

Apo-14 is required for digestive system organogenesis during fish embryogenesis and larval development

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ABSTRACT Apo-14 is a fish-specific apolipoprotein and its biological function remains unknown. In this study, *CagApo-14* was cloned from gibel carp (*Carassius auratus gibelio*) and its expression pattern was investigated during embryogenesis and early larval development. The *CagApo-14* transcript and its protein product were firstly localized in the yolk syncytial layer at a high level during embryogenesis, and then found to be restricted to the digestive system including liver and intestine in later embryos and early larvae. Immunofluorescence staining in larvae and adults indicated that *CagApo-14* protein was predominantly synthesized in and excreted from sinusoidal endothelial cells of liver tissue. Morpholino knockdown of *CagApo-14* resulted in severe disruption of digestive organs including liver, intestine, pancreas and swim bladder. Moreover, yolk lipid transportation and utilization were severely affected in the *CagApo-14* morphants. Overall, this data indicates that *CagApo-14* is required for digestive system organogenesis during fish embryogenesis and larval development.

KEY WORDS: *apolipoprotein, apo-14, digestive system, organogenesis, gibel carp*

Introduction

Apolipoproteins are the protein moieties of lipoproteins, and play critical roles in lipoprotein metabolism (Paolucci *et al.*, 1998) and in innate immune system (Concha *et al.*, 2003). Recent studies have revealed that high-density lipoprotein (HDL) in teleostean fish corresponds to the most abounding plasma protein and is constituted by two major apolipoproteins which are 28 kDa apolipoprotein and 14 kDa apolipoprotein (Kondo *et al.*, 2001; Concha *et al.*, 2004). The 14 kDa apolipoprotein was early identified as a major component of HDL from Japanese eel (Kondo *et al.*, 2001), and now also identified from common carp (Concha *et al.*, 2004), pufferfish (Kondo *et al.*, 2005), orange-spotted grouper (Zhou *et al.*, 2005), grass carp (Chang *et al.*, 2005) and fathead minnow (Wintz *et al.*, 2006). In common carp, the major constituents of HDL, 28 kDa ApoA-I and 13 kDa ApoA-II, were shown to display antimicrobial activity (Concha *et al.*, 2003) and to be potentially important effectors of innate immunity (Concha *et al.*, 2004). Kondo *et al.* (2001) characterized three main apolipoproteins Apo-28 kDa, Apo-27 kDa and Apo-14 kDa in pufferfish, and demonstrated that Apo-28 kDa and Apo-27 kDa were homologues of mammalian ApoA-I, whereas Apo-14 was a unique apolipoprotein, and specific to fish. The uniqueness sug-

gested that Apo-14 might be more important than other apolipoproteins (Kondo *et al.*, 2005), because most fish use lipids as the principal energy source in contrast to mammals which mainly use carbohydrates (Watanabe, 1982). On the other hand, the 28 kDa *ApoA-I* was demonstrated to be highly expressed during embryonic development in zebrafish several years ago (Babin *et al.*, 1997). However, little is known about expression patterns and physiological functions of the apolipoproteins during fish embryogenesis.

Gibel carp, *Carassius auratus gibelio*, because of its unique triploid genome and dual reproduction modes of gynogenesis and bisexuality (Gui, 1993; Zhou *et al.*, 2000; Zhou and Gui, 2002), has been used as a promising study model for reproductive and evolutionary mechanisms (Xie *et al.*, 2001; Dong *et al.*, 2004; Yang and Gui, 2004; Yin *et al.*, 2007). In order to identify some differentially expressed genes in early embryogenesis, two kinds of SMART cDNAs were respectively synthesized from the mature eggs and gastrula embryos, and the gastrula embryo SMART cDNA library was constructed. Following this program, some

Abbreviations used in this paper: MO, morpholino oligonucleotide; hpf, hours post fertilization; ORO, Oil Red O; YSL, yolk syncytial layer; aa, amino acid.

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differentially expressed genes at gastrula stage were screened, and one of them was found to be *Apo-14* (Liu *et al.*, 2005). The aim of this study was to characterize *Carassius auratus gibelio Apo-14* (*CagApo-14*) molecule, and to reveal its expression pattern and physiological functions during embryogenesis. We showed here that the gibel carp *Apo-14* was expressed in yolk syncytial layer (YSL) of early embryos from gastrula to hatching stage, and restricted to digestive system including liver and intestine in later embryos and early larvae. To reveal the physiological functions during embryogenesis, we used the specific antisense morpholino to abrogate *CagApo-14* protein during gibel carp embryogenesis. As a result, severe development abnormalities of digestive system were observed in the *CagApo-14* morphants.

Results

Molecular characterization and differential expression pattern of *CagApo-14* during embryogenesis

To reveal differentially expressed genes at gastrula embryos relative to mature eggs, about 1500 clones from the gastrula SMART cDNA library were screened with two probes prepared from gastrula first-strand cDNAs and mature egg first-strand cDNAs respectively. As shown previously (Liu *et al.*, 2005), 130 positive clones specific to gastrula embryos were obtained, and two of them were revealed to be full length cDNA clones of *CagApo-14* gene through sequencing analysis and database searches. This gene codes for a protein 141 amino acids in length (Fig. 1), with the first 19 amino acids representing a putative signal peptide by prediction with SignalP 3.0 program. It has a 48 bp of 5'-UTR and a 283 bp 3'-UTR including a typical polyadenylation signal sequence AATAAA located 17 bp upstream from the poly(A) tail. Glycosylation site analysis by NetNGlyc 1.0 and YinOYang 1.2 didn't find any potential N-glycosylation site or O-glycosylation site. NetPhos 2.0 analysis revealed four potential phosphorylation sites (Ser43, Ser57, Ser64, Ser74) (Fig. 1A).

Homology searches revealed five homologues from five fish species, which are grass carp (*Ctenopharyngodon idella*), Japanese eel (*Anguilla japonica*), pufferfish (*Takifugu rubripes*), European flounder (*Platichthys flesus*) and orange-spotted grouper (*Epinephelus coioides*). As shown in Fig. 1B, the amino acid identity between *CagApo-14* and other Apo-14 ranges from 51%

(grass carp) to 39% (European flounder), being the N-terminal region of the protein the most conserved.

RT-PCR analysis revealed differential expression pattern of *CagApo-14* during embryogenesis. No maternal *CagApo-14* transcript was found in eggs and early embryos, and the zygotic *CagApo-14* transcript was firstly detected at the gastrula stage, rapidly reaching a high level at neurula stage which was maintained until hatching (Fig. 1C).

Yolk syncytial layer and digestive system-specific expression of *CagApo-14* in embryos and early larvae

Expression pattern of *CagApo-14* was further analyzed by whole-mount *in situ* hybridization. As shown in Fig. 2, *CagApo-14* was expressed in a dynamic fashion during embryogenesis and early larval development. No positive signals were observed in early embryos before blastula stage (data not shown). *CagApo-14* transcript was detected at YSL of embryos from gastrula stage to hatching stage (Fig. 2A-C). At the just hatched larvae, *CagApo-14* transcript was restricted to primordial digestive tract (Fig. 2D),

A

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GGGACTCCAGCTCCTTTAATTCTCTCTACAGGAATCTCACTAGCACAAGATGAAGCTGACA 60
                                     M K L T
TTTGCCTCATTCTTGCCTCCAAAGTGTCAAGTGTGTGTTTGGGCACAGGATGGCCCCAG 120
F A L I L A L Q V S V C V W A Q E W P Q 5
CCTGACAAGGAGTTAGTAGAGGAATATGAGGGCTGAGGCGCTTTTCTTAAGAGGATC 180
P D K E L V E E Y E G L K A V F F K R I 25
GTCATGCTTGGGAAAAGACCAAAAGCTGCCCTTCAGCCAAACAATCGAGGGCTCACCAACA 240
V N A W E K T K A A L Q P T I E G S P T 45
GGAGATCAAGCCAAACAATCTTGAAGAAGCTGAGCAAAAGGCCAAGAGTGGAGAGTGCC 300
G D Q A K Q I L E E L S K R P R V E S A 65
ATCAAATCATCGGCGGTTGGCCAGTGTATGGAGCCTATGGTGGACAAGCTCGCATG 360
I K I I G G L A S D M E P M V D F K A R M 85
GCTCTTCTGGTGCTTATGGCCACTATCTGCGGCCCCACATCGGAGAATACCTGGACAGA 420
A L L G A Y G H Y L R P H I G E Y L D R 105
GCAATCACCACCAAGCCAGTGTGGTACTGCTGCTGCTCATGAGGGTTAGAGTCTG 480
A I T N I K P V L D T V L P H E G * 122
CAGCTCATCAACCCTGATTCCTCAACCCAGCAACTCAATAATAGATCTGGATGCTG 540
TGTGTCACAGCAGTCCGTCGAGATTTGGCATGTATATCTGATCTGTGATTTGATGACC 600
ATTCCTGTTGCTGAGCAAAAATAAATAACCCAGCCAGCATTAATGAGAGTAACT 660
ATGTGATTTTGTGCTCATAAAGAAACAACATGACCCATTCTAATAAATAAATCTGG 720
AAAATGCARAAAAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA 757
    
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B

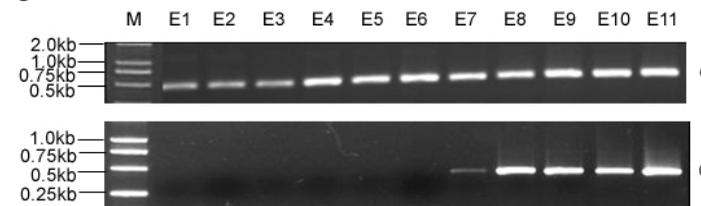
flounder	1	NNARKYVIALILALQVSVLCEVFAE--SKELDKYDAMRDFHRLRDAYGRDQEAAL
grouper	1	NNARYALALILALQVSVSLCEVFAE--SKELVNYKYLKAFYKRLDAYGRDQEAAL
pufferfish	1	NHVKYALALILALQVSVSLCEVFAE--SKELDKYKELKAFYKRLDAYGRDQEAAL
eel	1	MSKLVVALILALQVSVLCEVFAE--SKELVNYKYLKAFYKRLDAYGRDQEAAL
grasscarp	1	--MKLTBALILALQVSVLCEVFAE--IKELVNYKYLKAFYKRLDAYGRDQEAAL
gibelcarp	1	--MKLTBALILALQVSVLCEVFAE--IKELVNYKYLKAFYKRLDAYGRDQEAAL

flounder	59	AAKTESEBEGCCAKDYVDDCAKPAYCAVVKVCSGLVEAGPLVDKARDSELGVVE---
grouper	59	VEKVGDSAGCCAKDYIEELQKPELCAVVKVATGLCCAGPLVDKARDSELGVVE---
pufferfish	59	TEHFSQSEBEGCCAAALAGSSVQKGYCAAKKAAABALEPLVDKARDSELGVVE---
eel	59	AOHTESS--CAAKDYVEELQKPHVCSVVKVARGVASELPLVDKARDSELGVVE---
grasscarp	57	TESSPTG--EKAKEIFENRQSPRVEAVKILSGLASELPLVDKARDSELGVVE---
gibelcarp	59	TESSPTG--DQAKDIEELSKPRVESAANKIIGLASDMPPLVDKARDSELGVVE---

flounder	116	---YMRPQVGYLSEVYDHIKRYLDRVMPAE--	Identity	39%
grouper	116	---YLRFPYGNLSEVSDTAKVLRDREMPAE--		41%
pufferfish	116	---YLRFPYGNLSEVSDTAKVLRDREMPAE--		41%
eel	113	---YMRPQVGYLSEVYDHIKRYLDRVMPAE--		44%
grasscarp	114	ALRHEPQHSVDEQCSAGYFPAE-----		51%
gibelcarp	112	---YLRFPYGNLSEVSDTAKVLRDREMPAE--		100%

Fig. 1. Molecular characterization and expression pattern of *CagApo-14* during embryogenesis. (A) Nucleotide sequence and deduced amino acid sequence of *CagApo-14*. The N-terminal putative signal peptide is underlined. The asterisk indicates the stop codon and putative polyadenylation signal is bolded. Potential serine phosphorylation sites are shadowed. The nucleotide sequence has been submitted to GenBank databases under the accession number AY773183. (B) Amino acid alignment of 6 complete Apo-14 polypeptides from 6 species of fish, including gibel carp (this study), grass carp (GenBank accession no. AY445924), eel (GenBank accession no. AB046209), pufferfish (GenBank accession no. AB183288), flounder (GenBank accession no. AJ843093) and grouper (GenBank accession no. DN552074). Black boxes: identical residues, gray boxes: conservative substitutions. (C) RT-PCR detection of *CagApo-14* transcript during embryogenesis. E1-mature eggs, E2-fertilized eggs, E3-two cell stage, E4-multi-cell stage, E5-morula, E6-blastula, E7-gastrula, E8-neurula, E9-tailbud stage, E10-hatching embryos, E11-hatched larvae. Ubiquitous expression of α -tubulin was used as the endogenous control (C, top).

C



and then, high concentration of *CagApo-14* transcript appeared on liver, intestinal bulb and swim bladder (Fig. 2E-H) along with the larva development. Through 3–5 dpf, *CagApo-14* expression was specifically enriched in intestine, liver, swim bladder, and pharynx (Fig. 2I–L), especially in liver (Fig. 2L). The data suggested that *CagApo-14* might be specifically expressed in YSL and digestive system during fish embryogenesis and early larval development.

Liver endothelial cell expression and ubiquitous distribution of *CagApo-14* protein in larvae and adults

We also generated polyclonal antibodies against *CagApo-14* from rabbit. With the polyclonal antibodies, Western blot analysis was performed on protein lysates from embryos at different developmental stages. A specific band at 14 kDa was verified in mature eggs (Fig. 3A, lane 1), suggesting the presence of maternal storage of *CagApo-14* protein. As fertilization and cleavage occurs, no maternal *CagApo-14* was detected in early embryos (Fig. 3A, lane 2–7). The immunoreactive bands reappeared from neurula stage (Fig. 3A, lane 8), which should be the *CagApo-14* protein translated from zygotic transcript.

To further test the specificity of anti-*CagApo-14* antibodies, the antiserum was pre-adsorbed with the purified recombinant *CagApo-14* protein for 16 h at 4 °C and set as negative control for both Western blot detection and immunofluorescence staining. Indeed, the single band at 14kDa could not be recognized by the pre-adsorbed antiserum (Fig. 3B), and no brilliant green immunofluorescence but only faint diffuse background was observed in the section stained by the pre-adsorbed antiserum (Fig. 3D) in comparison with Fig. 3C. Control was also done with normal rabbit serum, and no signal could be detected in Western blot analysis and immunofluorescence staining (data not shown). The data demonstrated the specificity of anti-*CagApo-14* antiserum.

Then the anti-*CagApo-14* antiserum was used to trace the localization and distribution of *CagApo-14* protein during embryogenesis and larval development. Fig. 3E–H showed its dynamic distribution during embryogenesis. In early embryos at 5-somite stage, *CagApo-14* protein was detected in YSL, and formed two continuous fluorescence rings around the whole embryo body and YSL (Fig. 3E, F). After hatching, predominant localization of *CagApo-14* protein was obviously observed in the primordial digestive organs including liver and intestine bulb (Fig. 3G). In 5 day larva after hatching, the liver had expanded greatly. At that time, numerous hepatocytes with *CagApo-14* immunofluorescence invaded into the yolk sac, and characteristic fingerlike intestinal villi with positive signal appeared on the intestinal bulb (Fig. 3H).

Moreover, double staining of DAPI for nucleus (blue) and green immunofluorescence for *CagApo-14* further revealed predominant localization and differential distribution in developing liver. As shown in Fig. 4A–F, the *CagApo-14* positive signal was predominantly seen in liver, intestinal gut and swim bladder of the 6dph larva, but obviously differential distribution was observed in liver (Fig. 4A, E). Interestingly, a magnified observation revealed an island-like distribution (Fig. 4B, F), and the cells stained with strong immunofluorescence could be distinguished by their prolate nuclei, which were clearly different from numerous hepatocytes in the liver tissue (Fig. 4D). Obviously, *CagApo-14* protein was synthesized in and excreted from these special endothelial cells, since the liver tissue is basically composed of hepatocytes

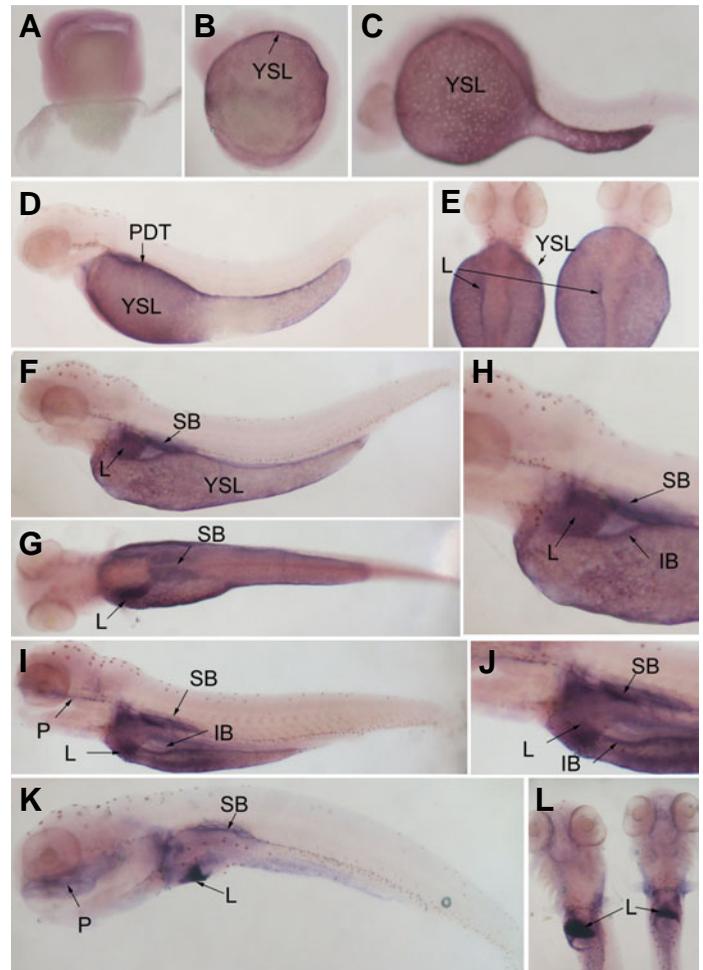


Fig. 2. Expression pattern of *CagApo-14* during embryogenesis determined by whole-mount *in situ* hybridization. (A) Gastrula embryo (8 hpf), (B) tail bud embryo (14 hpf), (C) hatching embryo (24 hpf), (D) just hatched larva (48 hpf), (E) dorsal view of just hatched larvae, (F) lateral view of 1dph larva (72 hpf), (G) dorsal view of 1 dph larva, (H) partial magnification of (F), (I) 3 dph larva, (J) partial magnification of (I), (K) 5 dph larva, (L) ventral view of 5 dph larvae. YSL, yolk syncytial layer; PDT, primordial digestive tract; IB, intestine bulb; L, liver; SB, swim bladder; P, pharynx; dph, days post hatching; hpf, hours post fertilization.

and invading endothelial cells (Zaret, 2002; Lee *et al.*, 2007).

Following the above observation, we further examined the cellular distribution of *CagApo-14* protein in adult liver tissue by immunofluorescence staining. As shown in Fig. 4G–L, *CagApo-14* immunofluorescence was regularly distributed as dots and lines in liver section (Fig. 4G–I). Higher magnification revealed that the immunostained dots and lines were the transverse and sagittal sinusoid capillaries, respectively (Fig. 4J–L). It was consistent with the above observations in larval liver that *CagApo-14* protein was localized in the liver sinusoidal endothelial cells. It is well known that the unusual sinusoid capillary is composed of a discontinuous layer of endothelial cells with large, irregular openings or fenestrations, and the basement membrane under these endothelial cells is also incomplete. Blood plasma easily percolates through the sinusoidal fenestrations and so makes intimate contact and facilitating exchange with the neighboring hepatocytes.

Tissue distribution of *CagApo-14* was further examined in adult

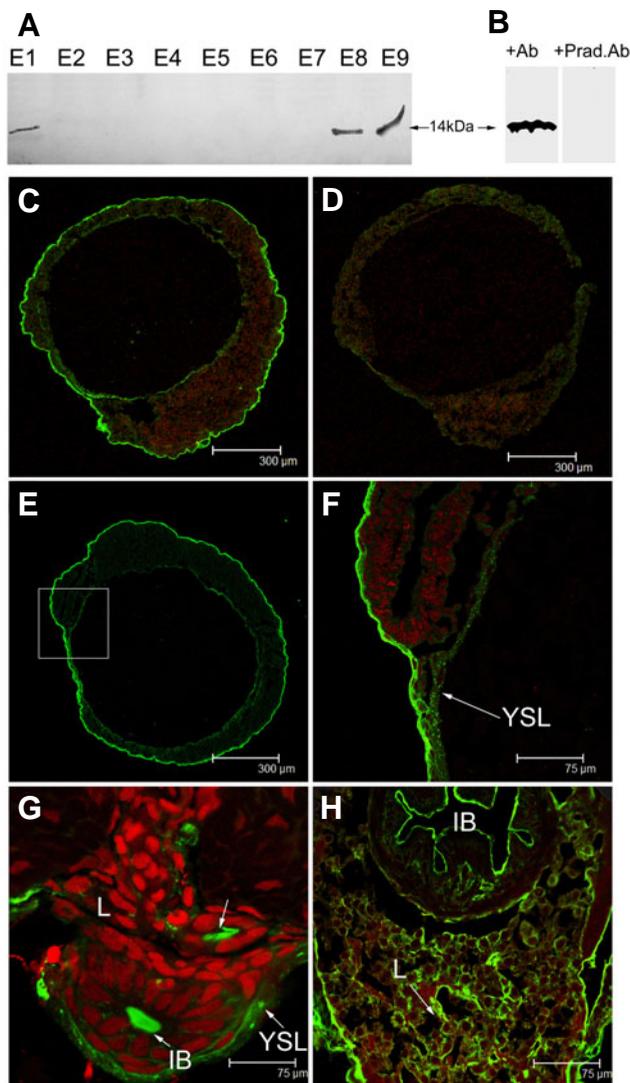


Fig. 3 (Left). Cellular localization of CagApo-14 protein during embryogenesis. (A) Western blot detection of CagApo-14 protein during embryogenesis. E1 to E9 respectively represents protein samples from mature eggs and different stage embryos. E1, mature eggs; E2, fertilized eggs; E3, two cell stage; E4, multi-cell stage; E5, morula; E6, blastula; E7, gastrula; E8, neurula; E9, tailbud stage. (B-D) Specificity of anti-CagApo-14 antiserum in Western blot and immunofluorescence staining. (B) Western blot detection of egg extracts by the anti-CagApo-14 antiserum (Ab, left) and by the pre-adsorbed antiserum (Prad. Ab, right). (C) Immunofluorescence staining of 5-somite stage embryo section by the anti-CagApo-14 antiserum. (D) Control of the same 5-somite stage embryo section stained by the pre-adsorbed antiserum. (E-H) Immunofluorescence staining of CagApo-14 protein in embryo and larva sections. Green fluorescence was immunostained by anti-CagApo-14, and red fluorescence stained by PI showing the nuclear position. (E) 5-somite stage embryo. (F) Magnification of the framed area in (E). (G) Cross section through primordial intestine bulb and liver of the 1 dph larva. (H) Cross section through intestine bulb and liver of the 5 dph larva. YSL, yolk syncyrial layer; L, liver; IB, intestine bulb.

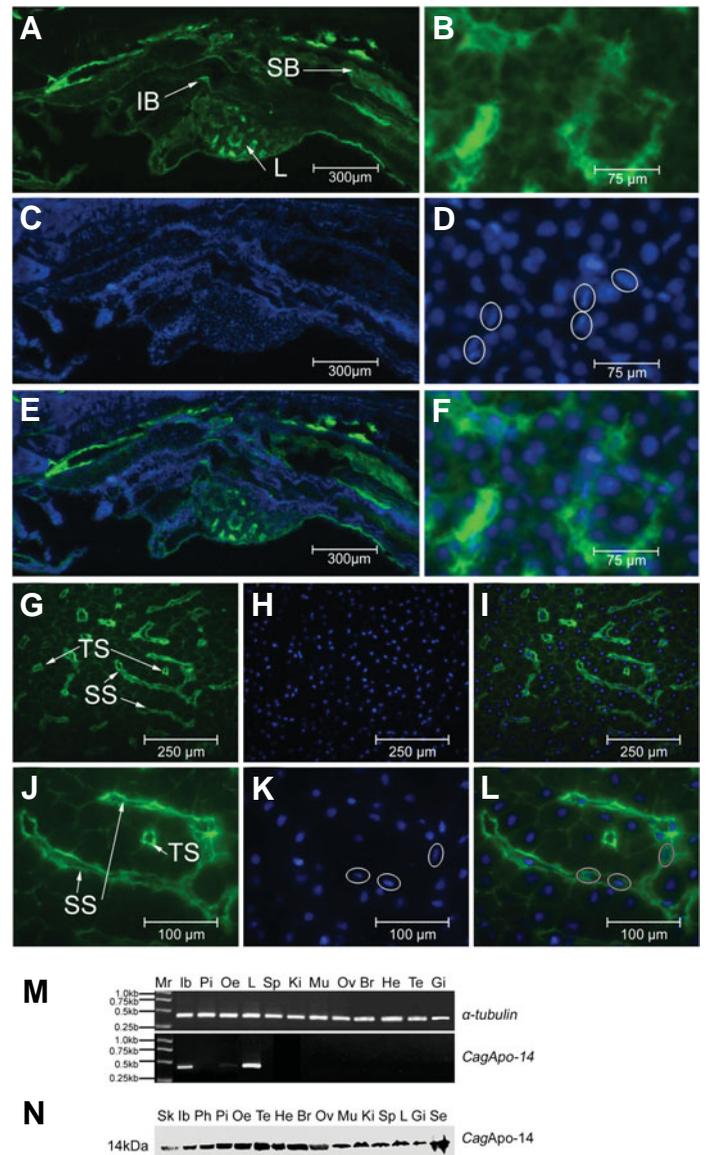


Fig. 4 (Right). Cellular localization and distribution of CagApo-14 protein in larvae and adults. (A-F) Double staining of DAPI for nucleus (blue) and immunofluorescence for CagApo-14 (green) in liver tissue of 6 dph larvae. The top line (A,B) shows the immunofluorescence staining of CagApo-14, middle line (C,D) shows the results stained by DAPI, and bottom line (E,F) shows the merge of the CagApo-14 green immunofluorescence and nucleus blue fluorescence. The circles highlight special endothelial cells in the liver tissue. IB-intestine bulb, L-liver, SB-swim bladder. (G-L) Double staining of DAPI for nucleus (blue) and immunofluorescence for CagApo-14 (green) in adult liver tissue. (J-L) are the partial magnification of (G-I). The left row (G,J) shows the immunofluorescence staining of CagApo-14, middle row (H,K) shows the results stained by DAPI, and right row (I,L) shows the merge of the CagApo-14 green immunofluorescence and nucleus blue fluorescence. The circles highlight special endothelial cells in the liver tissue. TS-transverse sinusoid, SS-sagittal sinusoid. (M) RT-PCR detection of CagApo-14 expression in different adult tissues. Ubiquitous expression of α -tubulin was used as the endogenous control (top). (N) Western blot detection of CagApo-14 in different adult tissues. Mr, marker; Ib, intestine bulb; Pi, posterior intestine; Oe, oesophagus; L, liver; Sp, spleen; Ki, kidney; Mu, muscle; Ov, ovary; Br, brain; He, heart; Te, testis; Gi, gills; Sk, skin; Ph, pharynx; Se, serum.

fish by RT-PCR and Western blot analysis. As shown in Fig. 4M, *CagApo-14* was restrictedly transcribed in liver and intestinal bulb, but *CagApo-14* protein seemed to be distributed in a much more ubiquitous manner (Fig. 4N). In all of the examined tissues, including skin, intestinal bulb, pharynx, posterior intestine, oesophagus, testis, heart, brain, ovary, muscle, kidney, spleen, liver, and serum, a positive 14 kDa band was revealed by Western blot detection, and its content was the most abundant in serum (Fig. 4N). The ubiquitous existence of *CagApo-14* protein was consistent with the presumed role as a lipid transporter in the bloodstream, and also indicated the existence of a ubiquitous receptor for Apo-14 in all the examined tissues. The cloning and characterization of this receptor still awaits further scrutiny.

Disruption of digestive system organogenesis in *CagApo-14* morphants

To get a better insight into its biological function during embryogenesis, we sought to abrogate *CagApo-14* protein in developing gibel carp embryos using antisense morpholino oligos. A translation-blocking morpholino (MO-*CagApo-14*) that specifically targets to a 25 base-pair sequence around the start codon AUG was designed, and injected into fertilized eggs of gibel carp at the one-cell stage. Firstly, the optimal delivering dose was determined by injections of MO-*CagApo-14* in a dilution series, that is, 1mM, 0.5mM and 0.3mM. In the later experiments, 0.3mM was adopted as the optimal dose, since working with this dose resulted in anticipated phenotypes in most but not all of the injected embryos. No obvious off-target effects, such as early necrosis and serious developmental delay, were displayed in all the morphants. Translation blocking of *CagApo-14* transcript in the morphants was detected by Western blot. As shown in Fig. 5A, the *CagApo-14* protein was not detected in the morphant embryos at 16 hpf, 24 hpf and 48 hpf, whereas a specific band of about 14 kDa was observed in the age-matched control embryos. In comparison with high concentration of *CagApo-14* protein in the control larvae, a small quantity of protein was also detected in the morphant larvae at 2 dph (72 hpf) and 3 dph (96 hpf). The data suggested that the injection of MO-*CagApo-14* did completely block the translation of *CagApo-14* transcript during embryogenesis, and only small quantity of *CagApo-14* protein was synthesized in the later larvae, probably resulting from the gradually dilution of MO-*CagApo-14* concentration in the developing larvae.

Moreover, morphological changes were inspected in detail in the *CagApo-14* morphants. In comparison with control embryos, no morphological changes were seen in *CagApo-14* morphants before hatching (data not shown), but obviously phenotypic differences were observed in early larvae after hatching. Significantly, the *CagApo-14* morphants showed loss and disruption of all the organs of digestive system, and no swim bladder was observed. As shown in Fig. 5B-I, at 1 day after hatching, about 40% of the *CagApo-14* morphants displayed severe morphological defects, such as unabsorbable yolk, kinking tail, reduced head and eyes, and pericardial edema (Fig. 5B), and they tended to death within 3 days. At 3 days after hatching, when control larvae inflated the swim bladder, and the liver underneath the swim bladder was getting apparent (Fig. 5E), both the swim bladder and the liver tissue

were not observable in the rest of *CagApo-14* morphants, whereas the blood circulation seemed normal (Fig. 5D), indicating that the development of vasculature was not impaired. Along with larval development and organogenesis, while the fading region of yolk absorption was occupied with developing compact tissues in control larvae at 4 dph (Fig. 5G), the *CagApo-14* morphants with mild symptom consumed some yolk, but the digestive system was strongly suffered, and no digestion organs were observed (Fig. 5F). At 6 dph, when control larvae had formed complete digestive system and ingested exoteric food (Fig. 5I), the *CagApo-14* morphants had almost exhausted the yolk, and a linear thin digestive tract was evident in the enlarged and edemic body cavity (Fig. 5H). Notably, many other structures in the dorsal trunk, such as macula, notochord, fin, and musculature, seemed to be grossly normal. Finally, the *CagApo-14* morphants died within a week depending on the yolk nutrition.

To clarify developmental details of internal organs, the *CagApo-14* morphants at 4dph and 6dph were used to perform histological analysis. As shown in Fig. 6, in comparison with normal digestion organs in control larvae (Fig. 6A-C, G-I), intestine bulb, liver and pancreas showed severe defects in the *CagApo-14* morphants (Fig. 6D-F, J-L). Intestine bulb lacked normal epithelial folds, and was just composed of one layer of polarized epithelial cells without characteristic fingerlike intestinal villi (Fig. 6E, K). Liver tissue and pancreas tissue were severely reduced to a small patch of cells (Fig. 6D-F, J-L).

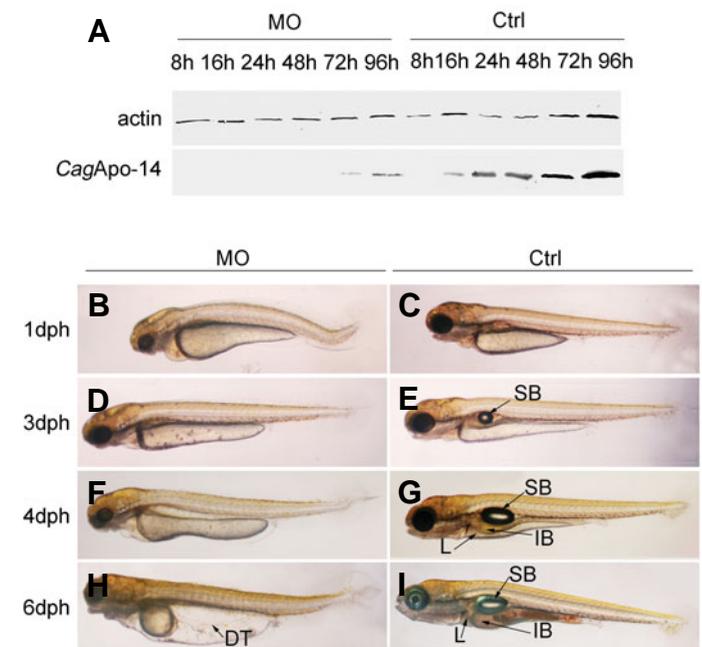


Fig. 5. *CagApo-14* knockdown results in severe disruption of digestive system in morphant larvae. (A) Western bolt detection of *CagApo-14* in *CagApo-14* morphants (MO) and control (Ctrl) experiments during embryogenesis and early larval development. Ubiquitous expression of actin was used as the endogenous control. **(B-I)** Representative phenotypes of *CagApo-14* morphants (MO, left row) in comparison with corresponding age-matched control larvae (Ctrl, right row), at 1 dph **(B,C)**, 3 dph **(D,E)**, 4 dph **(F,G)**, and 6 dph **(H,I)**. SB, swim bladder; L, liver; IB, intestine bulb; DT, digestive tract.

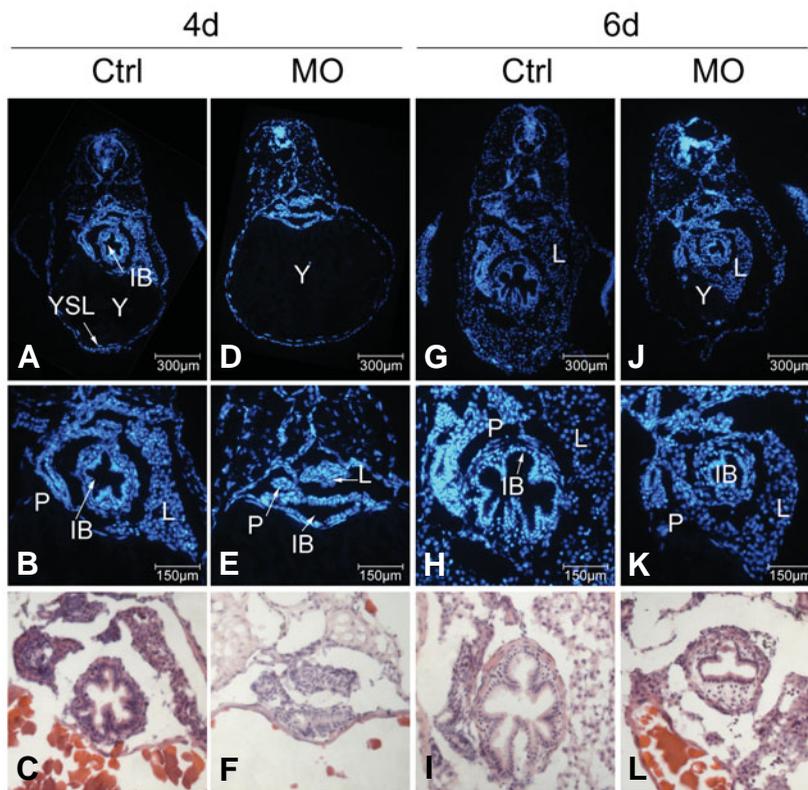


Fig. 6. Representative transverse sections through intestine bulb (IB), liver (L) and pancreas (P) in control (Ctrl) and *CagApo-14* morphant (MO) larvae at 4dph (A-F) and 6 dph (G-L). The top line (A,D,G,J) shows the whole sections with staining of DAPI for nucleus (blue). The middle line (B,E,H,K) shows the partial magnification of top line (A,D,G,J). The bottom line (C,F,I,L) shows the corresponding sections stained with Hematoxylin-Eosin. L, liver; P, pancreas; Y, yolk; YSL, yolk syncytial layer; IB, intestine bulb.

To confirm specificity of the MO-*CagApo-14* phenotypes, we further performed rescue experiments in which we co-injected MO-*CagApo-14* with a plasmid (pEGFP-N3-*CagApo-14*) bearing the *CagApo-14* coding region under CMV promoter control. The target sequence of MO-*CagApo-14* was absent in the plasmid construct. Three independent experiments were carried out in duplicate, and the data were then analyzed using paired Student's t-test. For the percentage of severely malformed embryos, the difference was statistically significant ($P < 0.01$) between the group injected with MO alone and the rescue group. As shown in Fig. 7, the loss and disruption of digestive system caused by MO-*CagApo-14* could be rescued by the co-injection of pEGFP-N3-*CagApo-14*. As a further control, co-injection with pEGFP-N3 did not rescue the phenotype (Fig. 7A). A typical rescue experiment was shown in Fig. 7A. In the group injected with MO-*CagApo-14* alone, only 14.0% (8/57) of embryos appeared normal, and the severely suffered embryos accounted for 40.4% (23/57). In the two independent experiments co-injected with two different dilutions of plasmid, the proportion of normal embryos respectively raised to 41.5% (27/65) and 51.3% (39/76). In comparison with intact digestive system of control larvae (Fig. 7B) and digestive system loss in *CagApo-14* morphants (Fig. 7C), the rescued larvae largely recapitulated the wild-type phenotype (Fig. 7D). At 7 dph, all of the morphants were died, but there were

about 50% survivals in the rescue groups, and most of them could open their mouths and live on. These data suggested that the loss and disruption of digestive system in *CagApo-14* morphants should be specifically resulted from the knockdown of *CagApo-14*.

Defection of yolk lipid transportation in vasculature of *CagApo-14* morphants

To investigate whether yolk lipid transportation and utilization are affected in *CagApo-14* morphants, we monitored neutral fat by staining the fixed larvae with Oil Red O (ORO) (Schlegel and Stainier, 2006). In wild-type embryos at 36 hpf, staining of yolk lipid was found in vasculature (Fig. 8A, C) and head structures (Fig. 8A), but almost no staining signal was observed in the corresponding morphants (Fig. 8B, D). At 50 hpf, even though blood circulation had become quite prosperous in both control and morphant embryos and high concentration of yolk lipid had appeared in control embryo (Fig. 8E, G), only a small quantity yolk lipid was detected in vasculature of the morphants (Fig. 8F, H).

Discussion

Apo-14, as a fish-specific apolipoprotein, has been suggested to have more significant roles than other apolipoproteins in fish (Kondo *et al.*, 2005), but its expression pattern has been studied only in one fish species, the orange-spotted grouper (*Epinephelus coioides*) (Zhou *et al.*, 2005), and its biological functions during fish embryogenesis and organogenesis remain largely unclear. In the present study, *CagApo-14* has been screened as a differentially expressed gene in early embryogenesis of gibel carp, and its expression patterns have been characterized in embryos and adults by RT-PCR, WISH, Western blot and immunofluorescence staining. During embryogenesis, *CagApo-14* gene began to transcribe from gastrula stage, and rapidly reached a high level at neurula stage. Western blot detection demonstrated the existence of maternal *CagApo-14* protein in mature gibel carp eggs, and the zygotic *CagApo-14* protein was detected from neurula stage. Sequentially, its expression characterization was further analyzed during the progress of embryogenesis and larval development. Interestingly, *CagApo-14* was expressed in YSL of early embryos, and restricted to the digestive system including liver and intestine bulb after hatching.

An intriguing finding in this study is the association of *CagApo-14* with digestive system organogenesis. Knockdown of *CagApo-14* resulted in severe disruption of digestive organs including liver, intestine, pancreas, and swim bladder. Recently, morphogenesis and organogenesis of digestive system have been extensively investigated in zebrafish (Field *et al.*, 2003a; Field *et al.*, 2003b; Wallace and Pack, 2003). In the current study, we have demonstrated that the fish-specific apolipoprotein Apo-14 is an important factor required for digestive system organogenesis during fish embryogenesis and larval development (Fig. 5). We found that *CagApo-14* transcript and protein product were firstly located in YSL at a very high level during embryogenesis, and then in

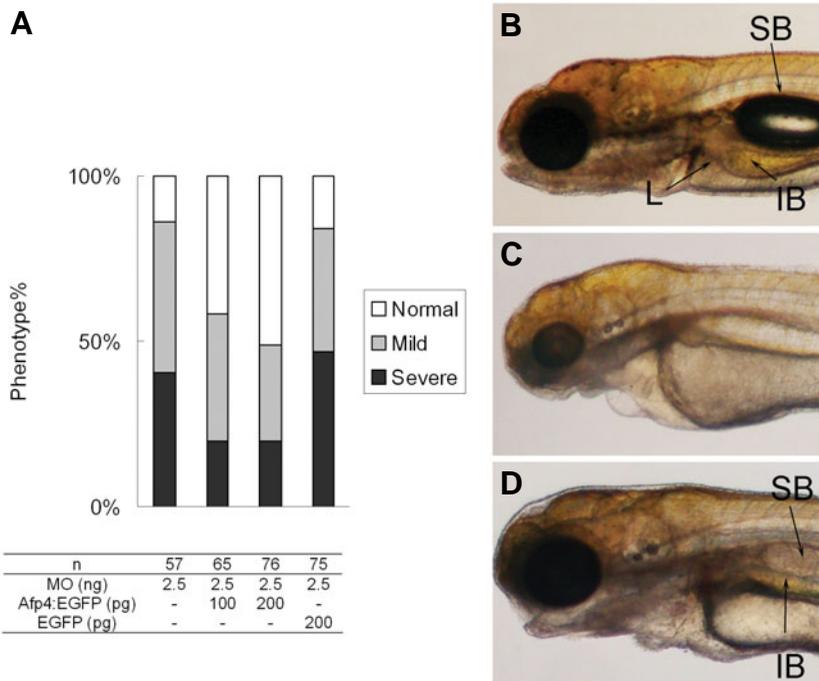


Fig. 7. Rescue experiments. (A) Percentage histogram of three phenotypes, including normal (white bar), mild (grey bar) and severe (black bar), in indicated rescue and control experiments (bottom). (B-D) Three typical phenotypes of normal (B), severe (C) and mild (D) larvae, which highlight the digestive and related organs. SB, swim bladder; IB, intestine bulb; L, liver.

digestive organs including liver and intestine after hatching (Fig. 2 and 3). Immunofluorescence staining in larvae and adults indicated that *CagApo-14* protein was predominantly synthesized in and excreted from the sinusoidal endothelial cells of liver tissue (Fig. 4). It is well known that liver sinusoidal endothelial cells constitute unique sinusoid capillaries that differ from other capillaries in the body (Braet and Wisse, 2002; Lee *et al.*, 2007; Straub *et al.*, 2007), and are necessary for liver morphogenesis, even before the formation of vasculature (Matsumoto *et al.*, 2001). The current study not only revealed predominant existence of *CagApo-14* in the endothelial cells (Fig. 4), but also found loss and disruption of the endothelial cells in intestine and liver of *CagApo-14* morphants (Fig. 6). Therefore, *CagApo-14* protein might be a potential molecular marker for tracing morphogenesis and organogenesis of digestive system, especially the endothelial cells in digestive organs.

Most apolipoproteins are expressed in liver, but how the spatially-restricted expression is regulated remains unknown. It has been reported that ApoE is upregulated by Bmp-2 in mesenchymal progenitors and is highly expressed in many organs including liver and heart (Bachner *et al.*, 1999). Bmp2 has been

shown to play instructive role in pancreas and liver development in chick (Rossi *et al.*, 2001) and mouse (Lemaigre and Zaret, 2004). Therefore, it would be a good idea to test whether Bmp or other signaling pathways regulate Apo-14 in fish.

Recently, molecular crosstalk between gut and the inhabited microbes has become a hot topic in vertebrates. In the absence of microbes, differentiation of gut epithelium is arrested in zebrafish (Bates *et al.*, 2006; Cheesman and Guillemin, 2007). On the other hand, previous reports have confirmed that apolipoproteins have antibacterial activities in mammalian and fish (Srinivas *et al.*, 1990; Singh *et al.*, 1999), and are potentially important effectors of innate immunity in teleost fish (Concha *et al.*, 2004). The involvement of fish *Apo-14* in immune response was reported fragmentarily. In grass carp, the transcription of both *Apo-14* and *ApoE* was up-regulated in response to parasite infection (Chang *et al.*, 2005). Similar up-regulation of *Apo-14*, *Apo A-1* and *Apo B* was also observed in fathead minnow when exposed to the toxicant 2, 4-DNT (Wintz *et al.*, 2006). It would be of great interest to investigate the detailed regulatory network concerning the microbes, the *Apo-14* expression, and the gut development.

YSL is an embryonic structure unique to fish embryos, and has been thought to be involved in nutrient transfer from the yolk to the blastoderm (Babin *et al.*, 1997; Poupard *et al.*, 2000). Kudoh *et al.* (2001) reported a screen of gene expression patterns in zebrafish embryogenesis, and found several genes expressed in the YSL. Moreover, spatial and temporal complexity of the gene expression patterns was revealed by *in situ* hybridization screening approach. Analogously to most of the YSL-specific genes in zebrafish embryos, *CagApo-14* was expressed in the YSL and continued its expression throughout all developmental stages of embryogenesis. Furthermore, we found that the *CagApo-14* transcript was restricted to the digestive system in the later embryo and larval development (Fig. 2), and its protein product was observed in primordial digestive tract and related organs (Fig. 3). The anterior intestine in fish participates in lipid absorption (Bates *et al.*, 2006) and expresses many genes involved in this process

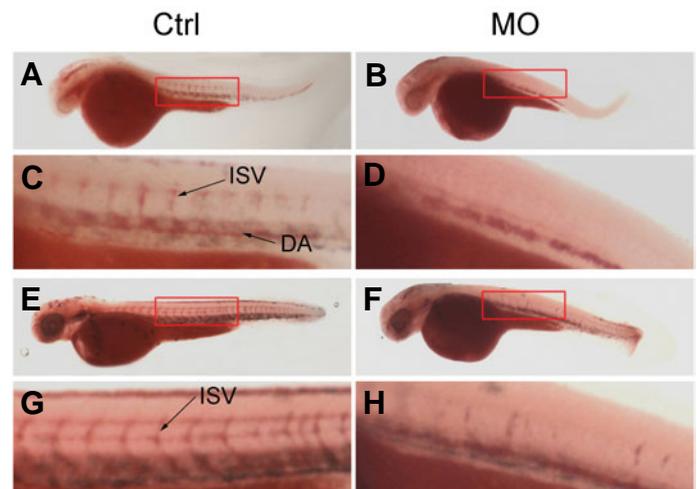


Fig. 8. Yolk lipid transportation defect detected by Oil Red O (ORO) staining of the vasculature of *CagApo-14* morphants. (A) Control embryo at 36 hpf. (B) *CagApo-14* morphant embryo at 36 hpf. (C) Magnification of the framed area in (A). (D) Magnification of the framed area in (B). (E) Control larvae at 50 hpf. (F) *CagApo-14* morphant larva at 50 hpf. (G) Magnification of the framed area in (E). (H) Magnification of the framed area in (F). ISV, intersegmental vessels; DA, dorsal aorta.

such as intestinal fatty acid binding protein (i-fabp) (Andre *et al.*, 2000). The concentrated localization of *CagApo-14* transcript and its protein product is similar to that of ApoE and ApoA-I, two apolipoproteins reported previously in zebrafish (Babin *et al.*, 1997). Both of them were considered to play important roles in the transportation of yolk nutrients to the developing embryos. Indeed, the yolk lipid transportation and utilization were demonstrated to be severely affected in the *CagApo-14* morphants (Fig. 8).

This study also revealed very rapid morphological changes around the time of hatching in gibel carp, including the shrinking yolk sac, the inflating swim bladder (Fig. 5), and the rapidly developing primordia of the gills, the jaws, and the gut and its associated organs (Fig. 6). In teleostean fish, after emerging from their chorions, the larvae encounter the outside environment directly, and obtain nutrition from the environment instead of the internal yolk supply. Three coherent trophic periods have been distinguished during embryogenesis and early larval development (Poupard *et al.*, 2000). During the endotrophic period, from fertilization to mouth opening, the endogenous yolk nutrients are the only energy source for the larvae (Kimmel *et al.*, 1995). After mouth opening, larvae undergo a transitional endo-exotrophic period, and then the exotrophic period starts. YSL plays important roles during endotrophic period, since YSL forms a barrier between the yolk cell and the developing embryo, and as a result all nutrients from the endogenous reserves must pass through the YSL to reach the embryo and the larva. The transition from endotrophic period to exotrophic period is a major change requiring the development of organs that can digest exogenous food. The digestive organs function co-operatively not only to digest and absorb nutrient, but also to provide a barrier against environmental toxins, and have important roles in immune defense (Wallace and Pack, 2003; Stainer, 2005; Bates *et al.*, 2006). The requirement of *CagApo-14* for yolk lipid transportation and utilization suggested that *CagApo-14* might play significant role at endogenous trophic period and endo-exotrophic transitional period. Additionally, *CagApo-14* protein was demonstrated to be ubiquitously distributed in adult fish tissues, and liver and intestinal bulb was revealed as main tissues for synthesizing *CagApo-14* protein (Fig. 4M). Thus, the extensive distribution should be attributed to the transportation of blood circulation, because high concentration of *CagApo-14* protein exists in the gibel carp serum (Fig. 4N).

In summary, we have characterized the fish-specific apolipoprotein *CagApo-14*, and revealed its expression pattern and physiological function in morphogenesis and organogenesis. We have demonstrated that *CagApo-14* is required for digestive system organogenesis during fish embryogenesis and larval development. Therefore, the fish-specific Apo-14 identified in this study provides a potential marker molecule to understand morphogenesis and organogenesis of digestive system.

Materials and Methods

Embryo and adult tissue collection

Gibel carp were bred in Guanqiao Experimental Station of the Institute of Hydrobiology, Chinese Academy of Sciences. Eggs and embryos were sampled in the spawning season according to previous

report (Xie *et al.*, 2003). Different tissues were sampled from 1-year-old gibel carp cultured in the Station. Total RNAs were isolated with SV total RNA Isolation System (Promega). SMART cDNA synthesis, library construction, PCR amplification, and dot-blot hybridization were performed as described previously (Liu and Gui, 2005).

Full length cDNA cloning and sequence analysis

The positive clones containing *CagApo-14* full length cDNA were screened from the gastrula SMART cDNA library as described previously (Liu *et al.*, 2005). DNA sequencing was carried out in Bioasia sequencing company. Homology searches were performed by BLAST at web servers of USA National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Homology comparison was performed using the ClustalW1.8 program. Prediction of signal peptide, glycosylation site, and phosphorylation site was done using software at the ExpASY Molecular Biology Server (<http://expasy.pku.edu.cn>).

RT-PCR analysis

Total RNAs were isolated by SV total RNA Isolation System (Promega) from the different tissues including brain, heart, kidney, liver, ovary, spleen, muscle, testis, oesophagus, intestinal bulb, gills and posterior intestine of 1-year-old gibel carp, and from embryos at different developmental stages including mature eggs, 5 minutes after fertilization, two cell stage, multi-cell stage, blastula, gastrula, neurula, tail bud, heartbeat, hatching and hatched larvae stage. The quality of RNA was measured by electrophoresis on 1% agarose gels. The isolated RNAs were, respectively, reverse-transcribed with M-MLV Reverse Transcriptase and oligo(dT) primer (Promega) as described by the manufacturer. The fragments representing *CagApo-14* and α -tubulin (as endogenous control) were respectively amplified by RT-PCR with specific primers:

RT-F (5'-ACAAGGAGTTAGTAGAGGAA-3') and

RT-R (5'-TCTATTATTGAAGTTTGCTG-3'),

α -tubulinF (5'-GTGCACTGGTCTTCAGGGGTT-3') and

α -tubulinR (5'-GGGAAGTGGATGCGTGGGTAT-3'). PCR conditions were as followed: pre-denaturation at 94 °C for 3 minutes, 30 cycles of 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1 min. Final extension at 72 °C for 5 minutes.

Riboprobe synthesis and whole-mount *in situ* hybridization

Embryos were fixed in 4% (w/v) paraformaldehyde according to a previous report (Yang *et al.*, 2001). The fragment amplified by RT-PCR was cloned to the pGEM-T vector (Promega) and linearized by *Not*I and *Nco*I, respectively. 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). The procedure of whole-mount *in situ* hybridization was performed as described previously (Yang *et al.*, 2001).

Fusion protein expression and antiserum preparation

The cDNA fragment coding for *CagApo-14* protein (141 aa) was subcloned into pET-32a expression vector (Novagen). Induced with 1 mM isopropyl-1-thio-B-D-galactopyranoside (IPTG), a soluble His-fusion protein of about 26 kDa was expressed in BL21 (DE3) *E. coli*. To acquire the antiserum, the expressed proteins were purified with His-tag purification kit (Novagene, USA) and applied to immunize white rabbit as described previously (Dong *et al.*, 2004).

To help establish whether a particular antiserum is specific to the antigen, the pre-adsorption was carried out as follows. The optimal ration of purified *E. coli* expressed antigen to anti-*CagApo-14* antiserum was determined by serial array of dilution. 0.2% diluted anti-*CagApo-14* antiserum was incubated with 10 μ g/ml purified antigen at 4 °C for 16 h in 0.5% non-fat milk/TBST. After centrifuging the antigen-antibody mixture at 16,000 \times g for 20 min, the supernatant was carefully pipetted into a clean vial and used in place of the normal antiserum in

the following Western blot detection and immunofluorescence analysis as controls.

Western blot detection

Western blot analysis was performed as described previously (Dong *et al.*, 2004). In brief, different tissues and embryos in different developmental stages were homogenized in 1 ml of chilled extract buffer (EB) (100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES pH 7.7, 1 mM DTT, 0.1 mM PMSF, 25 µg/ml aprotinin). Protein concentration was determined by staining with Coomassie brilliant blue R-250 and measured by Eppendorf Biomater. Protein samples were electrophoresed through a 12% SDS-polyacrylamide gel, and electroblotted onto a polyvinylidene difluoride membrane using a wet electroblotter (BioRad, USA). Membranes were blocked in non-fat milk powder in Tris-buffered saline with Tween (TBST; 5% skim milk powder, 25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20, pH 7.4) for 1 h at room temperature. After blocking, membranes were incubated with either anti-CagApo-14 antiserum (1:500 dilution) or anti-actin antiserum (Santa Cruz) for control at 4 °C for 16 h in 1.0% non-fat milk powder/TBST solution. The membranes were washed three times for 15 min each in TBST buffer and incubated with 1:2000 diluted alkaline phosphatase conjugated goat anti-rabbit IgG (Vector, USA). After washing three times for 10 min each in TBST buffer, detection was performed using BCIP/NBT and scanned with an Amersham scanner (Amersham, USA).

Immunofluorescence staining

Embryos at blastula, 5-somite stage, larvae of 1 day, 4 days and 6 days after hatching, and liver tissues from one year-old adult gibel carp, were fixed with 4% paraformaldehyde in PBS at 4 °C overnight. After washing with PBS (pH7.0) three times, the samples were immersed in 30% saccharose-PBS buffer overnight at 4 °C, 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 minutes, and blocked for 1 hour with 5% milk in PBS at room temperature to prevent non-specific binding of antibodies. The sections were then incubated with anti-CagApo-14 antibodies (1:400 dilution) for 16 hours at 4 °C, washed five times with PBS (10 minutes each), subsequently incubated for 1 hour 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 minutes each). After propidium iodide (PI, 5 µg/ml) or DAPI (1 µg/ml) staining of nuclei for 10 minutes, the sections were washed 3 more times with PBS (10 minutes each), and finally observed with a Leica confocal laser scanning microscope.

Morpholino design, plasmid construction and microinjection

Antisense Morpholino oligonucleotide (MO) (Gene Tools LLC) was designed to target the 5'-UTR of *CagApo-14* transcript. MO-*CagApo-14* and standard control morpholino (Gene Tools, Philomath, OR) were dissolved in water at a concentration of 2 µM and diluted to 0.3 µM in 1 × Danieau's buffer before injection. Morpholinos used in this study have the following sequences:

MO-*CagApo-14*: 5'-TGAGAGCAAATGTCAGCTTCA7CTT-3';

Control MO: 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

Bases in italics indicate the complement of the initiation codon for *CagApo-14* transcript. The ORF of *CagApo-14* was amplified with 4-base mismatch forward primer

misORF-F (5'-CTCGAGATGAAACTCACITTCGCTC-3', mismatch nucleotides leading to synonymous substitutions are underlined.) and ORF-R (5'-GGATCCACCCTCATGAGGCAGGAC-3'), and subcloned into pEGFP-N3 vector (clontech) in frame with EGFP. MOs or plasmids were injected into the yolk of 1–2 cell dechorionated embryos. 4 hours post-injection embryos were sorted, the unfertile or damaged removed and the rest allowed to grow at 24 °C for further observation.

Oil Red O staining

Embryos and larvae were fixed in 4% paraformaldehyde in PBS overnight at 4 °C, washed in 60% 2-propanol, and stained with freshly filtered 0.3% Oil Red O in 60% 2-propanol for 2 hours (Schlombs *et al.*, 2003).

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Int. J. Dev. Biol. (2006) 50: 553-560

Generation of the germ layers along the animal-vegetal axis in *Xenopus laevis*.

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