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Molecular characteristic of activin receptor IIB and its functions in growth and nutrient regulation in *Eriocheir sinensis*

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Activin receptor IIB (*ActRIIB*) is a serine/threonine-kinase receptor binding with TGF-β superfamily ligands besides myostatin to play the role in regulation of muscle growth in vertebrates. However, information about the function of the receptor in *Eriocheir sinensis* with specific molting to growth is scarce. In this study, the 4916 bp full-length cDNA of *EsActRIIB* was cloned, including a 1683 bp ORF encoding 560 amino acids with characteristic domains of TGF-β type II receptor superfamily. *EsActRIIB* expressions detected in all tested tissues suggested that there were changes in different molting stages, with a higher expression in the hepatopancreas tissue and peak at the premolt stage in muscles. RNA interference with *EsActRIIB* during whole molting cycle showed that a higher weight gain rate (WGR), higher specific growth rate (SGR) and lower hepatopancreas index (HI) were achieved in individuals with injection of dsRNA, while comparing to the control group ($P<0.01$). Nutrition ingredient analysis showed that the ~~contents of~~ amino acid (TAA, TEAA and TNEAA) increased significantly in hepatopancreas and muscle; and fatty acid composition (SFA, PUFA and MUFA) was affected in hepatopancreas ($P<0.05$). Furthermore, the expression levels of some target genes (*ActRI*, *FoxO*, *CPT1β*, *PL*, *FAS* and *FAE*) were affected in hepatopancreas and muscle, yet no change was noticed in *Smad3*, *Smad4* and *mTOR*. These results indicated that *EsActRIIB* negatively regulate muscle growth during the molting process, most probably through controlling protein synthesis and lipid metabolism in *Eriocheir sinensis*.

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Abstract

Activin receptor IIB *(ActRIIB)* is a serine/threonine-kinase receptor binding with TGF-β superfamily ligands besides *myostatin* to play the role in regulation of muscle growth in vertebrates. However, information about the function of the receptor in *Eriocheir sinensis* with specific molting to growth is scarce. In this study, the 4916 bp full-length cDNA of *EsActRIIB* was cloned, including a 1683 bp ORF encoding 560 amino acids with characteristic domains of TGF-β type II receptor superfamily. *EsActRIIB* expressions detected in all tested tissues suggested that there were changes in different molting stages, with a higher expression in the hepatopancreas tissue and peak at the premolt stage in muscles. RNA interference with *EsActRIIB* during whole molting cycle showed that a higher weight gain rate (WGR), higher specific growth rate (SGR) and lower hepatopancreas index (HI) were achieved in individuals with injection of dsRNA, while comparing to the control group ($P<0.01$). Nutrition ingredient analysis showed that ~~the contents~~ of amino acid (TAA, TEAA and TNEAA) increased significantly in hepatopancreas and muscle; and fatty acid composition (SFA, PUFA and MUFA) was affected in hepatopancreas ($P<0.05$). Furthermore, the expression levels of some target genes (*ActRI*, *FoxO*, *CPT1β*, *PL*, *FAS* and *FAE*) were affected in hepatopancreas and muscle, yet no change was noticed in *Smad3*, *Smad4* and *mTOR*. These results indicated that *EsActRIIB* negatively regulate muscle growth during the molting process, most probably through controlling protein synthesis and lipid metabolism in *Eriocheir sinensis*.

Introduction

The transforming growth factor-β (TGF-β) superfamily consists of a large number of structurally and functionally related cytokines subfamilies, including TGF-βs, bone morphogenetic proteins (BMPs), activins and growth differentiation factors (GDFs), which regulate a series of biological processes of cell differentiation, muscle growth and embryonic development (Santibanez, Quintanilla & Bernabeu, 2011; Morikawa, Derynck & Miyazono, 2016). The TGF-β family members exert their cellular functions via two heteromeric complexes of transmembrane proteins, type I and type II receptors, and activate the *Smad*-dependent or *Smad*-independent signaling pathways (Hata & Chen, 2016; Nickel, Ten & Mueller, 2018). Among the TGF-β receptors, activin receptor type IIB (*ActRIIB*) belongs to serine/threonine kinase transmembrane receptor, which encode about 500 amino acids characterized by an extracellular ligand-binding domain, a transmembrane domain and an intracellular serine/threonine kinase domain (Thompson, Woodruff & Jardetzky, 2003; Sako et al., 2010). *ActRIIB* mediates TGF-β signaling pathways with high affinity for ligands like activins and *myostatin* (Sako et al., 2010). *ActRIIB* has been known in regulating muscle growth, embryonic development and reproduction in vertebrates (Mathews, Vale & Kintner, 1992; Chen et al., 2015; Morvan et al., 2017).

Myostatin is well-known as a negative regulator of muscle growth, acting as inhibitor of muscle growth functioning predominantly via *ActRIIB* receptor (Han et al., 2013). Since the discovery of the *myostatin*/*ActRIIB* signaling pathway in 1997 (McPherron, Lawler & Lee; 1997), the

strategies to regulation of muscle growth by blocking *myostatin* ligands signaling through *ActRIIB* were developed greatly (Amthor & Hoogaars, 2012). Experimental evidences demonstrate that interference of *ActRIIB* signaling can increase muscle mass, reverse muscle wasting and muscle fibrosis in animal models for diseases related to muscular atrophy (Busquets et al., 2012; Babcock, Knoblauch & Clarke, 2015; Bayarsaikhan et al., 2017; Morvan et al., 2017). It is now clear that the mechanism of the *myostatin*/*ActRIIB* signal pathway is critical in regulating muscle protein balance (Han et al., 2013). In addition to activation of *Smad2/3/4* transcription, *myostatin* binding to *ActRIIB* also stimulates *FoxO*-dependent transcription to enhance muscle protein catabolism and suppresses *Akt*/*mTOR* signaling to inhibit muscle protein synthesis (Han et al., 2013; Hulmi et al., 2013).

The *ActRIIB* signaling, beside its effect on muscle growth, can also regulate (improve or inhibit) adipogenesis (Bielohuby, 2012; Li et al., 2016). *Myostatin* and *ActRIIB* mRNA levels would change in obese animals compared with normal or lean individuals (Allen et al., 2008; Morrison et al., 2014). Fat contents were reduced drastically in high-fat-diet mice by blockade of *ActRIIB* signaling (Akpan et al., 2009; Koncarevic et al., 2012). Furthermore, the interference with *ActRIIB* improved lipid profiles and prevented hepatic fat accumulation when compared with mice fed the same high-fat diet but treated with vehicle only (Bielohuby, 2012). Therefore, inhibition of *ActRIIB* signaling becomes a novel therapeutic approach in enhancing obesity and obesity-linked metabolic diseases (Koncarevic et al., 2012). Certainly, this strategy induced many changes in the expression patterns of fat metabolism-related genes in adipose tissues (Koncarevic et al., 2012; Xin et al., 2019). However, the molecular mechanism underlying *ActRIIB*-mediated metabolic cross-talk remains poorly understood in animals.

In crustaceans, the achievement of their growth and development is closely related to molting, which is an essential biological process (Huang et al., 2015). The TGF-β superfamily member, especially *myostatin*, which is known as a negative growth regulator in mammals, has also been identified to remain involved in regulation of molt-related growth in crustaceans (Lee et al., 2015; Abuhagr et al., 2016; Zhuo et al., 2017). As an important receptor of *myostatin*, *ActRIIB* gene has become a powerful therapeutic target especially for improvement of individual weight in mammals, and may also provide a useful approach to increase aquaculture production of economical aquatic crustaceans. However, its structure and functions on growth and development in crustaceans has remained unknown in crustaceans so far.

The Chinese mitten crab (*Eriocheir sinensis*) is an important economic crab species with huge aquaculture industry in China, but also significant ecological impact in its invaded regions (Wang et al., 2018). Unfortunately, in *E. sinensis* aquaculture, the weight gain rates of crab individuals after molting display significant difference even in the same culture environment, which results in low production of this highly demanded crab (Huang et al., 2014; Zhang et al., 2015). The present research was performed to acquire the full-length of *ActRIIB* cDNA from *E. sinensis* (*EsActRIIB*) and analyze its structural characteristics; to investigate the expression

patterns of the gene in different molting stages and different tissues; and to figure out the impacts of the gene interference on the growth characteristics, the composition of the crab as a nutrition source and changing patterns of some growth related genes; and for a better understanding of the role of *ActRIIB* in growth and nutrient regulation during molting processes, and thus, to provide a molecular basis to improve its growth and nutritional quality with further guiding significance in production of Chinese mitten crab.

Materials & Methods

Animal and tissue collection

Healthy Chinese mitten crab (*E. sinensis*) juveniles were collected from the Nanhui Experimental Station of Shanghai Ocean University, Shanghai, China. To detect the tissue expression levels of *EsActRIIB* at the premolt (PrM), molt (M), postmolt (PoM) and intermolt (InM) stages (Chen et al., 2017). The eyestalk, hepatopancreas, heart, gill, stomach, intestine, walking leg muscle, claw muscle, pectoral muscle, thoracic ganglia and epidermis were sampled from four healthy individuals of crab at the four mentioned molting stages. All collected tissue samples were flash-frozen in liquid nitrogen and subsequently stored at -80 °C for further analysis. Sampling procedures complied with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Shanghai Ocean University (SHOU-DW-2017021) on the care and use of animals for scientific purposes.

RNA extraction and cDNA synthesis

The total RNA was isolated using the RNA iso-Plus (Takara, Japan) according to the manufacturer's instructions. The concentration of extracted RNA was detected by a spectrophotometer (Eppendorf BioSpectrometer® basic, Hamburg, Germany) and he integrity of RNA was detected by 1% agarose gel electrophoresis. The RNA with an OD 260/280 value ranging from 1.8 to 2.0 were used for cDNA synthesis. The template used for full-length cDNA sequence cloning were synthesized by the SMARTer RACE 5′/3′ cDNA Kit Components (Clontech, USA). The template used for quantitative real time PCR (qRT-PCR) was synthesized by using the PrimeScript™ RT reagent Kit (TaKaRa, Japan) in accordance with the manufacturer’s instructions.

Full-length cDNA cloning of *EsActRIIB*

The full-length cDNA sequence of *EsActRIIB* was amplified from cDNA template that synthesized from muscle tissue RNA. Primers for cDNA cloning of *EsActRIIB*, which were designed based on transcriptome annotation results of *E. sinensis* in our previous study (Huang et al., 2015) by Primer Premier 5.0 software. To obtain full-length cDNA sequences of *EsActRIIB* gene, specific primers of ActRIIB-5′outer and ActRIIB-5′inner, ActRIIB-3′outer and ActRIIB-3′inner, and Universal Primer A Mix (**Table S1**), were used for the amplification of 5′ and 3′ ends full-length sequences, respectively. Total volume of the PCR reaction was 20 μL, including 10 μL 2×Hieff® Master Mix, 1 μL cDNA template, 0.5 μL 10 pmol/μL forward primer and 0.5

μL pmol/μL reverse primer, 8 μL ddH_2O. The PCR programs were run as follows: 94℃ for 5 min; 30 cycles of 94 ℃ for 30 s, 60℃ for 30 s; 72 ℃ for 1 min; 72 ℃ for 7 min. The PCR products were extracted from the 1% agarose gel using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit, then the purified PCR products were ligated with pMD19-T Vector (TaKaRa, Japan) and transformed into *Escherichia coli* DH5α competent cell (TaKaRa, Japan). The positive colonies were sent to Sangon Biotech Company (Shanghai, China) for sequencing.

Bioinformatics analysis of *EsActRIIB*

The amino acids of *EsActRIIB* was deduced by ExPASY-translate tool (http://www.expasy.org/). The sequence alignment analysis was performed by NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast). The protein domains were predicted by SMART (http://smart.embl-heidelberg.de/). The protein structure was modeled by I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). A phylogenetic tree was constructed by MEGA 5.1 software using neighbor joining methods with a bootstrap value of 1000 (**Table S2**).

Tissue expression detection of *EsActRIIB*

The qRT-PCR reaction system, which quantify the expression levels of *EsActRIIB* in eleven different tissues at the four molting stages, was consisted of 10 μL Hieff UNICON® qPCR SYBR Green Master Mix, 1 μL cDNA, 1 μL primer Mix, and 8 μL ddH_2O. The programs were run as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s. Temperature increased by 0.5°C per 5 s from 60°C to 95°C for the melting curve with 30 s elapse time per cycle. *β-actin*, *S27*, and *UBE*, were selected as reference genes (Huang et al., 2017), and relative expression levels of target genes were estimated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The primers for qRT-PCR were shown in **Table S1**.

RNA interference conduction

To obtain effective double-stranded RNA (dsRNA) of *EsActRIIB*, the target segment contained more functional short-interfering RNA (siRNA) sites predicted using siDirect version 2.0 (http://sidirect2.rnai.jp/). The designed primers included a T7 RNA polymerase-binding site at 5′-end (Table 1). The dsRNA was synthesized using T7 RiboMAX™ Large Scale RNA Production Systems (Promega, P1300) according to the manufacturer′s instructions. In order to test the efficiency of designed dsRNA, 6 crab individuals were injected with 3 μg/g dsRNA (1 μg/mL) used as experiment group, and 6 crab individuals were injected with PBS (3 μL/g) used as control group, respectively. qRT-PCR was conducted to test the expression of *EsActRIIB* to make sure the interference efficiency. In order to explore the function of *EsActRIIB* on growth during the molting process, 60 crab individuals (4.62 ± 0.78 g) were collected immediately after their molting, and divided randomly and equally into two groups, experimental (injected with dsRNA) and control (injected with PBS) groups. All carbs were cultured in the same condition. The first injection was at 5^{th} days after crab molting, and then injected every five days. After the second molt, all surviving crabs were collected for further analysis.

Basic growth character measurement

Body weight (BW) of each carb was measured on the second day after the first molt, the molting time was recorded daily for individual crabs, and the hepatopancreas was collected and weighted after the second molt. The parameters were calculated as following formulae: Weight gain rate (WGR, %) = [body weight after the second molt (BW_2) - body weight after the first molt (BW_1)]/BW_1 ×100; Specific growth rate (SGR, %/d) = ($LnBW_2$ - $LnBW_1$) ×100/molting interval time (MI); Hepatopancreas index (HI, %) = hepatopancreas weight (HW) /BW_2 ×100.

Nutrition ingredient analysis

At the end of the RNA interference (RNAi) experiment, the hepatopancreatic and whole muscle tissues of each crab were sampled for fatty acid and hydrolytic amino acid ingredients analysis. The compositions were measured by using Gas chromatography and mass spectometry (GC-MS) technique and amino acid automatic analysis apparatus, respectively (Wei et al., 2018). Three biological repetitions for each group were performed to significant difference analysis.

Expression of target genes

To determine the expression of target genes including *ActRI*, *Smad3*, *Smad4*, *FoxO* and *mTOR* (Han et al., 2013; Guru et al., 2019), which involve in *ActRIIB* signaling pathway, and pancreatic lipase (*PL*), carnitine palmitoyltransferases 1β (*CPT1β*), fatty acid synthase (*FAS*) and fatty acid elongation (*FAE*) (Liu et al., 2016; DeBose-Boyd, 2018; Liu et al., 2018; Wei et al., 2019), which are related to lipid metabolism, the hepatopancreas and muscle (from third walking leg muscle) tissues were collected from six individuals treated with dsRNA and PBS for 48 h. qRT-PCR was used to analyze the transcription of eleven target genes, as same method described above.

Statistical analysis

All data were statistically analyzed by one-way ANOVA followed by Duncan′s Multiple Range Test with SPSS 20. The P value < 0.05 was considered statistically significant. The P value < 0.01 was considered to be a highly significant difference.

Results

The full-length cDNA sequence of *EsActRIIB*

The full-length cDNA sequence of *EsActRIIB* was 4916 bp (GenBank accession number: MN832896), which was consisted of a 618 bp 5′ terminal untranslated region (UTR), an open reading frame (ORF) of 1683 bp, and a 2615 bp 3′ UTR. The ORF encoded 560 amino acids (aa) with a predicted molecular weight of 62.86 kDa, and a theoretical isoelectric point of 6.00 (**Fig. 1**). The predicted domains of *EsActRIIB* contain an activin receptor domain (46-140aa) and a Serine/Threonine protein kinases domain (251-539aa), which are characteristic domains of TGF-β type II receptor superfamily. A signal peptide and a transmembrane region were located at 1-

28aa and 198-220aa, respectively (**Fig. 2A**). The tertiary structure of EsActRIIB consists of thirteen α-helices and seven β-sheets (**Fig. 2B**).

cDNA sequence alignment and phylogenetic analysis

The BLAST analysis showed that EsActRIIB amino acid sequence shared high identities of 92% with that of *Portunus trituberculatus* (GenBank: MPC26231.1), 69% with that of *Penaeus vannamei* (GenBank: ROT74806.1), 54% with that of *Daphnia magna* (GenBank: JAL80963.1) and identities of 50%~60% with that of *Nasonia vitripennis* (GenBank: XP_001603863.1) and other insects. The phylogenetic tree of ActRIIB could be divided into two large branches. ActRIIBs from arthropods were clustered together in one large branch, in which the EsActRIIB was clustered closely with those of crustaceans. ActRIIBs of fishes, amphibians, mammals, reptiles and bird species were clustered together in the other branches (**Fig. 3**).

Tissue expression levels of *EsActRIIB*

The relative expression levels of *EsActRIIB* could be detected in all eleven tissues during different molting stages, which varied with the molting stages. The mRNA expression of *EsActRIIB* in the hepatopancreas was significantly higher than the other detected tissues in general ($P<0.05$), with the highest during premolt stage (PrM) ($P<0.05$). Generally, the expression levels of *EsActRIIB* were relatively lower in muscle tissues. However, the expression levels of *EsActRIIB* in the walking leg muscle, claw muscle and pectoral muscle also reached the highest at PrM stage ($P<0.05$) (**Fig. 4**).

Growth characteristics after RNAi of *EsActRIIB*

After injection of synthesized dsRNA, the expression level of *EsActRIIB* significantly decreased compared with control group at 48 h ($P<0.01$), with 60.85% of interference efficiency (**Fig. 5**). After a whole molting cycle interference, dsRNA group showed higher weight gain rate (WGR) and specific growth rate (SGR) with the significant difference, which increased 53.55% and 64.71% against control group ($P<0.01$), respectively. In addition, lower hepatopancreas index (HI) showed in dsRNA group with the significant difference compared to control group ($P<0.01$), which decreased 34.79% against control group. However, the molt cycle had no significant difference between dsRNA and control groups ($P>0.05$) (**Table 1**).

The effect of *EsActRIIB* RNAi on nutrient composition

After *EsActRIIB* interference, the contents of total essential amino acid (TEAA), total non-essential amino acid (TNEAA) and total amino acid (TAA) compared to those of the control group was increased by 9.57%, 8.47%, 8.92% in hepatopancreas and 12.53%, 8.56%, 10.07% in muscle, respectively ($P<0.05$). The ratio of TEAA/TNEAA in muscle increased by 5.08% ($P<0.05$) (**Table 2**). The contents of saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) increased by 2.53% and 4.46%, and polyunsaturated fatty acid (PUFA) decreased by

9.65% in hepatopancreas of dsRNA group (P<0.05). The same trends were observed in muscle, but with no significant differences (P>0.05) (**Table 3**).

Expression levels of target genes after RNAi of *EsActRIIB*

After dsRNA interference, the expression levels of eleven target genes (*ActRI*, *Smad3*, *Smad4*, *FoxO*, *mTOR*, *CPT1β*, *PL*, *FAS* and *FAE*) were compared to control group. For *ActRIIB* pathway related genes, the expression levels of *ActRI* and *FoxO* were down-regulated in hepatopancreas and muscle after treated with dsRNA (P<0.05). However, no significant differences were found among the relative expressions of *Smad3*, *Smad4* and *mTOR* (P>0.05). For lipid metabolism related genes, the expression level of *CPT1β* was significantly down-regulated in hepatopancreas but up-regulated in muscle, *PL* was up-regulated in hepatopancreas but down-regulated in muscle (P<0.05), while *FAS* and *FAE* were down-regulated in the hepatopancreas and muscle tissues after dsRNA injection compared to control group (P<0.05) (**Fig. 6**).

Discussion

In the present study, first of all, we cloned the full-length cDNA of *ActRIIB* from *E. sinensis*. Its encoded amino acid sequence has characteristic structural domains of TGF-β type II receptor members (Thompson, Woodruff & Jardetzky, 2003). In vertebrates, *ActRIIB* binds to activins and *myostatin* ligand through its extracellular activin receptor domain, and its intracellular Serine/Threonine protein kinases domain phosphorylates down-steam *Smad* signaling factors to exert in signal transduction (Shi & Massagué, 2003). These domains highly conserved in *E. sinensis*, it is presumed that *EsActRIIB* might play a role in TGF-β signal transduction, similar in vertebrates.

ActRIIB is widely distributed in various tissues and different developmental stages in mouse, zebrafish and other vertebrates (Garg et al., 1999; Rebbapragada et al., 2003). In this study, *EsActRIIB* was also widely expressed in all tested tissues at different molting stages. Molting is the special biological process of *E. sinensis* to achieve growth, and the transcription levels of numerous related genes in vivo changes with molting cycle (Huang et al., 2015). The expression levels of *EsActRIIB* in different tissues had differences in four molting stages, which revealed that it involved in molting-related growth regulation. In general, *EsActRIIB* was highly expressed in the hepatopancreas tissue in all four molting stages, which might be relevant to the important roles of hepatopancreas in carbohydrate and lipid metabolism, nutritional status, energy storage and breakdown in crustaceans (Wang et al., 2008). The expression of *EsActRIIB* in all three types of muscles were peaked at PrM, it might be related to muscle atrophy induced by molting (Tian & Jiao, 2016). These results indicated that hepatopancreas and muscles are the target ograns that *EsActRIIB* playing a role in molt-related growth.

In order to explore the function of *EsActRIIB* in growth regulation, we knocked-down the transcription level of *EsActRIIB* successfully. The *EsActRIIB* mRNA knocked-down crabs in this

study showed faster weight gain rate (WGR) and specific growth rate (SGR). Similar interference with *ActRIIB* positive regulation in muscle growth has been confirmed in dystrophic mdx mice (Dumonceaux et al., 2010). Moreover, higher contents of hydrolytic amino acids occurred in muscles and hepatopancreas indicating that the achievement of faster growth in *EsActRIIB* knocked-down individuals was caused by acceleration of protein synthesis. The *myostatin*/*ActRIIB* signal pathways regulating muscle protein balance has illuminated in mammals (Han et al., 2013). *ActRI* is the interacting protein of *ActRIIB* binds to *myostatin* (Hata & Chen, 2016), its lower expression was observed with interference of *EsActRIIB* in this study. But the expression of downstream transcription factors *Smad3* and *Smad4* was not influenced, which would be the fact that the Smads as core and versatile cytokines are active in whole TGF-β pathway, not only in *myostatin*/*ActRIIB* pathway (Massague, Seoane & Wotton, 2005). *ActRIIB* signal pathway also stimulates *FoxO*-dependent transcription to enhance muscle protein catabolism and suppresses *Akt*/*mTOR* signaling to inhibit muscle protein synthesis (Han et al., 2013; Hulmi et al., 2013). The expression of *mTOR* after *EsActRIIB* RNAi was not influenced, but the result of lower expression of *FoxO* could be explained. These results indicated the interference of *EsActRIIB* accelerated individual growth by regulation of protein catabolism and synthesis pathway.

It was proven that hepatopancreas is the main organ for lipid storage and lipid processing in crustaceans (Wang et al., 2008). The lower hepatopancreas index (HI) indicated that the inhibition in lipid metabolism. This phenotype is consistent with previous studies that the interference with *EsActRIIB* regulate adipogenesis to reduced adiposity (Koncarevic et al., 2012; Morrison et al., 2014). The corresponding result is that, the fatty acid composition (higher SFA and MUFA, lower PUFA) significantly changed in hepatopancreas after *EsActRIIB* interference. It is indicated that the lipid metabolism *in vivo* might be blocked. The further transcription levels of lipid metabolism related genes were verified. *PL* is responsible for lipodieresis (Grove et al., 2012) and *FAE* controls fatty acid elongation (Igarashi et al., 2019), higher expression of *PL* and lower expression of *FAE* in hepatopancreas after *EsActRIIB* interference might be the cause of less hepatopancreas weight. In addition, the expression of *CPT1β* was down-regulated in hepatopancreas but up-regulated in muscle. This result might due to the fact that *CPT1β* is the key enzyme of β-oxidation of fatty acid which acts on muscle (Liu et al., 2018), and, hepatopancreas might require to reduce energy consumption for muscle growth (Huang et al., 2015). Moreover, the expression levels of *FAS*, which is responsible for lipid synthesis (Loftus et al., 2000) and *FAE* were down-regulated in muscle after *EsActRIIB* interference. It indicated that the lipid synthesis was slowed down n muscle; but the expression of *PL* was also down-regulated in muscle. The balance of lipolysis and lipid synthesis was affected, but not broken. That might be the cause of no significant difference in fatty acid composition of the muscle tissue. Anyway, these results indicated that the RNA interference of *EsActRIIB* effected the balance of lipid metabolism.

Conclusions

In conclusion, *ActRIIB* of *E. sinensis* is a conservative functional gene belonging to TGF-β superfamily receptors, and the RNA interference of *EsActRIIB* positively regulated muscle growth after molting by effecting on protein synthesis and lipid metabolism. Our study was first to prove the negative regulatory function of *EsActRIIB* in *E. sinensis*. Further research may focus on the molecular mechanism of *ActRIIB* signal transduction related to in *E. sinensis*.

References

Abuhagr AM, MacLea KS, Mudron MR, Chang SA, Chang ES, Mykles DL. 2016. Roles of mechanistic target of rapamycin and transforming growth factor-beta signaling in the molting gland (Y-organ) of the blackback land crab, Gecarcinus lateralis. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, 198: 15-21. DOI: 10.1016/j.cbpa.2016.03.018

Akpan I, Goncalves MD, Dhir R, Yin X, Pistilli EE, Bogdanovich S, Khurana TS, Ucran J, Lachey J, Ahima RS. 2009. The effects of a soluble activin type IIB receptor on obesity and insulin sensitivity. *International Journal of Obesity*, 33: 1265-1273. DOI: 10.1038/ijo.2009.162

Allen DL, Cleary AS, Speaker KJ, Lindsay SF, Uyenishi J, Reed JM, Madden MC, Mehan RS. 2008. Myostatin, activin receptor IIb, and follistatin-like-3 gene expression are altered in adipose tissue and skeletal muscle of obese mice. *American Journal of Physiology and Endocrinology Metabolism*, 294: E918-927. DOI: 10.1152/ajpendo.00798.2007

Amthor H, Hoogaars WM. 2012. Interference with myostatin/ActRIIB signaling as a therapeutic strategy for Duchenne muscular dystrophy. *Current Gene Therapy*, 12: 245-259. DOI: 10.2174/156652312800840577

Babcock LW, Knoblauch M, Clarke MS. 2015. The role of myostatin and activin receptor IIB in the regulation of unloading-induced myofiber type-specific skeletal muscle atrophy. *Journal of Applied Physiology*, 119: 633-642. DOI: 10.1152/japplphysiol.00762.2014

Bayarsaikhan O, Kawai N, Mori H, Kinouchi N, Nikawa T, Tanaka E. 2017. Co-administration of myostatin-targeting siRNA and ActRIIB-Fc fusion protein increases masseter muscle mass and fiber size. *Journal of Nutritional Science and Vitaminology*, 63: 244-248. DOI: 10.3177/jnsv.63.244

399 Bielohuby M. 2012. Fat news: A novel ActRIIB decoy receptor in the BAT-tle for obesity.
400 *Endocrinology*, 153: 2939-2941. DOI: 10.1210/en.2012-1465
401 Busquets S, Toledo M, Orpi M, Massa D, Porta M, Capdevila E, Padilla N, Frailis V, Lopez-
402 Soriano FJ, Han HQ, Argiles JM. 2012. Myostatin blockage using actRIIB antagonism in
403 mice bearing the Lewis lung carcinoma results in the improvement of muscle wasting and
404 physical performance. *Journal of Cachexia Sarcopenia and Muscle*, 3: 37-43. DOI:
405 10.1007/s13539-011-0049-z
406 Chen JL, Walton KL, Al-Musawi SL, Kelly EK, Qian H, La M, Lu L, Lovrecz G, Ziemann M,
407 Lazarus R, El-Osta A, Gregorevic P, Harrison CA. 2015. Development of novel activin-
408 targeted therapeutics. *Molecular Therapy*, 23: 434-444. DOI: 10.1038/mt.2014.221
409 Chen X, Wang J, Yue W, Huang S, Chen J, Chen Y, Wang C. 2017. Structure and function of
410 the alternatively spliced isoforms of the ecdysone receptor gene in the Chinese mitten
411 crab, Eriocheir sinensis. *Scientific Reports*, 7: 12993. DOI: 10.1038/s41598-017-13474-1
412 DeBose-Boyd RA. 2018. Significance and regulation of lipid metabolism. *Seminars in Cell and*
413 *Developmental Biology*, 81: 97. DOI: 10.1016/j.semcdb.2017.12.003
414 Dumonceaux J, Marie S, Beley C, Trollet C, Vignaud A, Ferry A, Butler-Browne G, Garcia L.
415 2010. Combination of myostatin pathway interference and dystrophin rescue enhances
416 tetanic and specific force in dystrophic mdx mice. *Molecular Therapy*, 18: 881-887. DOI:
417 10.1038/mt.2009.322
418 Garg RR, Bally-Cuif L, Lee SE, Gong Z, Ni X, Hew CL, Peng C. 1999. Cloning of zebrafish
419 activin type IIB receptor (ActRIIB) cDNA and mRNA expression of ActRIIB in embryos
420 and adult tissues. *Molecular and Cellular Endocrinology*, 153: 169-181. DOI:
421 10.1016/s0303-7207(99)00044-1
422 Grove KA, Sae-tan S, Kennett MJ, Lambert JD. 2012. (-)-Epigallocatechin-3-gallate inhibits
423 pancreatic lipase and reduces body weight gain in high fat-fed obese mice. *Obesity*, 20:
424 2311-2313. DOI: 10.1038/oby.2011.139
425 Guru VP, Bhattacharya TK, Bhushan B, Kumar P, Chatterjee RN, Paswan C, Dushyanth K,
426 Divya D, Prasad AR. 2019. In silico prediction of short hairpin RNA and in vitro
427 silencing of activin receptor type IIB in chicken embryo fibroblasts by RNA interference.
428 *Molecular Biology Reports*, 46: 2947-2959. DOI: 10.1007/s11033-019-04756-0
429 Han HQ, Zhou X, Mitch WE, Goldberg AL. 2013. Myostatin/activin pathway antagonism:
430 molecular basis and therapeutic potential. *The International Journal of Biochemistry and*
431 *Cell Biology*, 45: 2333-2347. DOI: 10.1016/j.biocel.2013.05.019
432 Hata A, Chen YG. 2016. TGF-beta signaling from receptors to smads. *Cold Spring Harbor*
433 *Perspectives in Biology*, 8: a022061. DOI: 10.1101/cshperspect.a022061
434 Huang S, Chen X, Wang J, Chen J, Yue W, Lu W, Lu G, Wang C. 2017. Selection of appropriate
435 reference genes for qPCR in the Chinese mitten crab, Eriocheir sinensis
436 (Decapoda, Varunidae). *Crustaceana*, 90: 275-296. DOI: 10.1163/15685403-00003651
437 Huang S, Wang J, Yue W, Chen J, Gaughan S, Lu W, Lu G, Wang C. 2015. Transcriptomic
438 variation of hepatopancreas reveals the energy metabolism and biological processes

associated with molting in Chinese mitten crab, Eriocheir sinensis. *Scientific Reports*, 5: 14015. DOI: 10.1038/srep14015

Huang S, Wang ZQ, Mao HC, Wang CH. 2014. Observation on molting and growth of adult Chinese mitten crab reared in the laboratory condition (in Chinese). *Journal of Shanghai Ocean University*, 23: 259-365.

Hulmi JJ, Oliveira BM, Silvennoinen M, Hoogaars WM, Ma H, Pierre P, Pasternack A, Kainulainen H, Ritvos O. 2013. Muscle protein synthesis, mTORC1/MAPK/Hippo signaling, and capillary density are altered by blocking of myostatin and activins. *American Journal of Physiology. Endocrinology and Metabolism*, 304: E41-50. DOI: 10.1152/ajpendo.00389.2012

Igarashi M, Watanabe K, Tsuduki T, Kimura I, Kubota N. 2019. NAPE-PLD controls OEA synthesis and fat absorption by regulating lipoprotein synthesis in an in vitro model of intestinal epithelial cells. *The FASEB Journal*, 33: 3167-3179. DOI: 10.1096/fj.201801408R

Koncarevic A, Kajimura S, Cornwall-Brady M, Andreucci A, Pullen A, Sako D, Kumar R, Grinberg AV, Liharska K, Ucran JA, Howard E, Spiegelman BM, Seehra J, Lachey J. 2012. A novel therapeutic approach to treating obesity through modulation of TGFbeta signaling. *Endocrinology*, 153: 3133-3146. DOI: 10.1210/en.2012-1016

Lee JH, Momani J, Kim YM, Kang CK, Choi JH, Baek HJ, Kim HW. 2015. Effective RNA-silencing strategy of Lv-MSTN/GDF11 gene and its effects on the growth in shrimp, Litopenaeus vannamei. *Comparative Biochemistry and Physiology Part B: Biochemistry Molecular Biology*, 179: 9-16. DOI: 10.1016/j.cbpb.2014.09.005

Li N, Yang Q, Walker RG, Thompson TB, Du M, Rodgers BD. 2016. Myostatin attenuation in vivo reduces adiposity, but activates adipogenesis. *Endocrinology*, 157: 282-291. DOI: 10.1210/en.2015-1546

Liu L, Long X, Deng D, Cheng Y, Wu X. 2018. Molecular characterization and tissue distribution of carnitine palmitoyltransferases in Chinese mitten crab Eriocheir sinensis and the effect of dietary fish oil replacement on their expression in the hepatopancreas. *PLOS ONE*, 13: e0201324. DOI: 10.1371/journal.pone.0201324

Liu ZH, Wu XJ, Long XW, Zhao L, Li JY, Cheng YX. 2016. Effects of fish oil replacement by vegetable oils in fattening diets on the related gene expression of fatty acid metabolism of about male Chinese mitten crab, Eriocheir Sinensis (in Chinese). *Acta Hydrobiological Sinica*, 40: 765-778. DOI: 10.7541/2016.101

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25: 402-408. DOI: 10.1006/meth.2001.1262

Loftus TM, Jaworsky DE, Frehywot GL, Townsend CA, Ronnett GV, Lane MD, Kuhajda FP. 2000. Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors. *Science*, 288: 2379-2381. DOI: 10.1126/science.288.5475.2379

478 Massague J, Seoane J, Wotton D. 2005. Smad transcription factors. *Genes and Development*, 19:
479 2783-2810. DOI: 10.1101/gad.1350705
480 Mathews LS, Vale WW, Kintner CR. 1992. Cloning of a second type of activin receptor and
481 functional characterization in Xenopus embryos. *Science*, 255: 1702-1705. DOI:
482 10.1126/science.1313188
483 McPherron AC, Lawler AM, Lee S-J. 1997. Regulation of skeletal muscle mass in mice by a
484 new TGF-p superfamily member. *Nature*, 387:83-90. DOI: 10.1038/387083a0
485 Morikawa M, Derynck R, Miyazono K. 2016. TGF-beta and the TGF-beta family: context-
486 dependent roles in cell and tissue physiology. *Cold Spring Harbor Perspectives in*
487 *Biology*, 8: a021873. DOI: 10.1101/cshperspect.a021873
488 Morrison PK, Bing C, Harris PA, Maltin CA, Grove-White D, Argo CM. 2014. Preliminary
489 investigation into a potential role for myostatin and its receptor (ActRIIB) in lean and
490 obese horses and ponies. *PLOS ONE*, 9: e112621. DOI: 10.1371/journal.pone.0112621
491 Morvan F, Rondeau JM, Zou C, Minetti G, Scheufler C, Scharenberg M, Jacobi C, Brebbia P,
492 Ritter V, Toussaint G, Koelbing C, Leber X, Schilb A, Witte F, Lehmann S, Koch E,
493 Geisse S, Glass DJ, Lach-Trifilieff E. 2017. Blockade of activin type II receptors with a
494 dual anti-ActRIIA/IIB antibody is critical to promote maximal skeletal muscle
495 hypertrophy. *Proceedings of the National Academy of Sciences*, 114: 12448-12453. DOI:
496 10.1073/pnas.1707925114
497 Nickel J, Ten Dijke P, Mueller TD. 2018. TGF-beta family co-receptor function and signaling.
498 *Acta Biochimica et Biophysica Sinica*, 50: 12-36. DOI: 10.1093/abbs/gmx126
499 Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L. 2003. Myostatin signals
500 through a transforming growth factor beta-like signaling pathway to block adipogenesis.
501 *Molecular and Cellular Biology*, 23: 7230-7242. DOI: 10.1128/mcb.23.20.7230-
502 7242.2003
503 Sako D, Grinberg AV, Liu J, Davies MV, Castonguay R, Maniatis S, Andreucci AJ, Pobre EG,
504 Tomkinson KN, Monnell TE, Ucran JA, Martinez-Hackert E, Pearsall RS, Underwood
505 KW, Seehra J, Kumar R. 2010. Characterization of the ligand binding functionality of the
506 extracellular domain of activin receptor type IIb. *Journal of Biological Chemistry*, 285:
507 21037-21048. DOI: 10.1074/jbc.M110.114959
508 Santibanez JF, Quintanilla M, Bernabeu C. 2011. TGF-beta/TGF-beta receptor system and its
509 role in physiological and pathological conditions. *Clinical Science*, 121: 233-251. DOI:
510 10.1042/CS20110086
511 Shi Y, Massagué J. 2003. Mechanisms of TGF-β signaling from cell membrane to the nucleus.
512 *Cell*, 113: 685-700. DOI: 10.1016/s0092-8674(03)00432-x
513 Thompson TB, Woodruff TK, Jardetzky TS. 2003. Structures of an ActRIIB:activin A complex
514 reveal a novel binding mode for TGF-beta ligand:receptor interactions. *EMBO Journal*,
515 22: 1555-1566. DOI: 10.1093/emboj/cdg156

Tian ZH, Jiao CZ. 2016. Muscle atrophy and growth induced by molting in crustacean: a review (in Chinese). *Fisheries Science*, 35: 603-606. DOI: 10.16378/j.cnki.1003-1111.2016.05.026

Wang J, Xu P, Zhou G, Li X, Lu Q, Liu X, Zhou J, Wang C. 2018. Genetic improvement and breeding practices for Chinese mitten crab, Eriocheir sinensis. *Journal of the World Aquaculture Society*, 49: 292-301. DOI: 10.1111/jwas.12500

Wang L, Yan B, Liu N, Li Y, Wang Q. 2008. Effects of cadmium on glutathione synthesis in hepatopancreas of freshwater crab, Sinopotamon yangtsekiense. *Chemosphere*, 74: 51-56. DOI: 10.1016/j.chemosphere.2008.09.025

Wei B, Yang Z, Cheng Y, Wang J, Zhou J. 2018. Effects of the complete replacement of fish oil with linseed oil on growth, fatty acid composition, and protein expression in the Chinese mitten crab (Eriocheir sinensis). *Proteome Science*, 16: 6. DOI: 10.1186/s12953-018-0135-7

Wei BH, Yang ZG, Cheng YX, Yang H, Yang XZ. 2019. Molecular cloning and expression analysis of pancreatic lipase in Chinese mitten crab *Eriocheir sinensis* (in Chinese). *Genomics and Applied Biology*, 38: 2466-2475. DOI: 10.13417/j.gab.038.002466

Xin XB, Yang SP, Li X, Liu XF, Zhang LL, Ding XB, Zhang S, Li GP, Guo H. 2019. Proteomics insights into the effects of MSTN on muscle glucose and lipid metabolism in genetically edited cattle. *General and Comparative Endocrinology*, 113237. DOI: 10.1016/j.ygcen.2019.113237

Zhang QY, Ma XZ, Wang Y, Wang W, Yu YQ. 2015. The research of individual growth of Eriocheir sinensis for Liaohe population juvenile crab in paddy field net cage (in Chinese). *Chinese Journal of Zoology*, 50: 112-121. DOI: 10.13859/j.cjz.201501014

Zhuo RQ, Zhou TT, Yang SP, Chan SF. 2017. Characterization of a molt-related myostatin gene (FmMstn) from the banana shrimp Fenneropenaeus merguiensis. *General and Comparative Endocrinology*, 248: 55-68. DOI: 10.1016/j.ygcen.2017.03.010

Figure 1(on next page)

The nucleotide and deduced amino acid sequence of *EsActRIIB*.

The start codon (ATG) and the stop codon (TAA) were indicated with bold letters in box. The mRNA instability motif (ATTTA) was showed by bold letters. The signal peptide sequence was double underlined. The activin receptor domain was shaded with light gray. The transmembrane region was indicated in box. Serine/Threonine protein kinases domain was showed with a bold underline.

```
1                                                                                                   AATACCCCATGGAAATTTTAAACAGCCAAA
31          TATACACCAGTAAAACCCATGTAATCACCCTTAAAAACCCGCAGCGGAGGTTTTTAGAGGACTCCCCGCCAGTGGATTATGTCT
115         CCTGAAAATACTCGCCATATTGGATTTCGCTAACAAATCGGGCCTGAAATACTGCTGCTGGGCGAGGCATTGCTTGACGCTGCA
199         CCCTGCGGGCCCCGAGGCGAGTGGCCCAGCGCACTGTGATCGGTGGGGGGGCACGAGCAGGTGATGGTGTGCTGCAGCTGCTGC
283         AACAAGTGTGACAGTGCTCAAAATTACCCTTTCCATGAAGAAGGATACAGTGAAAAATCTAGAACCCATGTGCTTTGTTTTACA
367         CAGTGAAGTTCCTCTTAAAATCTGTTGCTAAAGTGAAGGAAAAACAGCTTGATGAGAGCCCGAGAGGGTTGCCATCGATGCAGG
451         ATATGGACATGACACGGGTCTGTCCATGAGGACAGATGTGACATTTGGAGGAGCTGAGGTGCCACACATGCAAGACCTGACTGG
535         ATCCTGGCTGAGAGGGATCACCCAAGGACAAGTGAGGGGTGGCTGAAGCTGAGGAGACCCAAGGCAAGGTTGGCCAGCTGTGTG
619         ATGGCTGGCAGCAGAAGAGTCTTGTATCCTCCATCAACCATGATGATCCTGCTAACCCTGGCAGCCTTCCCTGCCATCTCGGCC
1             M  A  G  S  R  R  V  L  Y  P  P  S  T  M  M  I  L  L  T  L  A  A  F  P  A  I  S  A
703         CTAATACCTGAAGGGATGGCACAGCCTTCGGAAACCCCCCGGCCCCAGGTGACGCACTGTGAGCTATACAACAGCACACAGTGT
29            L  I  P  E  G  M  A  Q  P  S  E  T  P  R  P  Q  V  T  H  C  E  L  Y  N  S  T  Q  C
787         ACTGGGGACAACTCTCTCTCGCCTGTGTGTAAGAAGGAAAGCCATCCGTGTGAGGACCTAGAGAAGGCCAACCAGTGCTTTGTG
57            T  G  D  N  S  L  S  P  V  C  K  K  E  S  H  P  C  E  D  L  E  K  A  N  Q  C  F  V
871         GTGTGGAGTAACATGTCTGGCACGCCAGAAGTAATCTACAAGGGATGTTTATGGAATTATAAAACTTGCAAAGATGAGTGCATC
85            V  W  S  N  M  S  G  T  P  E  V  I  Y  K  G  C  L  W  N  Y  K  T  C  K  D  E  C  I
955         AGCACTGAGCCAGTGAACAGTCTTACAGAGCAGAAGCCTCACCTGTTTTGTTGCTGCAACAACAACAATTGTAATCAGAACTTC
113           S  T  E  P  V  N  S  L  T  E  Q  K  P  H  L  F  C  C  C  N  N  N  N  C  N  Q  N  F
1039        TCGTGGCAGCCTAAGGTTGAGGTCCCCAGCACCACCCCAGAGGGGCCTTTAAGAAACATGATCCCCGCAGCTACTGGGTTAATG
141           S  W  Q  P  K  V  E  V  P  S  T  T  P  E  G  P  L  R  N  M  I  P  A  A  T  G  L  M
1123        GCCCAGGGTAACAGGGCTTACACTGGAAAAAGGGATAAAAACCTTATAAACATCCTGCCACCCCAGGAACAGGACATGGTTGTA
169           A  Q  G  N  R  A  Y  T  G  K  R  D  K  N  L  I  N  I  L  P  P  Q  E  Q  D  M  V  V
1207        CAGACGGTGGCTTGGACCCTTGGGACCCTCATCCTGTTGGTGGTTACGGTGACAGTTCTCTTCTACCTCTATAGGAGACAGAAA
197           Q  T  V  A  W  T  L  G  T  L  I  L  L  V  V  T  V  T  V  L  F  Y  L  Y  R  R  Q  K
1291        ATGGCCAACTTTATGGCAATACCTCGAGTGGAGTCCACAGCCCTTGTTCCTCCCTCTCCACCGATGGGCTTGCGACCCATACAG
225           M  A  N  F  M  A  I  P  R  V  E  S  T  A  L  V  P  P  S  P  P  M  G  L  R  P  I  Q
1375        CTGAGGGAGATCAAAGCCAGAGGCCGGTTTGGTGCTGTGTGGAAGGCCAACCTTCACAATGACGTTATTGCTGTCAAGATCTTC
253           L  R  E  I  K  A  R  G  R  F  G  A  V  W  K  A  N  L  H  N  D  V  I  A  V  K  I  F
1459        CCAGTCCAGGATAAACAGTCGTGGCTGGTGGAGACGGAGGTGTACTCCCTCCCTCAGCTGTCCCATGAGAATATCCTACACTAC
281           P  V  Q  D  K  Q  S  W  L  V  E  T  E  V  Y  S  L  P  Q  L  S  H  E  N  I  L  H  Y
1543        ATTGGGGCAGAGAAGCGTGGCGATAGCCTTCAGGCTGAGTTTTGGCTTATTACAGCCTACCATGAGAGAGGCTCCTTGTGTGAC
309           I  G  A  E  K  R  G  D  S  L  Q  A  E  F  W  L  I  T  A  Y  H  E  R  G  S  L  C  D
1627        TTTCTAAAGGCCAACCTCGTGACATGGGATGAACTGTGCAAGATTGGCGAGTCAATGGCCCGGGGGTTAATGTACATGCATGAG
337           F  L  K  A  N  L  V  T  W  D  E  L  C  K  I  G  E  S  M  A  R  G  L  M  Y  M  H  E
1711        GAGCAACCGGCTTCCAAGTGTGAGGCCCTCAAGCCTGCCATTGCCCACCGAGACTTCAAAAGCAAAAATGTGTTGCTGAAGAAT
365           E  Q  P  A  S  K  C  E  A  L  K  P  A  I  A  H  R  D  F  K  S  K  N  V  L  L  K  N
1795        GACCTGACTGCCTGCATTGCTGACTTCGGCCTTGCTTTGACCTTCCACCCTGGACAGTCAACTGGTGACACTCATGGACAGGTG
393           D  L  T  A  C  I  A  D  F  G  L  A  L  T  F  H  P  G  Q  S  T  G  D  T  H  G  Q  V
1879        GGCACAAGGAGGTACATGGCCCCTGAAGTCTTGGAAGGGGCCATCAATTTCCAGCGTGATGCCTTCTTACGCATTGACATGTAT
421           G  T  R  R  Y  M  A  P  E  V  L  E  G  A  I  N  F  Q  R  D  A  F  L  R  I  D  M  Y
1963        GCCTGTGGCCTTGTGCTGTGGGAGCTGCTGTCCAGATGTTCAAGCCCAGATGGACCCATACCTGAGTACCACTTGCCATTTGAG
449           A  C  G  L  V  L  W  E  L  L  S  R  C  S  S  P  D  G  P  I  P  E  Y  H  L  P  F  E
2047        GAGGAAGTTGGCCAGCATCCAACATTGGACGACATGCAGGAGTGTGTCGTCACCCAAAAGGCTCGACCTGTCATCCACGACCAT
477           E  E  V  G  Q  H  P  T  L  D  D  M  Q  E  C  V  V  T  Q  K  A  R  P  V  I  H  D  H
2131        TGGCGGAAGAATGCTGCCATGATGGGATTGATAGACACCATGGAGGAGTGCTGGGACCATGATGCAGAGGCCCGTCTCTCAGCT
505           W  R  K  N  A  A  M  M  G  L  I  D  T  M  E  E  C  W  D  H  D  A  E  A  R  L  S  A
2215        TCATGTGTGGTGGAGAGGCTGGCCAGCTTCTCAAGGAACCCCCAGTTTTCCCCTACCTCAAATCCACAGAAGGAGTCGAGTATA
533           S  C  V  V  E  R  L  A  S  F  S  R  N  P  Q  F  S  P  T  S  N  P  Q  K  E  S  S  I
2299        TAAGGCCAAGGTGTGGTGGCCACGTACCTGGACAAGTACCTGTGCTGTAACCGCCTATGAGGGTCCTGGTGCGGGGGAGAGCCC
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561 *
2383 AGCCACCATGTCCCGCAGGCACCAGCTGTGTTGGGGGCCTCGTGTGTCGGCCTCAGTAACCCTCCCCCCTCCCTTCTCCAATCA
2467 GCTGCACACCACAGCCAGACACTTGGTCATATTCACAGTTACCCGGTGACACCAGTTTTGTCTGGTCAAAGAGGGTTGGGTTCA
2551 GGGAATTTCTCTTCACATTTGAAAAAAAAAAGTACTTTTACTCTCAGCATCTAAATTGGCACTGATGGGTGAGAGTCTCCGTTA
2635 GTTAGATAGATACGTCTTCCTGTAATTAATTAGTGCAATAGTTTGATATCCCTGGCGGTGCTTCAAATGCATTTTTATGGACTG
2719 ATGCAAAAATCATACTTAGCGGGAAGTAAATTTTGGGATCCTATAGTCCAACCATAATTGATCTGGCTTGTGTGGTTGGGAGAG
2803 GGGGGAGGCTTAAAGTGTGGTGCCAAAGGGGTCAACAGGGAAGTTCTGGGACTGTTAGATAGAGCCACCCAAGTGAAGGTAGCT
2887 TGTGGGAATTCACTCCATGGCCTCATGACTCCCCATAGGTTGAGATGCTCCATAACACTACCCTCTCCAGCATTATTGTTGGGA
2971 TCATGTTTCCTTGTGTTGGGCTTGAAAGGGTTAACAAT**ATTTA**GCTGTTATTATTACTGTCCAAAAAAGAAATTGATGTTCTTT
3055 GAAATACTTACACTTATTGGTATCGGCTGTTT**ATTTA**TGTTACGAACATGCAAGAAGGAAACCCTGTAC**ATTTA**GTGCAGACTA
3139 TAGCATGCGCAGCTGCTAACATAGAAGAATGAAAGAGGATCTCATGGTGCGTGTTGCCATAATGGAAGAAGCACCAAGAGGTAA
3223 AATTATTTTCGTAAAGTATCTAAATAAAATTTCAAGAGGAATGTTTTCACGTGCCACCCACACAGGGACCGTCTTGGGCATTCC
3307 TACCTCTGAATCCTCACTGTAAGTCTCCTGATGTGAGTTGAGGAGCTGTGGTGCTGCTTCCATCTTGTAGGACGGCTGTGGAAA
3391 GTGAATTCTGAATCTTCATCACCATCCGATTGTTTGGTGTGTACGATGGAGCTGGAGATCGTGCATGGAAAGTGGTGTTCACAG
3475 CTGTCAGTGCATTTCCTGTCAATGAGAAACATGGTGTCT**ATTTA**ACCATCAACAGACAATTGCATCCAGCCATTCAGTTTTGCA
3559 GCTCAGTGCAGCTTCAAGCTCAGTAACTCAGGCAAAAGGAAAACTATGGTTGTGCCTCCCTGGCAAGTGTCTAATGGCGTATGG
3643 GGAAGAGTGGCTGTGGGTTCGTCTGTCCTAAACTGAAATTTCCCATCTGTGTTGAAAACTGGATGGATTGCAGTGTTCTAATCA
3727 GTGAAGCTTTTGTTTTTAATTTTTGCTTAGAAAGACTCTATAGTGTGAATGATTTCTACATAAAAAGGAAACATAGTATTTCAG
3811 CCCACTATGTTCATTATCTGTGTGTCGTCAGTGCCAGCAGAGCTCCGACACTGTCTGCTGGGCTAGTAGGACACAAGACGAGCC
3895 CAGCCATTGTGAGGCCAGTGGAGGGCCACAATCGGGCTGCCCCTTTACTGTCAGCTCAGGACAAACTTCAGTGACCACCCTGTT
3979 ACCACTTTGCCCCCACACACGTACATACATACCCTCATCTTGTATATATTTGCGCCTTGTTTGTAGCATTACACAAGTTGTCGC
4063 CAACAAAATGAAACTAGTGATTATTTTAAATGTGGTTTATTTGTGAAACCAATGGTGCTTTTTTTCTTCTATTTCCAAGATGAA
4147 TGTTAATTGTTAAACACTGAATCAAGCCAGCATGAGTGTGGAAACAAAGAGGCACATTGCCCACTCAGCATTGTAAATACCTCA
4231 TTATTGTACACAGCATTATGACTTGTGACATCCAGACCTCACCATGACAGATCCTGAATGCTTATAAGCAGAATTCTATTACTT
4315 CTTGATTGTGTATAGTAAGTTCTCTCTTGTTTGGGAACTATAGTTGTTGGAAAGCCATAGAAAGGTCATTTTATTGATCCAGAA
4399 AACTTAAAAGAGGAAACTAAAGTAAACTCTGGTCTCTGCA**ATTTA**ATTCTTGTCATTGTAGTTAAGCCATTAAGAGGCTATGAG
4483 AGAGAAAGAGAGAGAGAGAGACTGTAAAATGAGAAAATAGATAAAAGCAAAGAGAAAATTGAACTTTGCAAAACTTTGACCTTG
4567 GGAGACTTGAGTTCATCTGCTGTGAGGCCTTCTGAGGCGAGGACGAGGCAGTGTGACTGTCAAACGAGGACTGGAGGCGTCATG
4651 TGATAGATTGGATACGTACAGGGACTTTTGGGCACAGGTACCGCCTGTGCCTTGGGGAACCACTTGTCACTGTTACAAGACTGT
4735 GTATGGGGGGAAATTTGCTATTTTCTTGTACTTCCTTGAATTA**ATTTA**ATTTTATTTTATGTTCATTACTTGTTAGGACACCCC
4819 CTTTATCCTTGTTGTTCCTTGCAGGCATGAATGTAATGTCTCATATATCTGTATCCCATGTAGTAGTATAAAGTAATCGATATT
4903 GAAAAAAAAAAAAA

Figure 2

Schematic diagram for structure prediction of EsActRIIB.

(A) Predicted domain structure of EsActRIIB. (B) Predicted protein structure of EsActRIIB. α-helix (red), β-sheet (yellow), the activin receptor domain (cyan) and the Serine/Threonine protein kinases domain (magenta) were shown.

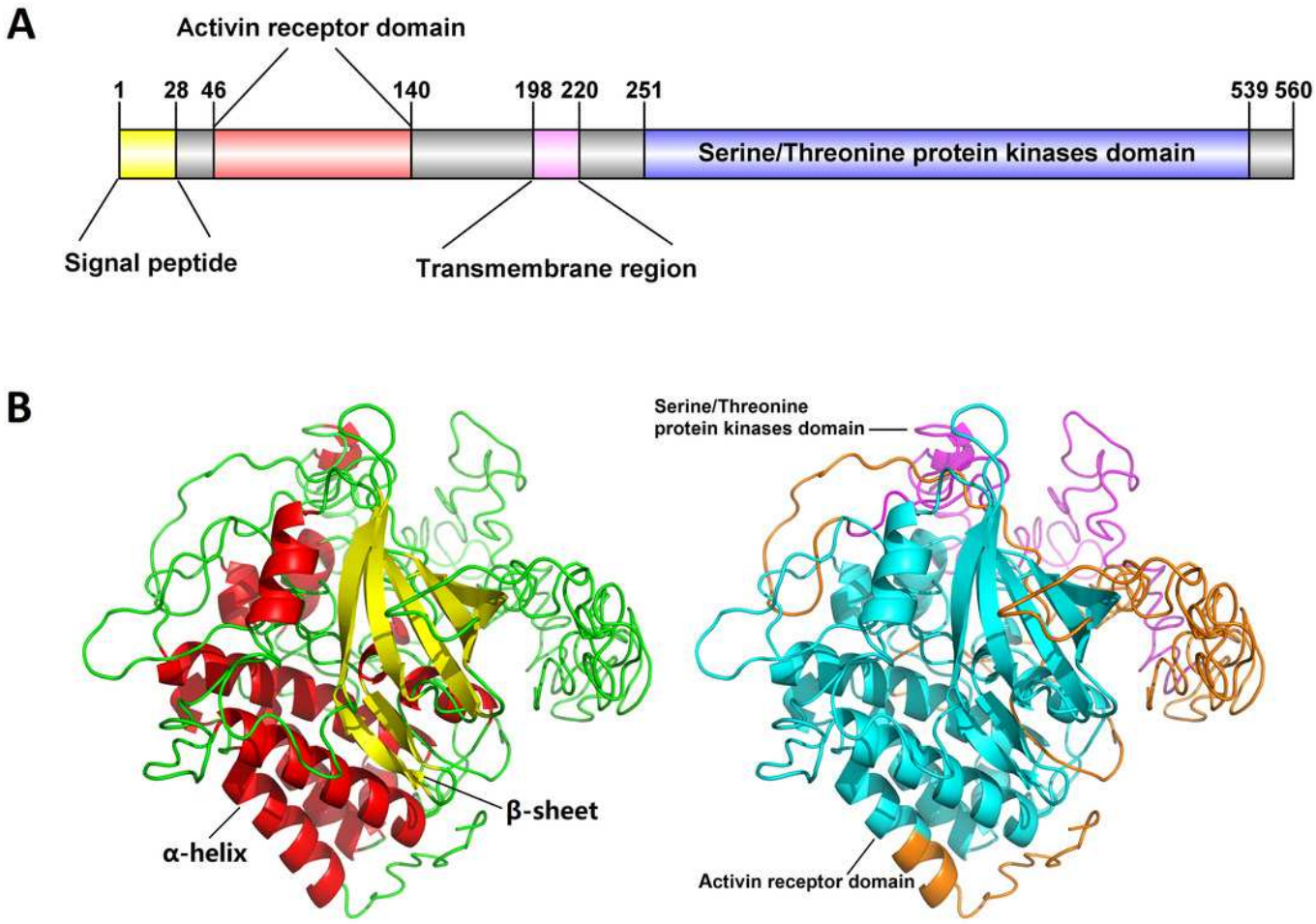

Figure 3

Phylogenetic tree based on ActRIIB amino acid sequences.

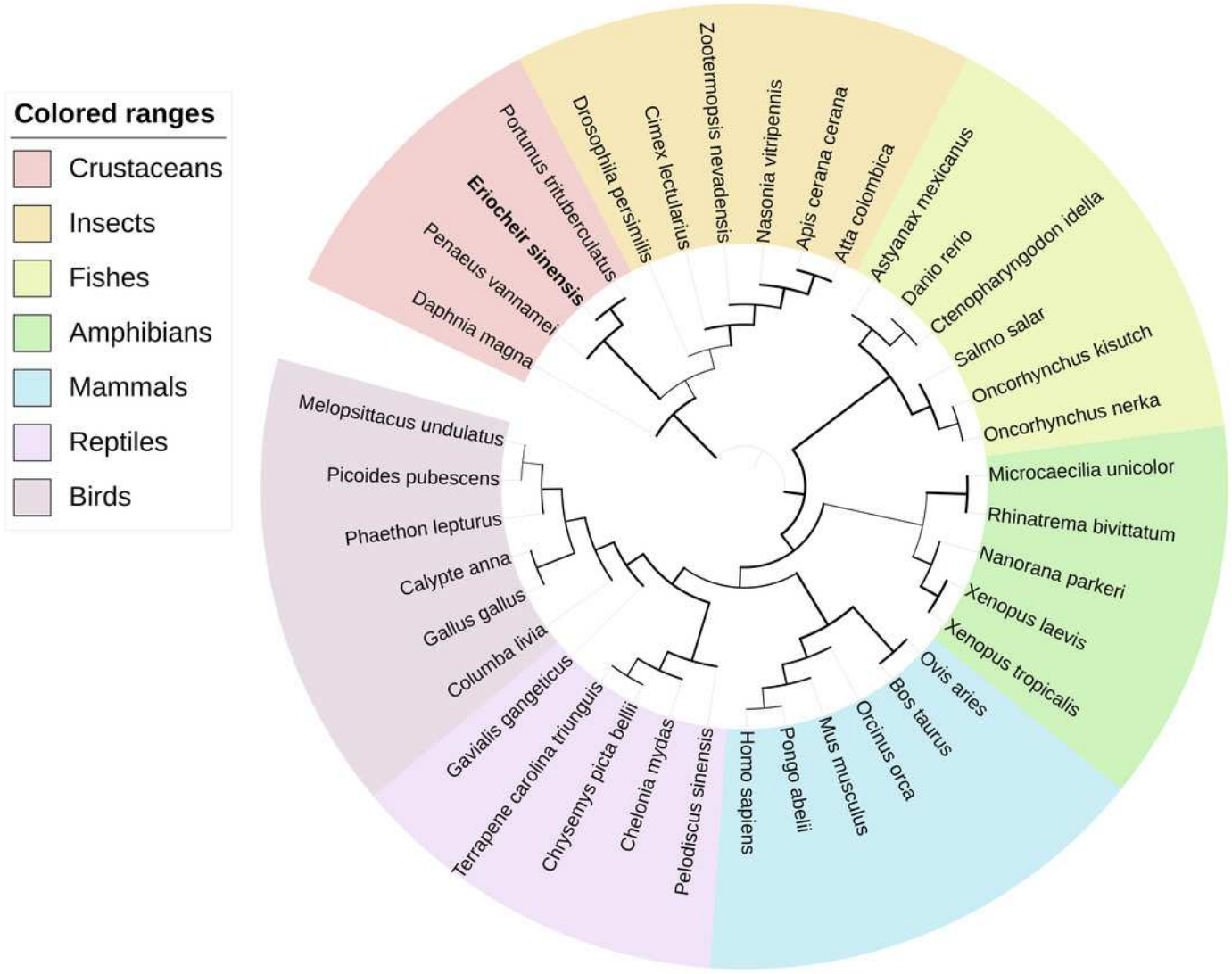

Figure 4

The expression levels of *EsActRIIB* in different tissues at four molting stages.

PoM: postmolt stage; InM: intermolt stage; PrM: premolt stage; M: molt stage; Es: eyestalk; Hp: hepatopancreas; Ht: heart; G: gill; S: stomach; I: intestine; Mw: walking leg muscle; Mc: claw muscle; Mp: pectoral muscle; Tg: thoracic ganglia; Ep: epidermis; Histogram plotted with mean and standard error; Different letters showed there were significant differences between groups ($P<0.05$).

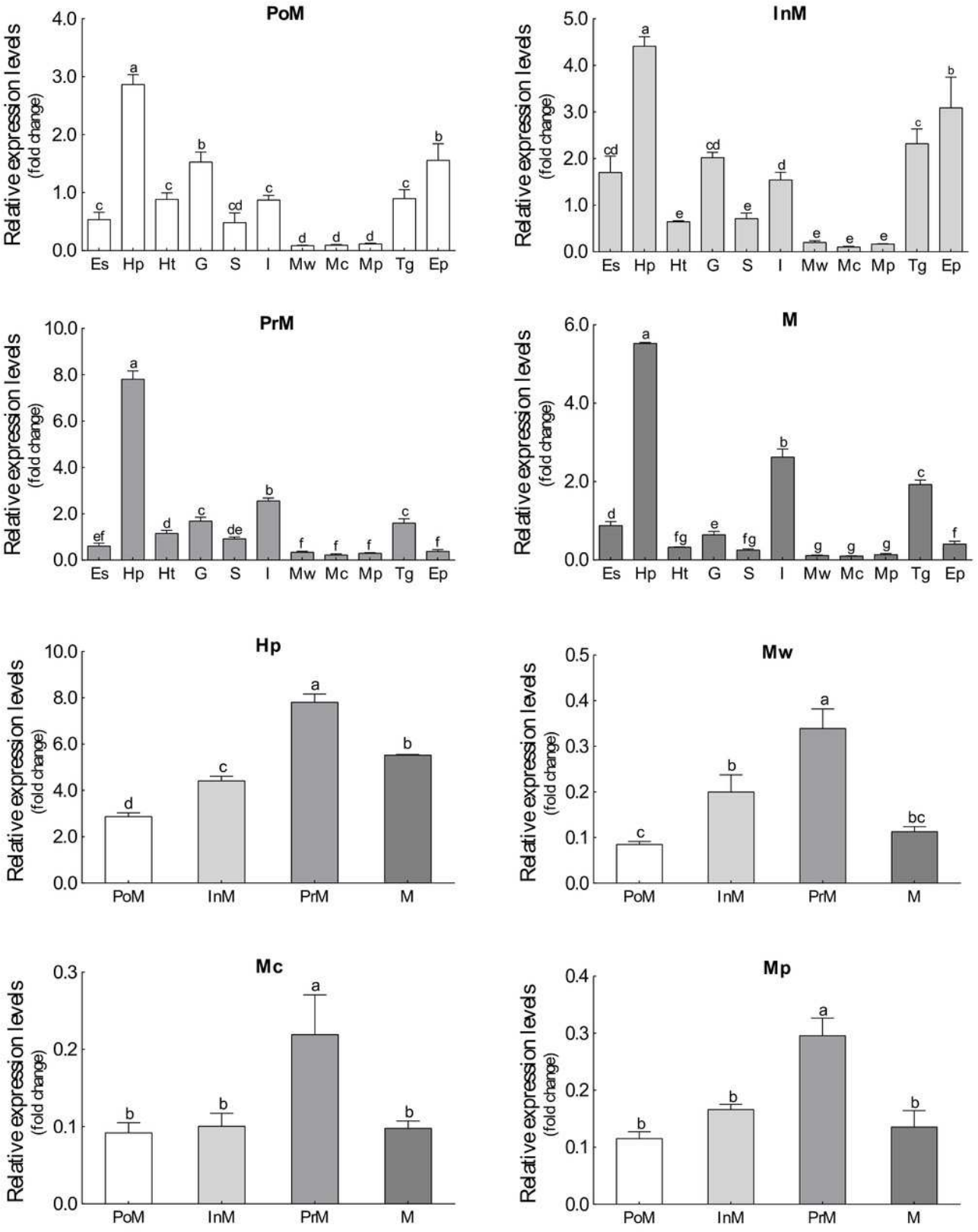
PoM
InM
PrM
M
Hp
Mw
Mc
Mp
Relative expression levels
(fold change)
Es Hp Ht G S I Mw Mc Mp Tg Ep
PoM InM PrM M

Figure 5

The interference efficiency of designed *EsActRIIB* dsRNA.

The different letters (a, b and c) indicated significant difference between the dsRNA group; "**" indicated extremely significant difference between two groups ($P<0.01$).

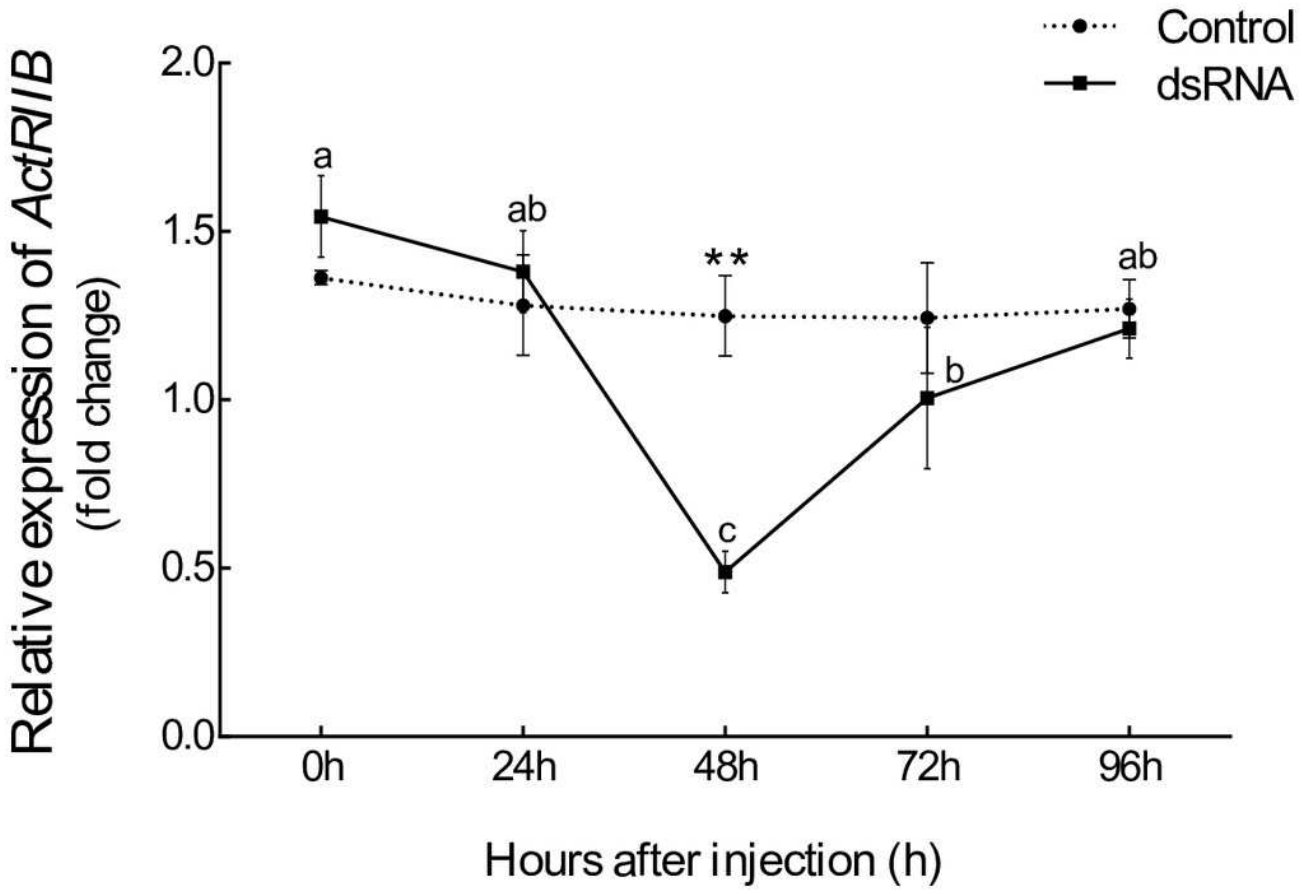

Figure 6

The expression levels of target genes after RNAi of *EsActRIIB*.

"*" indicated significant difference between two groups ($P<0.05$); "**" indicated extremely significant difference between two groups ($P<0.01$).

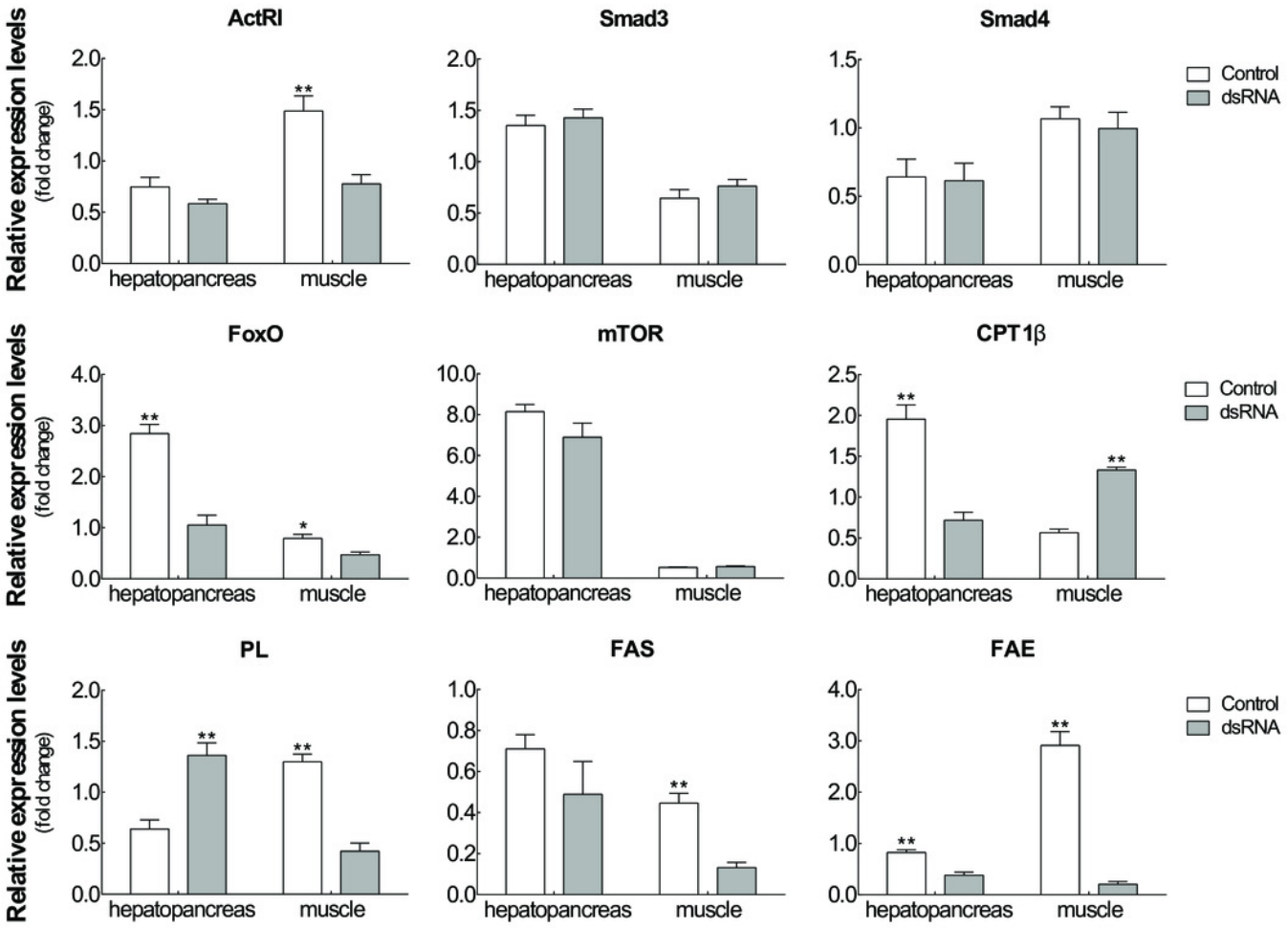

Table 1(on next page)

Effects of RNAi on growth performance of juvenile mitten crab (means ± SD).

BW_1: body weight after the first molt; BW_2: body weight after the second molt; WGR: weight gain rate; SGR: specific growth rate; MI: molting interval time; HI: hepatosomatic index; $WGR=[(BW_2-BW_1)/BW_1] \times 100$; $SGR=(LnBW_2-LnBW_1) \times 100/MI$; HI=weight of hepatopancreas/$BW_2 \times 100$.

Indicators of growth	Experimental groups		Statistical analysis
	dsRNA (n=14)	Control (n=14)	
BW_1 (g)	4.72±0.86	4.54±0.85	$P>0.05$
BW_2 (g)	6.76±1.16	5.86±1.28	$P>0.05$
WGR (%)	43.87±8.23	28.57±860	$P<0.01$
MI (d)	44.79±7.53	50.14±7.44	$P>0.05$
SGR (%/d)	0.84±0.26	0.51±0.16	$P<0.01$
HI (%)	5.26±0.58	7.09±0.55	$P<0.01$

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Table 2(on next page)

Amino acid composition in hepatopancreas and muscle of *E. sinensis* (g/100 g, dry weight).

"*" indicated significant difference between two groups ($P<0.05$); "**" indicated extremely significant difference between two groups ($P<0.01$); "A": essential amino acid; TEAA: total essential amino acid; TNEAA: non-essential amino acid; TAA: total amino acid.

Amino acid	Hepatopancreas		Muscle	
	Control	dsRNA	Control	dsRNA
Asp	2.49±0.04	2.70±0.02**	3.52±0.13	3.89±0.13*
Thr[A]	1.13±0.02	1.23±0.01**	1.60±0.04	1.78±0.07*
Ser	0.92±0.01	1.04±0.01**	1.54±0.05	1.69±0.05*
Glu	2.68±0.05	2.92±0.01**	5.02±0.18	5.49±0.14*
Gly	1.16±0.01	1.27±0.01**	2.12±0.05	2.16±0.08
Ala	1.12±0.02	1.25±0.01**	2.23±0.06	2.39±0.08*
Val[A]	1.15±0.02	1.29±0.02**	1.89±0.05	2.01±0.08
Met[A]	0.28±0.20	0.35±0.10	0.24±0.04	0.66±0.15**
Ile[A]	0.83±0.04	0.88±0.02	1.54±0.04	1.67±0.02**
Leu[A]	1.61±0.03	1.75±0.01**	2.54±0.06	2.77±0.06**
Tyr	1.10±0.01	1.18±0.01**	1.50±0.13	1.68±0.10
Phe[A]	1.14±0.03	1.24±0.04*	1.36±0.15	1.49±0.10
His[A]	0.56±0.00	0.60±0.02*	1.03±0.05	1.13±0.03*
Lys[A]	1.45±0.03	1.58±0.00**	2.34±0.09	2.59±0.09*
Arg	1.54±0.02	1.58±0.02*	3.17±0.08	3.45±0.14*
Pro	1.05±0.06	1.11±0.02	2.02±0.01	2.19±0.10*
TEAA	8.15±0.22	8.93±0.08**	12.53±0.45	14.10±0.35*
TNEAA	12.04±0.11	13.06±0.05**	21.14±0.62	22.95±0.79*
TEAA/TNEAA	0.68±0.02	0.68±0.01	0.59±0.01	0.62±0.01**
TAA	20.19±0.33	21.99±0.11**	33.67±1.10	37.06±1.13*

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Table 3(on next page)

Fatty acid composition in hepatopancreas and muscle of *E. sinensis* (% of total fatty acids).

"*" indicated significant difference between two groups ($P<0.05$); "**" indicated extremely significant difference between two groups ($P<0.01$); SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.

Fatty Acid	Hepatopancreas		Muscle	
	Control	dsRNA	Control	dsRNA
C14:0	1.40±0.01**	1.33±0.02	1.37±0.03	1.30±0.05
C15:0	0.64±0.00**	0.61±0.01	0.59±0.08	0.60±0.04
C16:0	21.56±0.25	22.34±0.19*	21.86±0.0.43	22.68±0.49
C17:0	0.38±0.01	0.37±0.01	0.47±0.04	0.44±0.06
C18:0	2.79±0.04	2.83±0.03	3.71±0.32	3.32±0.20
C20:0	0.23±0.01	0.24±0.00*	——	——
C21:0	0.23±0.01	0.24±0.01	——	——
C22:0	0.24±0.00	0.25±0.01	——	——
C23:0	0.40±0.03	0.36±0.03	1.16±0.30	0.94±0.23
C24:0	0.16±0.00	0.15±0.01	——	——
SFA	**28.02±0.26**	**28.73±0.09***	**29.16±0.12**	**29.28±0.21**
C14:1	0.22±0.01	0.21±0.01	——	——
C16:1	9.54±0.05	9.80±0.04**	8.63±0.61	8.99±018
C17:1	0.68±0.00**	0.63±0.01	0.66±0.03**	0.54±0.01
C18:1n9t	0.23±0.04	0.18±0.02	——	——
C18:1n9c	30.81±0.33	32.47±0.28**	30.39±0.66	31.74±0.16*
C20:1n9	0.84±0.01	0.88±0.00**	0.94±0.04	0.87±0.02
C22:1n9	0.16±0.00**	0.12±0.00	——	——
C24:1n9	0.35±0.03	0.45±0.02**	——	——
MUFA	**42.82±0.37**	**44.73±0.23****	**40.62±1.21**	**42.15±0.33**
C18:2n6c	23.58±0.37**	21.63±0.05	23.29±0.48	22.95±0.16
C18:2n6t	0.12±0.00	0.12±0.00	——	——
C18:3n3	2.22±0.11**	1.82±0.07	2.39±0.06**	2.10±0.06
C18:3n6	0.30±0.00	0.37±0.02**	——	——
C20:2	0.83±0.01	0.84±0.03	1.05±0.07	1.05±0.05
C20:3n3	0.20±0.01	0.22±0.02	——	——
C20:3n6	0.08±0.05	0.17±0.01*	——	——
C20:4n6	0.15±0.01**	0.10±0.01	——	——
C20:5n3	0.84±0.07*	0.64±0.04	2.34±1.34	1.57±0.39
C22:2	0.15±0.00	0.14±0.03	——	——
C22:6n3	0.64±0.07*	0.48±0.06	1.14±0.23	0.91±0.25
PUFA	**29.10±0.58****	**26.54±0.31**	**30.21±1.09**	**28.57±0.54**
n-3	3.90±0.24*	3.17±0.19	5.87±1.52	4.57±0.61
n-6	24.22±0.33**	22.40±0.08	23.29±0.48	22.95±0.16

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