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IGFBP7 is involved in abalone metamorphosis

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ABSTRACT

Metamorphosis is the most critical life stage for abalone. There are morphogenetic transformations and habitat selection with metamorphosis. A large amount of larvae often die during this stage. Insulin/insulin-like growth factor signaling pathway (IIS) plays a role in the development. However, there is little knowledge of IIS function in metamorphosis. IGFBP7, a component of IIS, was found to be involved in metamorphosis of small abalone *Haliotis diversicolor*. The expression of *saIGFBP7* mRNA rapidly increased before metamorphosis, which were mainly localized in the velum and foot. There was a significant decrease of metamorphosis rate after competent larvae were exposed to 5 μg/mL dsRNA of *saIGFBP7* ($p = 2.50 \times 10^{-10}$, $F = 389.05$). The expressions of three downstream genes of IIS (*PI3K*, *ERKα* and *AKT*) as well as *saIGFBP7* were decreased in the dsRNA exposure experiment. There was no significant difference of metamorphosis rate ($p = 0.97$, $F = 0.071$) after competent larvae were induced in different concentrations of insulin. The expression of *saIGFBP7*, *PI3K* and *ERKα* also had no significant difference in insulin inducing assay. However, *AKT* expression level of 1 μmol/L insulin induced group was significantly higher than that of other concentrations ($p = 3.41 \times 10^{-7}$, $F = 26.68$). These results suggested that IGFBP7 is involved in metamorphosis of abalone larvae. Our study provides insight on developing new methods for increasing the metamorphosis rate in abalone aquaculture. However, it does not work as a negative regulator of IIS activity, or plays its role in an IGF- and insulin-independent manner during metamorphosis of abalone larvae.

1. Introduction

Life histories of marine invertebrates are extremely diverse. Still, the general theme of a pelago-benthic life cycle is metamorphosis, in which a free-swimming larval form feeds and/or disperses in the plankton for a time, and then settles into the benthic habitat where it undergoes a more or less dramatic transition both morphologically and physiologically to its juvenile/adult habitat on the sea floor (Heyland and Moroz, 2006).

Abalone is an ideal model for the study of marine invertebrate, because its larva is lecithotrophic and larval metamorphosis is strongly induced by chemical cues (Morse et al., 1979; Roberts, 2001). Many chemical cues are able to induce abalone larvae settlement and metamorphosis, such as γ-aminobutyric acid (GABA) (Morse et al., 1979), thyroid hormones (THs) (Fukazawa et al., 2001), serotonin (Wang et al., 2010), and chemical substances in certain crustose red algae (Morse and Morse, 1984). Small abalone (*Haliotis diversicolor*) is commercially important in the southeast coast of China as a primary cultured species. The hatchery-produced seeds are very important for abalone culture. However, a major problem in seeding production is poor larval metamorphosis resulting in low larvae survival rate. Higher metamorphosis rate of larval abalone is very essential in successful culture industry (Li et al., 2006). Unfortunately, there are few methods to keep high metamorphosis rate, because the mechanism of abalone metamorphosis remains largely unknown.

Apoptosis of larval tissues occurs in parallel with the differentiation of adult tissues during the metamorphic transition. Metamorphosis is a process that removes larval tissues and builds up adult tissue. For example, the entire larval musculature is replaced in the adult. On the level of the nervous system, adult-specific neurons establish new connections (reviewed by Heyland and Moroz, 2006). Therefore, signaling pathways involving in apoptosis/differentiation may play roles in metamorphosis.

Insulin/insulin-like growth factor signaling pathway (IIS) is well conserved from yeast to humans (Barbieri et al., 2003), and plays important roles in diverse biological processes, such as growth, energy metabolism, and development. IIS contains secreted ligands

Abbreviations: BC, blank control; Dilp, *Drosophila* insulin-like peptide; dsRNA, double-stranded RNA; FSW, filtered seawater; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; hpf, hours post-fertilization; IB, insulin-like growth factors binding; IIS, insulin/insulin-like growth factor signaling pathway; IGFBP7, insulin-like growth factor binding protein 7; IGFBPs, insulin-like growth factor binding proteins; IGFR, insulin-like growth factor receptor; ILPs, insulin-like peptides; Imp-L2, imaginal morphogenesis factor—late 2; InR, insulin receptor; MAPK, mitogen-activated protein kinases; NC, negative control; PBS, phosphate saline; PFA, paraformaldehyde; PI3K, phosphoinositide 3-kinase; PTTH, prothoracicotropic hormone; qRT-PCR, quantitative real time PCR; THs, thyroid hormones; WISH, whole mount in situ hybridization.

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(insulin-like peptides [ILPs]), tyrosine kinase receptors (insulin receptor [InR], insulin-like growth factor receptor [IGFR]), and ligands binding proteins (insulin-like growth factor binding proteins [IGFBPs]). IIS regulates neuronal proliferation, survival, and neurite outgrowth (Lau and Chalasani, 2014). In *Drosophila*, IIS regulates metamorphosis by controlling ecdysone production (Sarraf-Zadeh et al., 2013). These reports suggest that IIS may also play a role in abalone metamorphosis.

These secreted ligands of IIS transmit intercellular signals through the activation of InR or IGFR, leading to the activation of protein kinase signaling pathways, such as phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinases (MAPK), and p53 (Zhu et al., 2014). IGFBPs modulate the activity of IIS by binding to ILPs with high affinity. IGFBPs are a group of cysteine-rich proteins, which are primarily characterized by the presence of an N-terminal IGFs binding (IB) domain. They were grouped together, and named IGFBP-superfamily (Kim et al., 1997; Baxter et al., 1998; Hwa et al., 1999; Rosenfeld et al., 1999).

IGFBP7 is also known as IGFBP-related protein-1 (IGFBP-rP1), MAC25, and tumor-derived cell adhesion factor (TAF). It belongs to the IGFBP-superfamily, which is involved in the regulation of IIS. Unlike IGFBP1-6, IGFBP7 binds IGFs with low affinity, but recognizes insulin with a high affinity, and thereby modifies its metabolism, distribution, and ability to bind to the insulin receptor (Yamanaka et al., 1997). In vertebrate, IGFBP7 exits in a wide range of normal tissues, as well as in biological fluids (Degeorges et al., 2000; Lopez-Bermejo et al., 2003). Its biological functions have been focused on various cancers, and may become as a tumor suppressor gene through the induction of apoptosis and senescence (reviewed by Zhu et al., 2014).

There are few reports of invertebrate IGFBP7. Those reports mainly come from *Drosophila*, *Bombyx* and *Caenorhabditis*. There is only one IGFBP in *Drosophila*, Imp-L2 (Imaginal morphogenesis factor-late 2), which is the homolog of vertebrate IGFBP7 (Honegger et al., 2008). In situ hybridizations and immunohistochemistry revealed Imp-L2 expression in the corpora cardiaca and prothoracic portions of the ring gland, in enteroendocrine cells in the anterior midgut, and in distinct neurons in both brain hemispheres and the subesophageal ganglion (Honegger et al., 2008; Sarraf-Zadeh et al., 2013). Imp-L2 is involved in differentiation of adult-specific tissues (Honegger et al., 2008). The Imp-L2 production by prothoracicotropic hormone (PTTH) expressing neurons regulates *Drosophila* metamorphosis by adjusting ecdysone production (Sarraf-Zadeh et al., 2013).

There are two kinds of IGFBP7 homologs in *B. mori* (Ma and Zhang, 2010, Gao et al., 2012).The role of IGFBP7 in metamorphosis of *B. mori* has never been illuminated. There are 3 homologs of IGFBP7 in *Caenorhabditis elegans*. One of them was involved in development (Khatibzadeh et al., 2009). We found a homolog of IGFBP7, saIGFBP7, in small abalone *H. diversicolor*. The *saIGFBP7* mRNA and protein could be detected in all examined tissues, with the highest expression level in hemocytes, higher expression level in gills, and was up-regulated in hemocytes and gills after bacterial injection (Li et al., 2012). Moreover, we found that overexpression of *saIGFBP7* increased the hemocyte density, as well as, silencing *saIGFBP7* decreased the hemocyte density. This means that saIGFBP7 was involved in hemocyte proliferation of abalone (Wang et al., 2015). This suggests that saIGFBP7 may also play a role in proliferation of adult tissues during metamorphosis.

In this study we showed that *saIGFBP7* mRNA and protein were firstly detected in trochophore stage, increased during swimming larvae stage, reached a vertex in metamorphosis, and kept in juvenile stage. The expression pattern was similar to Hemp, a novel epidermal growth factor like protein, which plays a central role in ascidian metamorphosis (Eri et al., 1999). Down-regulation of *saIGFBP7* mRNA by RNAi significantly decreased metamorphosis rate. The analysis of *saIGFBP7* function will enhance our understanding of metamorphosis, and contribute to abalone seed production.

2. Materials and methods

2.1. Spawning and larval culture

Spawning of adult *H. diversicolor* and larval culture were carried out at Hongyun Aquaculture Co. Limited. On the night of a predicted spawning event, brood stock were placed into individual spawning aquaria with UV-irradiated seawater and allowed to spawn freely. Eggs were collected by 100-μm wet screens, and were back washed into a 1 L beaker and fertilized for 5 min with sperm collected from male spawning aquaria. Fertilized eggs were thoroughly washed with sand-filtered seawater (FSW), and put in a 50 L tub. The upper 2/3 seawater in tub was poured out, fresh seawater was poured in, and every 1 h until trochophore larvae were hatched. Larvae were poured into a 200 L aquarium until they were used in RNAi assays. A brood of larvae were derived from gamete contributions from at least three males and three females. There are six broods of larvae from different parents. The samples of each brood were collected at different development stages. The samples were put into liquid nitrogen, or into 4% paraformaldehyde (PFA). After trochophore stage, larvae were added dropwise with $MgCl_2$ saturated solution to paralyze muscles, before being put into 4% PFA with 1× phosphate saline (PBS: 0.8% NaCl, 0.02% Na_2HPO_4, pH 7.4). After 24 h, the samples in PFA were transferred to methanol by gradual dehydration (25%, 50%, 75%, 95% and 100% methanol; 5 min per step). The samples in methanol were maintained at −20 °C until use for in situ hybridization.

2.2. Double-stranded RNA (dsRNA) generations

The fragment of *saIGFBP7* (136 bp–819 bp of AEE01360.1) was amplified by PCR using ds-FT7 and ds-R primers or using ds-F and ds-RT7 primers. A 657 bp fragment of the green fluorescent protein (GFP) gene from the pEGFP-N1 vector was also amplified by PCR using ds-c-FT7 and ds-c-R or using ds-c-F and ds-c-RT7. The sequences of primers are shown in Table 1. The italics at 5′ ends are T7 promoter sequences.

After being purified and sequenced, the expected PCR products were used as templates to transcribe single-stranded RNA (ssRNA) by T7 phage RNA polymerases (Promega, USA). The DNA templates were then degraded by addition of DNase I (Promega, USA) at a ratio of 1 U/μg of template. The transcripts were then purified by organic solvent extraction using standard methods. The sense and antisense cRNA strands were mixed in 400 mM NaCl–10 mM Tris–Cl (pH 7.4) and annealed by incubation at 75 °C for 15 min, at 65 °C for 15 min, and at room temperature for 15 min. The formation of dsRNA was monitored by determining the size shift in agarose gel electrophoresis, and the concentration of dsRNA was measured by Ultrospec™ 2100 pro spectrophotometer (Amersham Biosciences, Sweden). The dsRNA of *saIGFBP7* was used to silence *saIGFBP7*. GFP dsRNA was used as control.

2.3. dsRNA exposure assay

Larvae were collected by 100 μm screens, and washed with 0.2 μm FSW, then transferred into 10 mL test tubes, until 80% larvae developed eyespots (competent larvae). The *saIGFBP7* dsRNA/GFP dsRNA was added in a test tube and adjusted to a final concentration of 5 μg/mL. The FSW with *saIGFBP7* dsRNA/GFP dsRNA was regarded as RNAi group/negative control (NC) group. The FSW without any addition was regarded as blank control (BC) group. After 2 h, larvae were transferred into a 1 L beaker covered by benthic diatom. 10 h after being transferred in a beaker, larvae were collected. Approximately 50 larvae were used to count the number of metamorphosed and dead larvae. Then these larvae were treated by PFA as 2.1 descriptions for in situ hybridization. Metamorphosed larvae lost their cilia and began formation of the juvenile shell. Dead larvae were indicated by deterioration of the