappeared in 15 day larvae (Fig. 2b).

To make further investigation into *CagAFPIV* expression pattern during embryogenesis, whole-mount in situ hybridization was also performed. As shown in Fig. 3, *CagAFPIV* transcripts was firstly detected by this technique at blastula stage in a small group of cells at the margin and marginal blastomere of the blastoderm (Fig. 3Aa), and in the corresponding sections of the hybridized blastula embryos, some cells of hypoblast also displayed purple staining (Fig. 3Ba, b). As epiboly and internalization started, the expression domain of *CagAFPIV* extended over the whole circumference of the margin of the blastoderm,

A

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1    GACCACCACAACATCAGGTCTAAAAACCCCTGGATACAAGGCAAGTCTTACTCAA
56   CCTGCAACAATGAAATTCTCTCTCATCACCATCCTTGTTGTTGCTCTGGCCATTGGCTCT
1                M  K  F  S  L  I  T  I  L  V  V  A  L  A  I  G  S
116  GAATCAGTATCTCTGGTCAAGAGAGATGCTCCTGCTGAGCTGGAGAAGATCACCAAG
18     E  S  V  S  L  V  K  R  D  A  P  A  E  L  E  K  I  T  K
173  TATTTTCAGGATCTTGTGGACAATCTAAAGCACGTCGAGGGCCCTGAGCTGGTCAGC
37     Y  F  Q  D  L  V  D  N  L  K  H  V  E  G  P  E  L  V  S
230  AAGGCCAACGCTTACTTTGAGCAGAGCAGAGCCCAGTTCCAGCCCATGGTTGAGAAG
56     K  A  N  A  Y  F  E  Q  S  R  A  Q  F  Q  P  M  V  E  K
287  CTCCAGGAGCAGCTGAAGCCCCTCTCCAGCAACATTGAAGACCACATCAAGCCTCTG
75     L  Q  E  Q  L  K  P  L  S  S  N  I  E  D  H  I  K  P  L
344  GCCGCCTCCGTCCAGGCTCAGGTCGCCCCCCTGGCCAGCATGATCCAGACTCATGTT
94     A  A  S  V  Q  A  Q  V  A  P  L  A  S  M  I  Q  T  H  V
401  GAAGATGTCCTCAAGTTTGTGGCTGACAAGAGTAAAGCCATCCTGCCTCCCCAGTAA
113    E  D  V  L  K  F  V  A  D  K  S  K  A  I  L  P  P  Q  ✦
458  AACCCACAGGAATAACCATTTCTAGTTTTCCTCTTCCATTTTGTTCTTTAAATTAATGTG
518  TGTTTATATAATAATAAAAGTGATTTAAAGGTCAGTGTTTTTCCTTTGCACATCTCATAG
578  TGAAACAGTGGAGAAACCT AATAA AAATGCATTCAAAAAAAAAAAAAAAAAAAAA
634  AAAAAAAA
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B

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MKFSLIT-ILVVALAIGSESVSLVKRDAPAELEKITKYFQDLVDNLKH-----VEGPELV
MKFSLIA-VIVVALAIGSESASLVKRDAPAELDKIAKYFQDLVDNLKN-----VEGAELA
MKFSLVATIVLLALAQGS------FAQGAADLESLGQYFEEMKTKLIQDMTEIIRSQDLA
*****:: ::::*** **       :..*:*:.: :**:::  :* :    :.. :*.

SKANAYFEQSRAQFQPMVEKLQEQLKPLSSNIEDHIKPLAASVQAQVAPLASMIQTHVED
NKANAYLEQSRAQFQPMVEKLQEQLKPFSGNIEEQIKPLAASVQSQVAPLAGMVQTHVED
NQAQAFVEDKKTQLQPLVAQIQEQMKTVATNVEEQIRPLTANVQAHLQPQIDNFQKQMEA
.:*:*:.*:.::*:**:* ::***:*..: *:*::*:**:*.**::: *  . .*.::*

VLKFVADKSKAILPPQ     gibel carp          100%
MIKFVADQAKAMLPPQ     zebrafish           82.3%
IIKKLTDQTMAIEN--     longhorn sculpin    33.1%
::* ::*:: *:
```

C

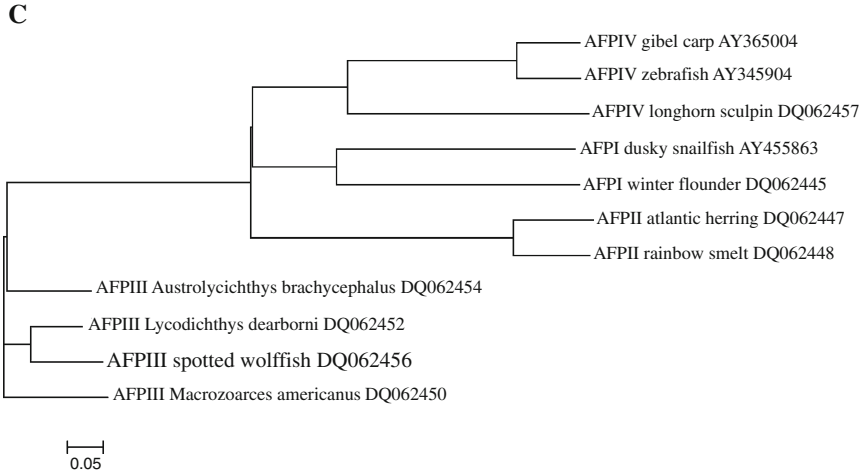

Fig. 1 **a** Nucleotide and deduced amino acid sequences of full-length cDNA of *CagAFPIV* cloned from gibel carp. The *asterisk* indicates the stop codon, and putative polyadenylation signal is in *bold*. **b** Amino acid sequence alignment of 3 *AFPIV* proteins from different fish including gibel carp (*Carassius auratus gibelio*), zebrafish (*Danio rerio*) and longhorn sculpin (*Myoxocephalus octodecemspinosus*). The signal peptides are boxed. **c** Phylogenetic tree of AFP family proteins, showing that *CagAFPIV* is clustered with its type IV antifreeze protein homologs

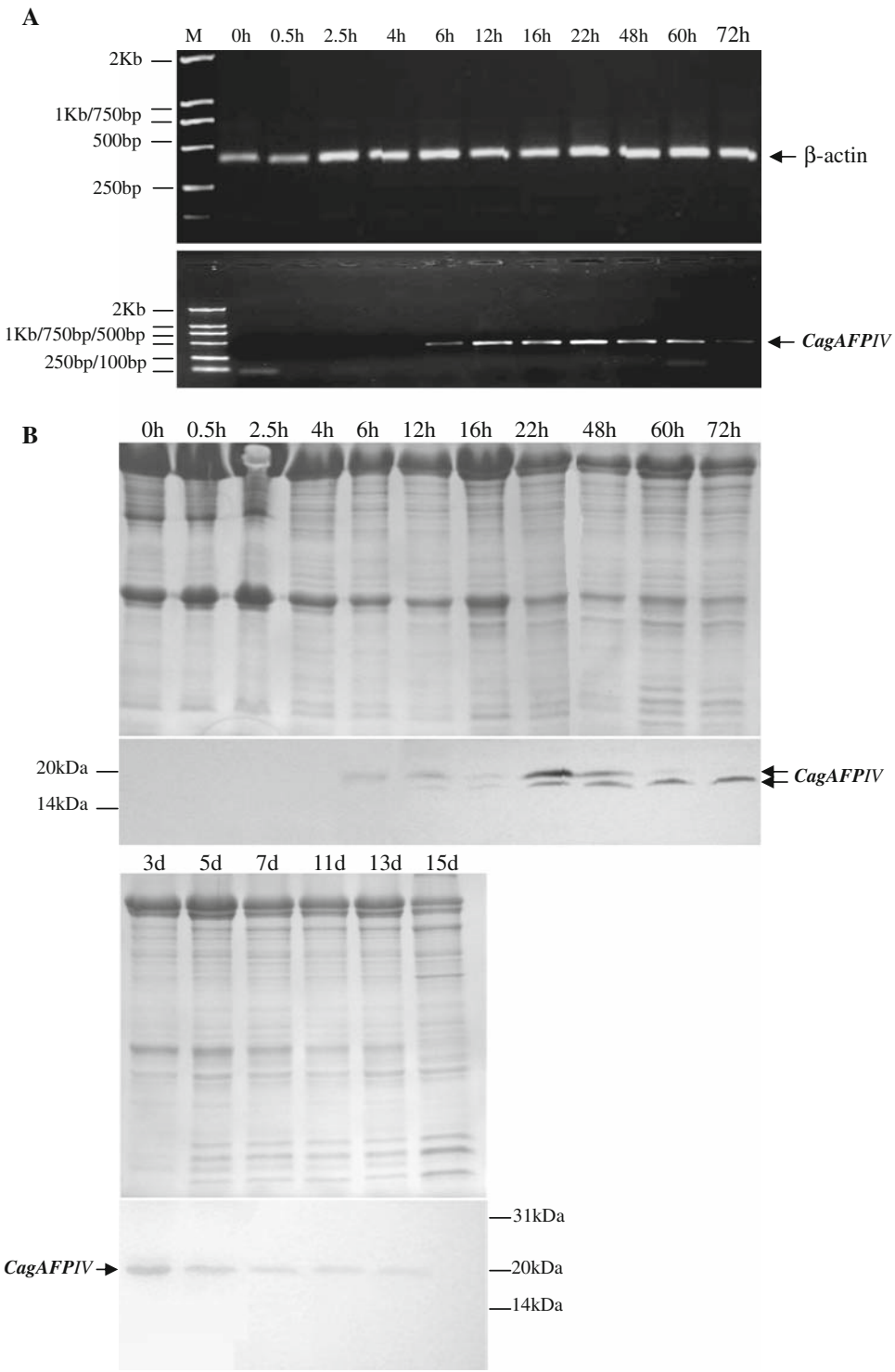

Fig. 2 **a** RT-PCR detection of *CagAFPIV* expression during embryogenesis (A, *down*). Ubiquitous expression of *β*-actin was used as the RT-PCR control (A, *upper*). 0–72 h define the hours post fertilization, respectively, indicates 11 samples from mature eggs (0 h), fertilized eggs (0.5 h), multi-cell stage embryos (2.5 h), morula embryos (4 h), blastula embryos (6 h), gastrula embryos (12 h), neurula embryos (16 h), tail bud embryos (22 h), heartbeat embryos (48 h), hatching embryos (60 h) and hatched larva (72 h). **b** Western blot detection of *CagAFPIV* expression during embryogenesis and in larva. 0–72 h define the hours post fertilization, respectively, indicate 11 samples from mature eggs (0 h), fertilized eggs (0.5 h), multi-cell stage embryos (2.5 h), morula embryos (4 h), blastula embryos(6 h), gastrula embryos (12 h), neurula embryos (16 h), tail bud embryos (22 h), heartbeat embryos (48 h), hatching embryos (60 h) and hatched larva (72 h). 3, 5, 7, 11, 13, and 15 day, respectively, indicate the days after larva hatched. The equal amount of proteins was loaded in each *line* for western blot detection

and the strong purple staining was also detected in the internalization cells (Fig. 3A(b–c)), and in the corresponding sections, the hypoblast became thickened and became a morphological layer, displayed a large, flat and bipoler shape (Fig. 3Bc), and the precursors of mesoderm and endoderm (endomesoderm) displayed strong positive signals (Fig. 3Bd). At later gastrula stage, the positive signals began to display a characteristic salt-and-pepper distribution in yolk syncytial layer (YSL), and in the following developmental embryos and in the larva the *CagAFPIV* transcripts all along restricted in YSL and the positive staining became more and more faint (Fig. 3A(d–i)).

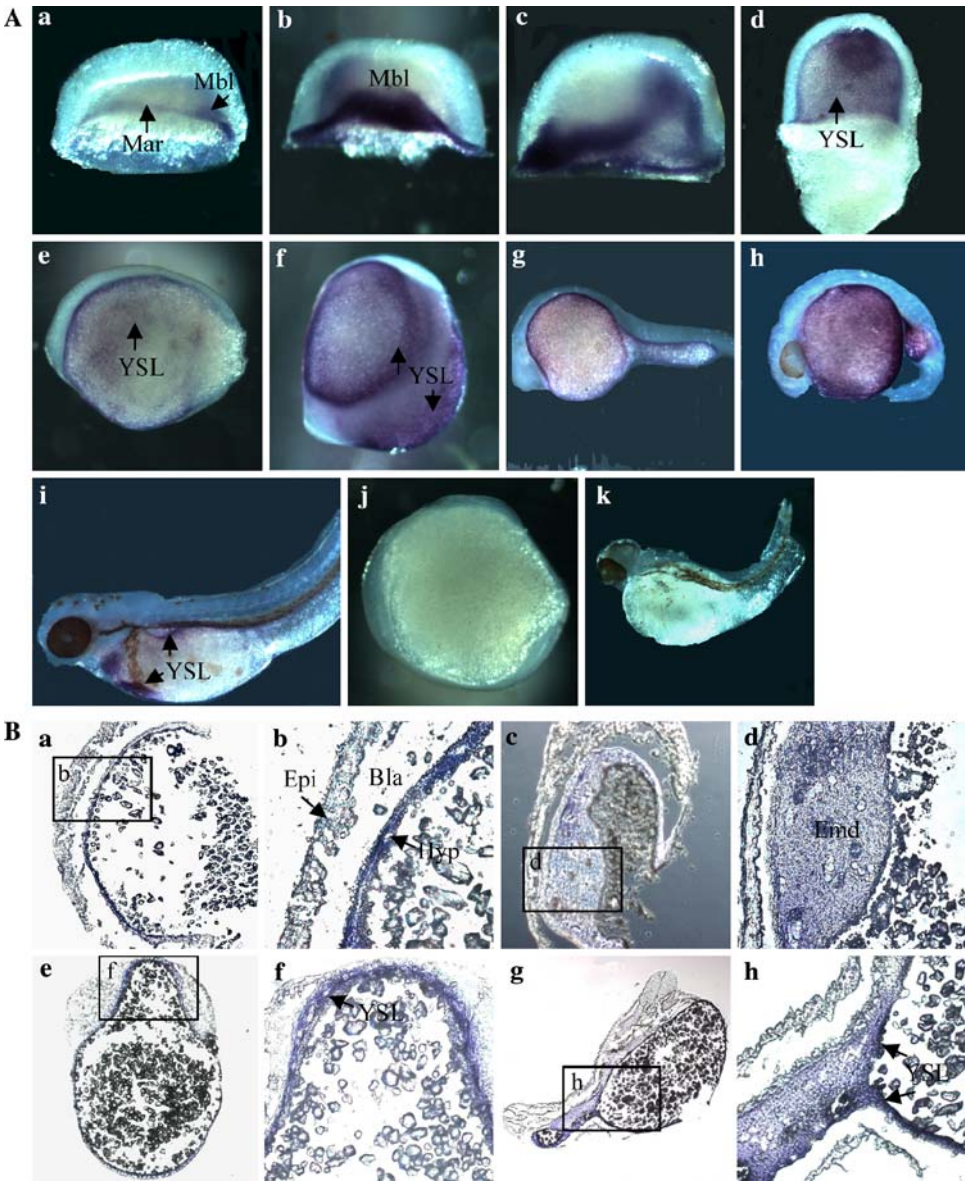

Fig. 3 **A** The whole-mount in situ hybridization of the *CagAFPIV* on gibel carp embryos of 40% epiboly (*a*, *b*), 50% epiboly (*c*, *d*), 70% epiboly (*d*), neurula stage (*e*), tail bud stage (*f*), heartbeat stage (*g*), hatching stage (*h*), 1 day larva (*i*) with antisense probes, and tail bud stage embryos (*j*) and 1 day larva (*k*) hybridized with sense probes as the control. **B** The corresponding sections of hybridized embryos of 40% epiboly (*a*, *b*), 50% epiboly (*c*, *d*), tail bud stage (*e*, *f*) and heartbeat stage (*g*, *h*). Mar-margin; Mbl-marginal blastomere; Epi, epiblast; Bla, blastocele; Hyp, hypoblast; Emd, endomesoderm; YSL, yolk syncytial layer

In the corresponding sections, only the cells of YSL shown the purple signals (Fig. 3B(e–h)). The negative probe does not generate any staining (Fig. 3A(j–k)).

Distribution of *CagAFPIV* protein in larva

The distribution of *CagAFPIV* protein in larva was investigated by immunofluorescence localization. As shown in Fig. 4, in the hatched larva, strong positive signals predominantly distributed in blood vessel (Fig. 4A, A′). The corresponding section shown the same location of the larva stained with HE, in the figures, the vascular endothelial cells lined the inside of blood vessels, surrounding the characterized cavity structure of the vessel. And the immunofluorescence localization approved that the *CagAFPIV* protein was secreted by YSL cells as a type of plasma protein in blood.

Discussion

In order to identify differentially expressed genes in specific stage during early embryogenesis, two kinds of SMART cDNAs have, respectively, been synthesized from mature eggs and gastrula embryos of *Carassius auratus gibelio*, and the gastrula embryo SMART cDNA library has been constructed. *CagAFPIV* was identified as one of the differentially expressed genes at gastrula stage in our screenings [23]. In this study, we report the expression pattern of *CagAFPIV* in gibel carp embryos and larva. In

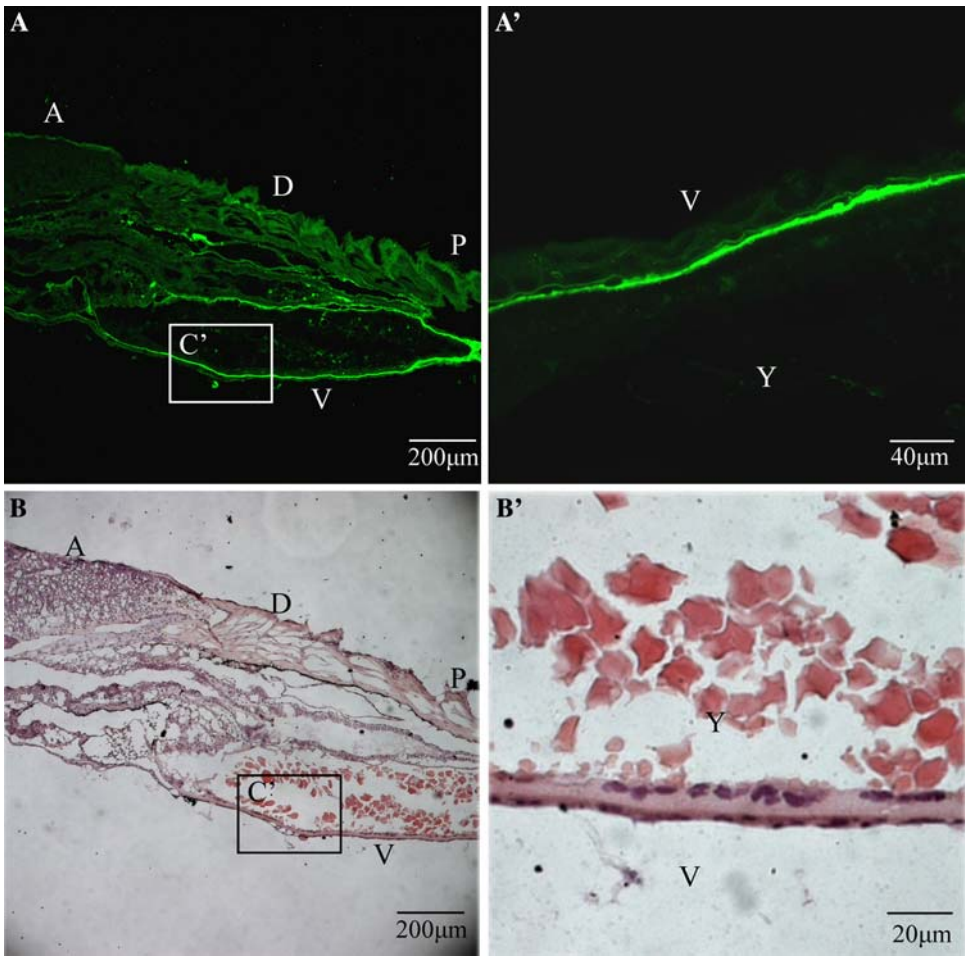

Fig. 4 Immunofluorescence localization of *CagAFPIV* protein in 2 day larva sections (A, A′). The corresponding HE staining sections are shown in Fig. B, B′. *A* anterior, *P* posterior, *V* ventral, *D* dorsal, *Y* yolk

the blastrula stage and the early-gastrula stage embryos, the positive *CagAFPIV* transcripts signals only distributed in the cells at the margin, marginal blastomere and the internalization cells, marked the precursors of mesoderm and endoderm.

At the late blastula stage, endomesoderm progenitors are located all around the margin, and they involuted during early-gastrulation and mid-gastrulation become a morphological layer [25–27]. The *CagAFPIV* expression pattern in blastula and in early-stage gastrula in gibel carp is very similar with the expression pattern of *no tail(ntl), cyclops* and *spt* in zebrafish [28–30]. And *no tail(ntl), cyclops* and *spt* have been identified to control the embryonic patterning and axial tissues formation, and to induct the mesodermal and endoderm formation [28–30]. The similar *CagAFPIV* expression pattern in gibel carp suggested that *CagAFPIV* possibly functioned in the embryonic patterning or in the mesodermal and endoderm induction.

In addition, by immunohistochemical staining, we demonstrate the predominant *CagAFPIV* distributed in blood vessel of the larva. AFPs have been identified with antifreeze activity and lower solution freezing point characterization, as plasma proteins to protect living organisms by freezing temperatures and protecting cell membranes from cold-induced damage. Our immunohistochemical staining of *CagAFPIV* in blood vessel proved plasma protein characterization of AFPs by another different technique.

In this paper, we not only demonstrated the expression pattern of *CagAFPIV* during embryogenesis for the first time, but also offered a new way to detect the AFPs functions in fish.

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