Molecular cloning and function analysis of insulin-like growth factor-binding protein 1a in blunt snout bream (*Megalobrama amblycephala*)

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Abstract: Insulin-like growth factor-binding protein 1 (IGFBP-1), a hypoxia-induced protein, is a member of the IGFBP family that regulates vertebrate growth and development. In this study, full-length IGFBP-1a cDNA was cloned from a hypoxia-sensitive Cyprinidae fish species, the blunt snout bream (*Megalobrama amblycephala*). IGFBP-1a was expressed in various organs of adult blunt snout bream, including strongly in the liver and weakly in the gonads. Under hypoxia, IGFBP-1a mRNA levels increased sharply in the skin, liver, kidney, spleen, intestine and heart tissues of juvenile blunt snout bream, but recovered to normal levels after 24-hour exposure to normal dissolved oxygen. In blunt snout bream embryos, IGFBP-1a mRNA was expressed at very low levels at both four and eight hours post-fertilization, and strongly at later stages. Embryonic growth and development rates decreased significantly in embryos injected with IGFBP-1a mRNA. The average body length of IGFBP-1a-overexpressed embryos was 82.4% of that of the control group, and somite numbers decreased to 85.2%. These findings suggest that hypoxia-induced IGFBP-1a may inhibit growth in this species under hypoxic conditions.

Keywords: *Megalobrama amblycephala*; IGFBP-1a; Hypoxia; Overexpression

The growth of vertebrates is primarily regulated by the insulin-like growth factors (IGF) signaling pathway (Baker et al, 1993; Duan & Xu, 2005; Reindl & Sheridan, 2012). Insulin-like growth factor-binding proteins (IGFBPs), with molecular weights of 24×10^3–50×10^3, bind IGFs and regulate their binding with corresponding receptors; thus, they regulate the biological functions of the IGF signaling pathway (Hwa & Rosenfeld, 1999; Firth & Baxter, 2002; Shimizu et al, 2005; Shen et al, 2012). All six IGFBP genes are known to exist in teleosts and have been characterized individually or as a group, including their phylogenetic relationships and the impact of genome duplication (Macqueen et al, 2013).

Currently, duplicate *igfbp-1*, i.e. *igfbp-1a* and *-1b*, have been found in zebrafish, Nile tilapia, fugu and medaka (Kamei et al, 2008). IGFBP-1 is the only one of the six IGFBP proteins that is regulated by hypoxia. Under hypoxia, IGFBP-1 expression increases, which suppresses the IGF signaling pathway (Kajimura et al, 2006; Kajimura & Duan, 2007). Hypoxia raises the expression levels of IGFBP-1 mRNA in hypoxia-tolerant grass carp embryos, which suppresses the growth and development rates of grass carp embryos (Sun et al, 2011). Here, we describe the molecular characterization and function analysis of IGFBP-1a in blunt shout bream (*Megalobrama amblycephala*), a hypoxia-sensitive and aquaculturally important species (Shen et al, 2010).

MATERIALS AND METHODS

Experimental fish

Blunt snout bream (*Megalobrama amblycephala*) adults and fertilized eggs were sampled from the Genetics and Breeding Center for Blunt Snout Bream of

Received: 29 September 2013; Accepted: 26 December 2013
Foundation items: This work was supported by grants from the Key Technologies Research and Development Program of China (2012BAD26B02), National Science Foundation of China (31272633; 31201760), Special Funding from the Ministry of Agriculture of China (201203081, 201203086), and Shanghai University Knowledge Service Platform (ZF1206).
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Shanghai Ocean University. Three ~1 000 g adult fish were sacrificed by immersion in MS-222 (tricaine methanesulfonate; Sigma, St. Louis, MO, USA) and their spleens, muscles, kidneys, eyes, hearts, gills, livers, intestines, brains, skins and ovaries or testes were removed, cryopreserved in liquid nitrogen, and then reserved at −80 °C. Fertilized eggs were obtained from artificial fertilization and hatched in embryo rearing solution (ERS) replaced every four hours. Meanwhile, 20 viable embryos were chosen, observed and photographed under a stereoscopic microscope (SMZ1500; Nikon, Japan) and then reserved in RNAstore preservation solution (TIANGEN BIOTECH, Beijing, China) at 4 °C.

Hypoxia treatments

Totally, 30 healthy juvenile blunt snout bream fish with weights of approximately 150–180 g were selected. After cultivation for one week in an automatic water cycle system at 20 °C, they were divided randomly into two experimental groups and a control group. The experimental facility was designed following Zhang et al (2003). With nitrogen bubbling continuously, the oxygen dissolved in the water was kept at 1.0±0.5 mg/L, a level of acute hypoxia. The control group was placed in an identical environment, that only differed in the dissolved oxygen level of 7.0±0.5 mg/L.

After experiencing hypoxia for four hours, five blunt snout bream from each group were chosen for dissection. The dissolved oxygen value for the anoxic groups was sequentially recovered over the course 60 min to a similar condition of the control group (7.0±0.5 mg/L). After 24 h later, five blunt snout bream from each oxygen-recovered group and the control group were chosen for dissection. Skin, muscles, eyes, gills, brains, intestines, spleens, livers, kidneys and hearts were extracted and then quick-frozen in liquid nitrogen and then preserved in a refrigerator at −80 °C.

Cloning of blunt snout bream IGFBP-1a cDNA

Total RNA was extracted from embryos 28 hours post-fertilization (hpf) using the TRIzol (Invitrogen) method, and randomly reverse transcribed into complementary DNA (cDNA) using M-MLV reverse transcriptase (TaKaRa, Japan) following the manufacturer's procedures. Based on the conserved region of IGFBP-1a mRNA of zebrafish (GenBank Accession No. NM_173-283.3) and grass carp (Sun et al, 2011), a pair of specific primers (IGFBP-1a-F, 5′-GCTGCCTCGCCTGTGCGT TGAAG-3′ and IGFBP-1a-R, 5′-TCCAGGATGACACA CACCAACAC-3′) were designed for blunt snout bream IGFBP-1a cDNA cloning, and reverse transcriptase polymerase chain reactions (RT-PCR) were performed. RACE and nested PCR procedures were performed using a SMART RACE cDNA Amplification Kit (Clontech, TaKaRa, Japan), while the primers (3′-IGFBP-1a-GSP3r, 5′-GCATGAA ATCCAAAGTCAACGCAATACG-3′, 3′-IGFBP-1a-GSP3n, 5′-AAA GTCAAACAGTGTGAATC GTCT C-3′, 5′-IGFBP-1a-GSP5r, 5′-GATTCACACTGT TTGACTTTG TAT-3′ and 5′- IGFBP-1a-GSP5n, 5′-GTGATTTAGT GATCTTGTCAAGGGC-3′; all synthesized by Sangon Biotech, Shanghai, China) were designed according to the kit's instructions. Detected by 1.5% agarose gel electrophoresis and purified using TIAN gel Midi Purification Kit (Tiangen Biotech, Beijing, China), the nested PCR product was ligated into the pMD19-T (TaKaRa, Japan) vector system.

Sequence analysis

Alignments of the 5-RACE, 3-RACE and intermediate region were performed using ClustalW within BioEdit 7.0. Primers at both ends were cut off and full-length IGFBP-1a cDNA of the blunt snout bream was assembled. Similarity searches using IGFBP-1a cDNA sequences and predicted amino acids using the BLAST n/p program were performed against the GenBank database, while multiple alignments of the homologous IGFBP-1 nucleotide and amino acid sequences of the vertebrates from the search were performed using the CLUSTAL W program. A neighbor-joining phylogenetic tree was constructed using MEGA 5.0 (Tamura et al, 2011) using bootstrap values based on 1 000 replications.

RT-PCR analysis

One microgram of DNAzyme digested total RNA from the tissues and different embryo developmental stages was reverse transcribed into cDNA using M-MLV reverse transcriptase (TaKaRa). The IGFBP-1a primers (IGFBP-1a-F and IGFBP-1a-R) and β-actin gene primers (β-actin RT-F, 5′-CCGCTGCCTCTTCTTCCTC-3′ and β-actin RT-R, 5′-CTACCTCCCTTTGCCA GTTTCCGC-3′) that crossed the introns were designed based on the β-actin full-length cDNA sequence of blunt snout bream and the genomic sequence of zebrafish. RT-PCR was performed using different tissues from adult and embryonic blunt snout bream using the above primer sets. The PCR conditions were as follows: an initial denaturation

of 5 min at 94 °C, 30 cycles of 30 sec denaturation at 94 °C and 30 sec annealing at 55 °C followed by 72 °C for 30 sec and a final extension at 72 °C for 10 min. The PCR products were detected using 1.2% agarose gel electrophoresis and then photographed.

qRT-PCR analysis

Total RNA was extracted from tissues of the hypoxia groups and the control group using TRIzol (Invitrogen) and reversed transcribed into cDNA using Oligo(dT)18 primer and M-MLV. Quantitative real-time PCR were performed in an iCycler iQ Multicolor using the cDNA template and the IGFBP-1a primers (IGFBP-1a-F and IGFBP-1a-R) and β-actin gene primers (β-actin RT-F and β-actin RT-R). Quantitative analysis was performed using the $2^{-\Delta\Delta Ct}$ method (Shen et al, 2010).

***in vitro* synthesis and microinjection of IGFBP-1a capping mRNA**

The IGFBP-1a open reading frame (ORF) was subcloned into pCS2+ vector to construct the pCS2-IGFBP-1a. Capped IGFBP-1a mRNA of the blunt snout bream as well as GFP mRNA were synthesized *in vitro* using a commercial kit (mMESSAGE mMACHINE Kit; Ambion, TX, USA) with linearized plasmid DNA as the template.

The IGFBP-1a mRNA (at a concentration of 500 pg/embryo) and EGFP mRNA (a concentration of 200 pg/embryo) were together injected into fertilized eggs at the 1–2-cells stage of blunt snout bream. The injection volume was 1–2 nL as previously described (Jiang et al, 2012). After injection, fertilized eggs were incubated in ERS at 25°C. The embryo injected with EGFP mRNA was used as a control. Growth status was observed under a Nikon SMZ1500 fluorescence microscope. Phenotypes of injected eggs was observed every 4 hours.

Statistical analysis

Data from qRT-PCR, somite number and body length are expressed as mean±*SE*). Differences among groups were analyzed with a one-way ANOVA followed by Fisher's post hoc tests or unpaired t-tests. Significance was accepted at the level of $P<0.01$ or 0.001.

RESULTS

Cloning of full-length cDNA of blunt snout bream IGFBP-1a

The full length of the cDNA of blunt snout bream IGFBP-1a is 1 178 bp, containing 116 bp of 5′-UTR and 273 bp of 3′-UTR, which contains a microsatellite sequence of 49 CA repeats at the 913–1 030 bp region (Figure 1). The Cys residues of the protein are the same as the IGFBPs of other vertebrates in both number and position. The N-terminal of the protein contains a representative IGFBP sequence motif (GCGCCXXC) and the C-terminal contains a thyroglobulin-1 sequence motif (CWCV), whereas the intermediate region is less conserved. Clustering analysis confirmed that duplicated *igfbp-1*s (*igfbp-1a* and *-1b*) exist in teleosts (Figure 2), and the *igfbp-1* gene we cloned fell into the *igfbp-1a* clade.

IGFBP-1a expression features in tissues and expression regulation under hypoxia

IGFBP-1a mRNA was expressed in most tissues of adult blunt snout bream, and the transcription was highest in the liver and very weak in the testis and ovary (Figure 3A). qRT-PCR analysis of IGFBP-1a mRNA in each tissue type was performed after hypoxia and oxygen recovery (Figure 3B). The IGFBP-1a transcription level in the skin, livers, kidneys, spleens, intestines and hearts of juvenile blunt snout bream increased sharply (18.5, 19.0, 8.8, 22.1, 16.9 and 29.5 times those of the control group, respectively). After 24-hour exposure to normal dissolved oxygen, transcription in the tissues returned to normal levels. The IGFBP-1a transcriptional levels in the eyes, muscles, brains and gills were not influenced by hypoxia.

IGFBP-1a overexpression significantly reduces blunt snout bream embryonic development and growth rate

Blunt snout bream IGFBP-1a mRNA was expressed throughout the embryonic development process. The expression level was very low at the 4 and 8 hpf embryonic development stages, increased significantly at 12 and 16 hpf and remained stable after 20 hpf (Figure 4A).

To further determine the function of blunt snout bream IGFBP-1a, capped IGFBP-1a mRNA and EGFP mRNA were injected together into fertilized eggs at the blunt snout bream 1–2-cell stage. Capped EGFP mRNA was injected as a control. Injection success was determined by detecting the expression of EGFP. The 24 hpf embryos injected with EGFP mRNA did not significantly differ from those of wild type embryos in terms of morphology, somite number and body length (Figure 4B, 4D). Compared with the wild type or EGFP mRNA injected group (Figure 4B, 4D, 4F), embryos injected with IGFBP-1a mRNA showed a decrease ($P<0.01$) in body length and somite number, 30% (18/60) of which were malformed to various

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1    AAGCAGTGGTATCAACGCAGAGTCATTTTGAATAGCGCAAAGACAGCGAGGAACTCCTCATTTCACTTCAGCTGATATTTATTTATTTAT
91   CTATTTATTCTGAGCCTTGCTTTGGGATGAACAGACTGCTTCTGAACTTTTTCTGGGTGGCAGCATTCAGCGCACTCCTTTCAGCGCCGG
                              M  N  R  L  L  L  N  F  F  W  V  A  A  F  S  A  L  L  S  A  P
181  GGCTCCGGGCTTCCCCAGTGGTTGGGCAGGAACCTATCCGCTGCGCCCCGTGCTCCCCGGAGCGGCTGGCCGAGTGTCCTGCGGTGGATG
     G  L  R  A  S  P  V  V  G  Q  E  P  I  R  C  A  P  C  S  P  E  R  L  A  E  C  P  A  V  D
271  CCGGCTGTGAGGAGGTGCTTCGAGAGCCGGGCTGCGGCTGCTGCCTCGCCTGTGCGTTGAAGAGAGGTGACCCGTGCGGGATCTACACTG
     A  G  C  E  E  V  L  R  E  P  G  C  G  C  C  L  A  C  A  L  K  R  G  D  P  C  G  I  Y  T
361  CGCCCTGCGGCTCGGGGCTCCGCTGCTTGCCGAAACCTGGAGAAGCCCGACCCCTGCACGCTCTCACCCGAGGACAGGCGGTGTGCACTG
     A  P  C  G  S  G  L  R  C  L  P  K  P  G  E  A  R  P  L  H  A  L  T  R  G  Q  A  V  C  T
451  AGACCCCCAAGCCTGATCAGAGCCAAAGCGACACAACGCCAGATCATCCTGAGTCCAACAACGGGGCAATGTCCGTGAATGAAGGCAGCT
     E  T  P  K  P  D  Q  S  Q  S  D  T  T  P  D  H  P  E  S  N  N  G  A  M  S  V  N  E  G  S
541  CAGCGGTCTTCGTGCCCGGGCACGGCAAGCCCTTCGACCCGCGGGTCATCACTGCTAAAGAGAGCATGAAGGCCAAAGTCAACGCTATAC
     S  A  V  F  V  P  G  H  G  K  P  F  D  P  R  V  I  T  A  K  E  S  M  K  A  K  V  N  A  I
631  GCAAGAAACTGGTGGAGCAGGGTCCTTGTCATATGGAACTGCAGACGGCCCTTGACAAGATCACTAAATCACAGCAGAAACTGGGAGACA
     R  K  K  L  V  E  Q  G  P  C  H  M  E  L  Q  T  A  L  D  K  I  T  K  S  Q  Q  K  L  G  D
721  AAATGACCAGATTCTACCTTCCCAATTGCGACAAACACGGTCTATACAAAGTCAACCAGTGTGAATCGTCTCTGGATGGTCAGAGGGGGA
     K  M  T  R  F  Y  L  P  N  C  D  K  H  G  L  Y  K  V  N  Q  C  E  S  S  L  D  G  Q  R  G
811  AGTGTGGGTGTGTCATCCTGGAATGGGAAGAAGATTCCTGGATCAAGTGACCTGCCACCAGATGCCGAGTGTCCCGAGGAACTCAACC
     K  C  G  C  V  S  S  W  N  G  K  K  I  P  G  S  S  D  L  P  P  D  A  E  C  P  E  E  L  N
901  ACTGATCTGTTTCACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACA
     H  *
991  CACACACATATAGGGGGACCTAAAGAGCCACACACACACATGTAATACCACTGAATTTTATCATTATATTTTTTAAAAATATCTTAGTAT
1081 TTATTTATTGTTAGTGGTGATATTTAATTCAGGTACACTGTTTTTGATTGGTAAATTCCCTGAACTTTCCAAAAAAAAAAAAAAAAAAA
1171 AAAAAAAA
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Figure 1 cDNA and encoding amino acid sequences in blunt snout bream *igfbp-1a*

The microsatellite sequence containing CA repeats is shown in the block. The PPD domain is shown in the dash block.

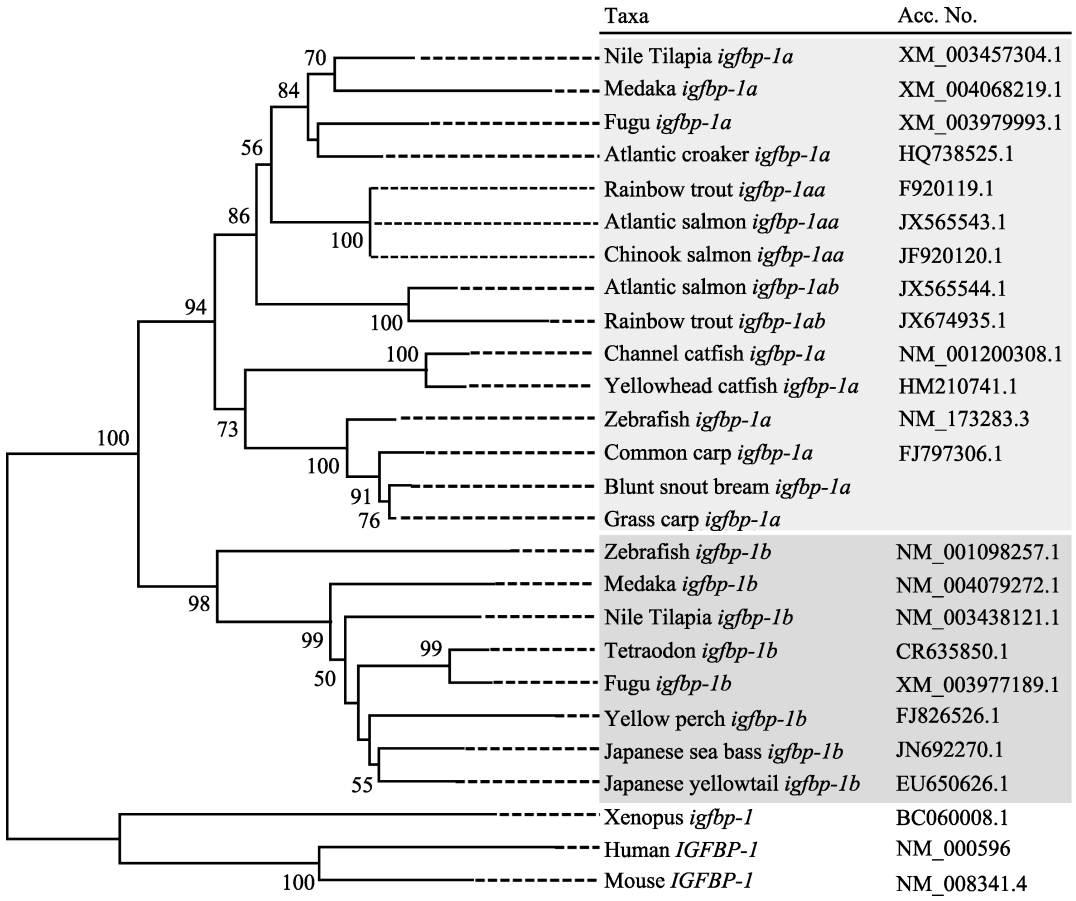

Figure 2 Clustering analysis of vertebrate *igfbp-1* amino acid sequences constructed using the neighbor-joining method

Accession numbers of sequences retrieved from GenBank are shown. Similarity searches and phylogenetic analysis revealed that the IGFBP-1a of blunt snout bream is most closely related to that of grass carp, common carp and zebrafish, with identities of 97%, 93% and 92%, respectively. The IGFBP-1a of blunt snout bream shows only about 60% identities with that of channel catfish (*Ictalurus punctatus*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynehus mykiss*) and Japanese yellowtail (*Seriola quinqueradiata*), and about 50% with that of mouse (*Mus musculus*) and human (*Homo sapiens*).

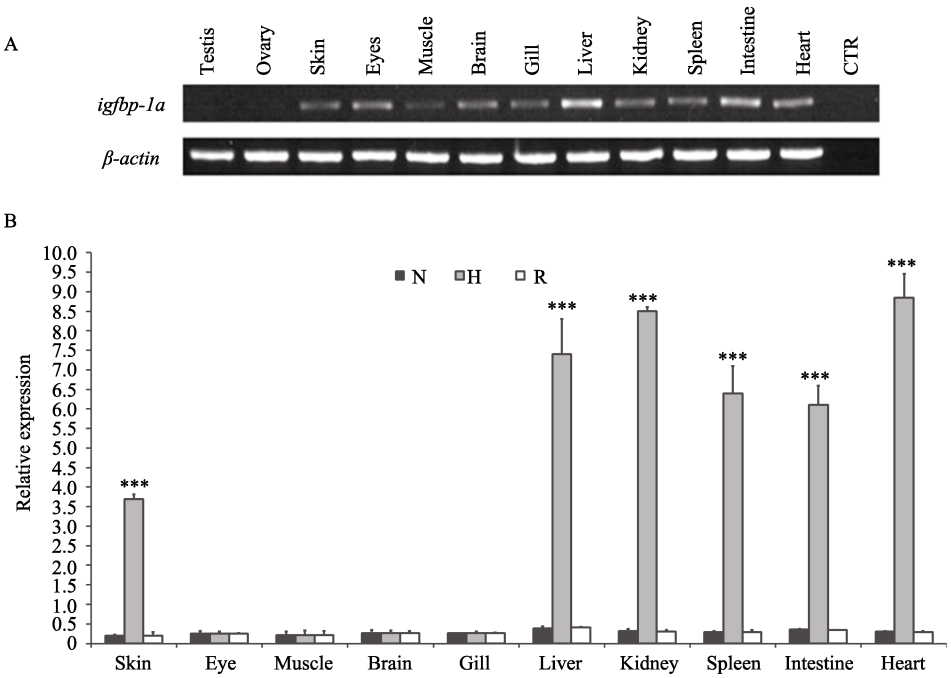

Figure 3 Expression of blunt snout bream IGFBP-1a mRNA in adult tissues and the influence of the hypoxia treatment on juvenile blunt snout bream

A: RT-PCR analysis of blunt snout bream IGFBP-1a in adult tissues; CTR, negative control without genomic DNA; B: qRT-PCR analysis of IGFBP-1a mRNA at different tissues of juvenile blunt snout bream during normoxic (N: 7.0±0.5 mg O_2/L), hypoxic (H: 1.0±0.5 mg O_2/L) and recovery (R) conditions. IGFBP-1a mRNA copy number was normalized as a ratio to the β-actin (a house keeping gene) mRNA copy number. qRT-PCR data are presented as mean±SE; ***: P<0.001.

degrees (Figure 4C, 4E, 4G). Blunt snout bream embryos in the IGFBP-1a mRNA injected group grew more slowly (P<0.01) than the wild type or EGFP mRNA injected group (Figure 4H). At 24 hpf, the average body length of the IGFBP-1a injected embryos was 2.8±0.3 mm, 82.4% of that of the EGFP mRNA injected group (3.4±0.1 mm). Additionally, 24.9±2.6 somites was found in embryos injected with IGFBP-1a mRNA at 24 hpf, which is only 85.2% of the EGFP mRNA injected control (29.2±1.6) (Figure 4I), equivalent to embryos at 17–20 hpf of the control group. These results indicate that increasing IGFBP-1a mRNA level in blunt snout bream embryos significantly decreased their growth and development rates.

DISCUSSION

We obtained the full-length IGFBP-1a cDNA in hypoxia-sensitive blunt snout bream, an aquaculturally important cyprinid fish. The 3′-UTR of the IGFBP-1a cDNA in blunt snout bream contains a microsatellite sequence of 49 CA repeats, which could be only found on IGFBP-1a in grass carp (39 CA) and IGFBP-1b in Japanese yellowtail (79 CA) and zebrafish (28 CA). Neither the cause or function of the microsatellite sequence of the CA repeats have been elucidated. Clustering analysis revealed that duplicated *igfbp-1*s (*igfbp-1a* and *-1b*) exist in teleost fish and should result from fish-specific 3rd whole genome duplication. In addition, duplicated *igfbp-1a*s exists in the genome of pseudo-tetraploid Atlantic salmon and rainbow trout, which are considered pseudo-tetraploid fish species (Macqueen et al, 2013). The duplicated *igfbp-1* genes were supposed to be the result of the 3rd or 4th genome duplication specific to fishes (Taylor et al, 2003; Jaillon et al, 2004; Crow et al, 2006; Ocampo Daza et al, 2011).

The expression of IGFBP-1a mRNA was detected in a wide range of tissues in adult blunt snout bream. Under hypoxia, the expression levels of IGFBP-1a mRNA increased greatly in many tissues in juveniles, and recovered to normal levels after 24-hour exposure to normal dissolved oxygen. Similar to our result, average expression levels in the liver of adult zebrafish under hypoxia is approximately 280 times that of the control group (Maures & Duan, 2002, Kajimura et al, 2005). Studies of grass carp and Atlantic croaker also show increases in IGFBP-1a mRNA expression levels under hypoxia (Sun et al, 2011; Rahman & Thomas, 2011).

In blunt snout bream embryos, IGFBP-1a is highly expressed at 12 hpf and only weakly at 4 and 8 hpf. This

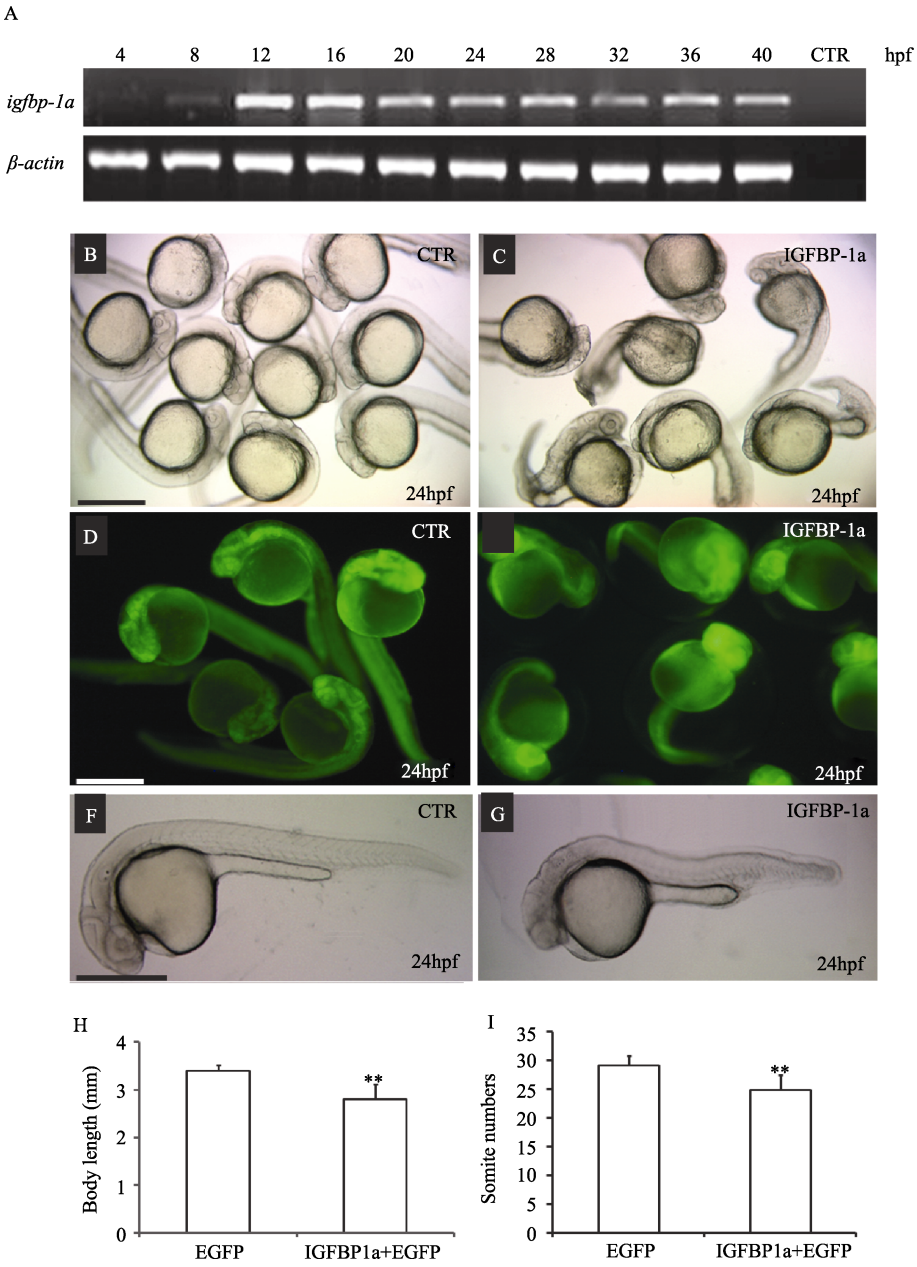

Figure 4 Overexpression of IGFBP-1a reduced growth and developmental rate in blunt snout bream embryos

A: RT-PCR analysis of IGFBP-1a mRNA in blunt snout bream during embryogenesis. Developmental stages are indicated above the figure. hpf, hour post-fertilization. CTR, negative control without genomic DNA; B–G: Blunt snout bream embryos are shown at the 24 hpf stage by visible light (B, C, F, G) and fluorescence (D, E). EGFP mRNA (200 pg) (B, D and F) or a mixture of EGFP mRNA (200 pg) with IGFBP-1a mRNA (500 pg) (C, E and G) was injected into each embryo at the 1–2 cell stage. The body length (H) and somite number (I) of IGFBP-1a and/or EGFP mRNA overexpressed blunt snout bream embryos at 24 hpf. The results are presented as mean ± SE; $^{**}P<0.01$. Scale bar=600 μm.

is different to hypoxia-tolerant grass cap embryos, in which IGFBP-1a mRNA expression was not detected at 4 or 8 hpf, and only trace amounts at 12 hpf (Tao & Zou, 2011). In zebrafish, transcription of the duplication IGFBP-1b gene could not be detected until the later embryo developmental stage, but IGFBP-1a is present at all stages (Kamei et al, 2008), similar to the expression characteristic of blunt snout bream. Fish eggs carry maternal mRNA and embryonic transcription of its own mRNA dates back to the 9th cleavage (Kane & Kimmel, 1993). The IGFBP-1a mRNA detected at early stages is likely from the maternal deposit and the significant increase in mRNA during the middle and later stages results from embryonic transcription.

Microinjection of capped IGFBP-1a mRNA caused significant developmental retardation and growth reductions

in blunt snout bream embryos. It will be interesting to test whether hypoxia induces IGFBP-1a expression in blunt snout bream embryos in the future. Similar to the present results, hypoxia-induced growth reduction was also caused by high IGFBP-1a expression in grass cap embryos (Sun et al, 2011). In zebrafish, hypoxia stimulates increased IGFBP-1a transcription during early embryonic development and causes embryo developmental retardation and growth reductions, whereas only IGFBP-1b is affected by hypoxia during later stages (Kamei et al, 2008). Accordingly, IGFBP-1a likely functions as a bridge between hypoxia and the inhibition of embryonic growth in fish.

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