Identification of MLST8, a Component of MTOR Complex 1 in *Eriocheir Sinensis*: CDNA Cloning and Its Expression Respond to Starvation

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Abstract: The mechanistic target of rapamycin (mTOR) signaling pathway is conserved among organisms from single-celled yeasts to complex multicellular ones such as human. Here we report the cDNA sequences and expression pattern of one gene encoding a component of mTOR complex 1 from crustacean *Eriocheir sinensis*, EsmLST8 (mammalian lethal with Sec13 protein 8). The deduced EsmLST8 from the cDNA sequence includes 317 amino acids (aa), which contains a WD40 superfamily region with WD40 repeats domains, forming a circularized beta-propeller structure. Expression analysis with qRT-PCR showed that in juvenile *E. sinensis*, the top three tissues with high mRNA levels for EsmLST8 are Y organ, stomach and hepatopancreas; the tissue with the lowest expression of mLST8 is eyestalk. After the animals' food deprivation, the expression of *EsmLST8* was significantly induced at 14d in claw muscles. These results provide basic information on the functions of mTOR signaling pathway in regulation of growth and nutritional metabolism in crustaceans.

1 INTRODUCTION

The mechanistic target of rapamycin (mTOR) is an conservative serine/threonine kinase of PI3K-related kinase (PIKK) family from single-celled yeast to multiple-celled complex animals such as human, which form two functionally distinct protein complexes of mTOR complexes: Complexes 1 (mTORC1) and 2 (mTORC2) (Yang et al., 2013). The mTORC1 responds to extracellular stimulus such as growth factors, energy, oxygen, amino acids and mechanical stimulus, and is involved in regulating protein, lipid, nucleotide, and glucose metabolism in mammals (Chen and Long, 2018). It plays a central role in controlling the balance between anabolism and catabolism of mammals in response to environmental conditions (Saxton and Sabatini, 2017).

The mLST8 (mammalian lethal with Sec13 protein 8), together with mTOR and raptor (regulatory protein associated with mTOR), are three core components of the mTORC1 (Yonezawa et al., 2004). The mLST8 plays critical roles in mTOR kinase activity by associating with its catalytic domain and stabilizing the kinase activation loop (Yang et al., 2013).

Crustaceans undergo periodic molting during their growth. The molting is controlled by steroid hormone ecdysteroids secreted by YO (Y-organ, crustacean molting gland) and molt-inhibiting hormone (MIH) neuropeptides secreted by X-organ/sinus gland complex in eyestalk (Mykles, 2011; Webster et al., 2012). Inhibited by MIH neuropeptides, the ecdysteroids are in low level in hemolymph at the stages of inter-molt and post-molt. The mTOR signaling pathway genes' transcripts were well present in YO by transcriptome analyses (Das et al., 2016); and the biosynthesis of ecdysteroids in YO requires mTOR-dependent protein synthesis in early pre-molt stage in crustacean (Abuhagr et al., 2016; Das et al., 2018). In black land crab, the mTOR signaling genes were up-regulated to activate YO and sustain ecdysteroidogenesis in mid- and late pre-molt stages (Abuhagr et al., 2014; Shyamal et al., 2018).

Investigating the different components of mTOR

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pathway is the necessary step to study the function of this pathway, while its most components in crustacean *Eriocheir sinensis* have not been intensively studied. In this study we cloned the cDNA sequence encoding a mTOR pathway component mLST8 (EsmLST8) in *E. sinensis*, and their transcriptional expression were investigated when animals under conditions of starvation.

2 MATERIALS AND METHODS

2.1 Animal Sampling

One-year-old juvenile crabs, weighing 10.95 ± 2.25 g, obtained from Gucheng fisheries farm (Jiangsu Province, China) in May, 2019. The crabs were maintained in tanks containing freshwater approximately 2 cm in depth with the nature photoperiod and the temperature of 25 ± 2°C. The water was renewed once each day and the crabs were fed with commercial pellets (Huaxu®, Xinxiang, Henan Province, China) of 10% body weight. The ingredients of the pellets include crud protein (≥30%), crud fat (≥3%), crude ash (≥18%), total phosphorus (≥1.0%), Ca (≥0.6%), lysine (≥1.3%) and water (≤12.0%). After acclimated to the laboratory conditions for 1 week, animals' hepatopancreas, eyestalk, gill, stomach, intestines, Y organ, heart and claw muscle tissues were collected and immediately stored at -80^{0}C for cDNA cloning and expression analysis. Moreover, the individuals in inter-molt stage, identified according to the criterion we previously reported (Tian et al. 2012) for starvation experiment. Animals were reserved individually in different tanks in order to avoid cannibalism. Hepatopancreas and claw muscles were collected at the 0, 7 and 14 days and stored at -80^{0}C for RNA isolation and qRT-PCR.

2.2 Cloning of cDNA Encoding mLST8 in *E. sinensis*

Total RNA was extracted from hepatopancreas of *E. sinensis* with TRIzol. RNA concentration and purity were measured with ultraviolet spectrophotometer. The cDNA was synthesized with Revert Aid kit (Fermentas, USA) and used as PCR template later. The PCR primers (Table 1) were designed based on the TSA (transcriptome shotgun assembly) sequence (GenBank accession no. **GBZW01005746.1**) in NCBI database.

Table 1: The primers for mLST8 amplification in *Eriocheir sinensis*.

Primer	Sequence (5'–3')	Application
EsmLST8-F1	ATTACCTGTCT TACCTGCC	RT-PCR
EsmLST8-R1	GTACCATCAC CTCCTGTG	RT-PCR
EsmLST8-F2	CGCAGACTCC CAACACATTA	qRT-PCR
EsmLST8-R2	GCTGTACTCG CTCTTGATCTC	qRT-PCR
27S -F	GGTCGATGAC AATGGCAAGA	qRT-PCR
27S -R	CCACAGTACT GGCGGTCAAA	qRT-PCR

The amplification of EsmLST8 cDNA with PCR in following system: 5.0μl cDNA template, 1.5μl upstream and downstream primers (10 uM), 1.0μl dNTP Mix (10 mM), 1.0μl Ex taq (Takara, Japan), 25.0μl 2×Ex taq Buffer (Takara, Japan), add sterile deionized water to a total volume of 50 μl. The reaction conditions were as follows: 94°C pre denaturation for 2 min; 94°C denaturation for 30 sec, 55°C annealing for 30 sec, and 72°C extension for 1 min 40 sec, run 35 cycles; 72°C extension for 10 min. The PCR products were detected by 1.2% agarose gel electrophoresis, recovered by gel cutting, ligated into the pUCm-T vector (Sangon, Shanghai), transformed into *E. coli* and cultured in LB-Amp plates with X-gal and IPTG. Positive colonies (white colonies) were selected and sequenced with a 3730xl DNA Analyzer (ABI) by Sangon Biological Co., Ltd. (Shanghai).

2.3 Bioinformatics Analysis of EsmLST8

The ORF finder of the NCBI website and the translate tool of Expasy were used to identify the reading frame and translate it into amino acid sequences. Sequence similarity analysis and multiple alignment were performed with BLAST and COBALT (constraint-based alignment tool for multiple protein sequences) from NCBI as well as Clustal from Bioedit. The editing of the cDNA and protein sequences was carried out with Bioedit. CD-search from NCBI was used for conserved functional domain prediction for the protein (Marchler-Bauer et al., 2017), SWISS-MODEL, Phyre2, PONDR and pyMOL software (Mura et al., 2010) were used to predict and analyze the structure and natural disorder regions of the proteins. The CLUSTAL and MUSCLE multiple alignment tools and the NJ (Neighbor-Joining) or UPGMA tree building tool of MEGA-X were used to build the cladogram of proteins (Kumar et al., 2018).

2.4 The Expression of *EsmLST8* in Different Tissues

The expression of *EsmLST8* was evaluated by the 2-ΔΔCt method with real-time PCR instrument (Applied Biosystems® QuantStudio® 3). The cDNA from different tissues was used as templates for qRT-PCR with the primers designed based on the cDNA sequence of ESmLST8 (Table 1). The 27s RNA was used as an internal reference as it is the most stable and suitable for *E. sinensis* (Huang et al., 2017). The reaction was as follows: 95°C for 3 min; 40 cycles of 95°C for 5 sec, 60°C for 15 sec, and 95°C for 15 sec. The melting curve generation from 65°C to 99°C in steps of 0.5°C/s. Three technical replicates were performed for qRT-PCR.

2.5 Statistical Analysis

Statistical analysis was performed using one-way ANOVA with SPSS 18.0 software. Post hoc comparisons of the means were performed using Duncan's least significant difference test with a significance level of $P \leq 0.05$. The values are presented as the mean ± standard error (SE).

3 RESULTS

3.1 Sequence Analysis on cDNA and the Deduced Protein of EsmLST8

The cDNA of 1001 nucleotides (nt) encoding a putative EsmLST8 was cloned and sequenced, which contains partial sequences of 5'- and 3'- untranslated regions and the complete coding sequence (CDS) of 954 nt (Figure 1). The deduced 317 amino acids (aa) sequence from the CDS was searched as query by blast in non-redundant GenBank proteins database (organisms were limited in crustaceans), the aligned sequence with the highest score was target of rapamycin complex subunit lst8-like in *Penaeus vannamei* (GenBank accession no. **XP027234568.1**, with the total score of 577 and the E-value of 0.0). We designated the proteins as EsmLST8 (MTOR associated protein, LST8 homolog in *E. sinensis*) and performed further analysis.

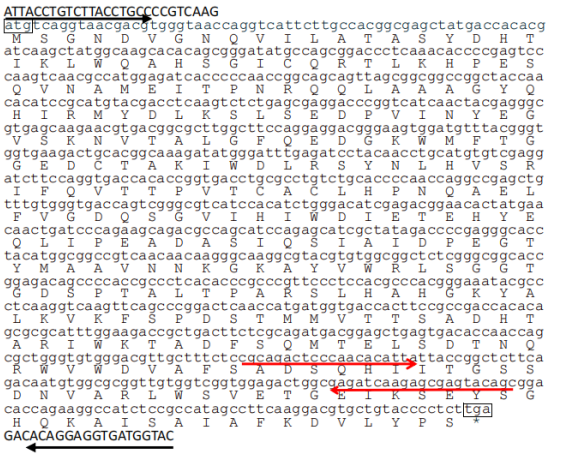

Figure 1: Nucleotide and deduced amino acid sequences of the *EsmLST8*, including a partial 5' and 3' untranslated region (UTR). The initiation codon is indicated with atg. The stop codons are boxed and indicated with an asterisks. The sequences corresponding primers for RT-PCR and qRT-PCR are underlined with black and red arrows respectively.

Predicated by the CD search tool, the EsmLST8 contains WD40 repeats structural motif, structural tetrad on conserved domain WD40, covering 11-288 aa (Figure 2). A three-dimensional structure of EsmLST8, which covered 10-312 aa, was predicted using SWISS-MODEL workspace based on the template (SMTL ID 5wbu.1.B) from human Crystal structure of mLST8-PRAS40 complex (Yang et al., 2017). The sequences of EsmLST8 and the template human mLST8 (H-mLST8) used for modeling showed 64.03% identity. The polypeptide backbones of the EsmLST8 (depicted in red) and H-mLST8 (green) are almost perfectly overlapped, except that Asp257-Trp262 of EsmLST8 form a shorter loop than the counterpart region in H-mLST8 (Figure 3). The EsmLST8 cDNA sequences have been deposited in GenBank with the accession number of **MN244302.**

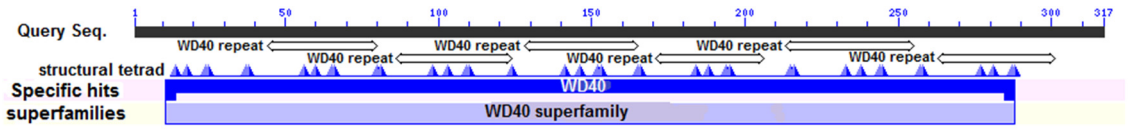

Figure 2: The conserved domains (WD40 domain) and function sites of the EsmLST8 covering 11-288 aa predicted by CD-search.

Figure 3: Predicted three-dimensional structure of the EsmLST8 (red) covered 10-312 aa and its template human mLST8(green). Val10, Asp312, Asp257-Trp262 were labelled.

3.2 Multiple Sequence Alignment and Phylogenetic Analysis on EsmLST8

EsmLST8 was aligned with homologs from *P. vannamei* (GenBank accession no. **XP027234568.1**), *Zootermopsis nevadensis* (GenBank accession no. **XP021941884.1**), *Cryptotermes secundus* (GenBank accession no. **XP023707874.1**) and *Cephus cinctus* (GenBank accession no. **XP015604398.1**) using COBALT on the NCBI web site. The sequence identity of EsmLST8 compared with above 4 sequences are 0.854, 0.670, 0.664 and 0.668 respectively. The WD40 domain, WD40 repeat motif and structural tetrad sites were showed in the alignment. The function sites and structural motifs are more conserved between two decapods or among three insects respectively; While there are several sites in **EsmLST8** that are different with that in *P. vannamei* as well as other 3 insects (Figure 4, marked with the star symbol).

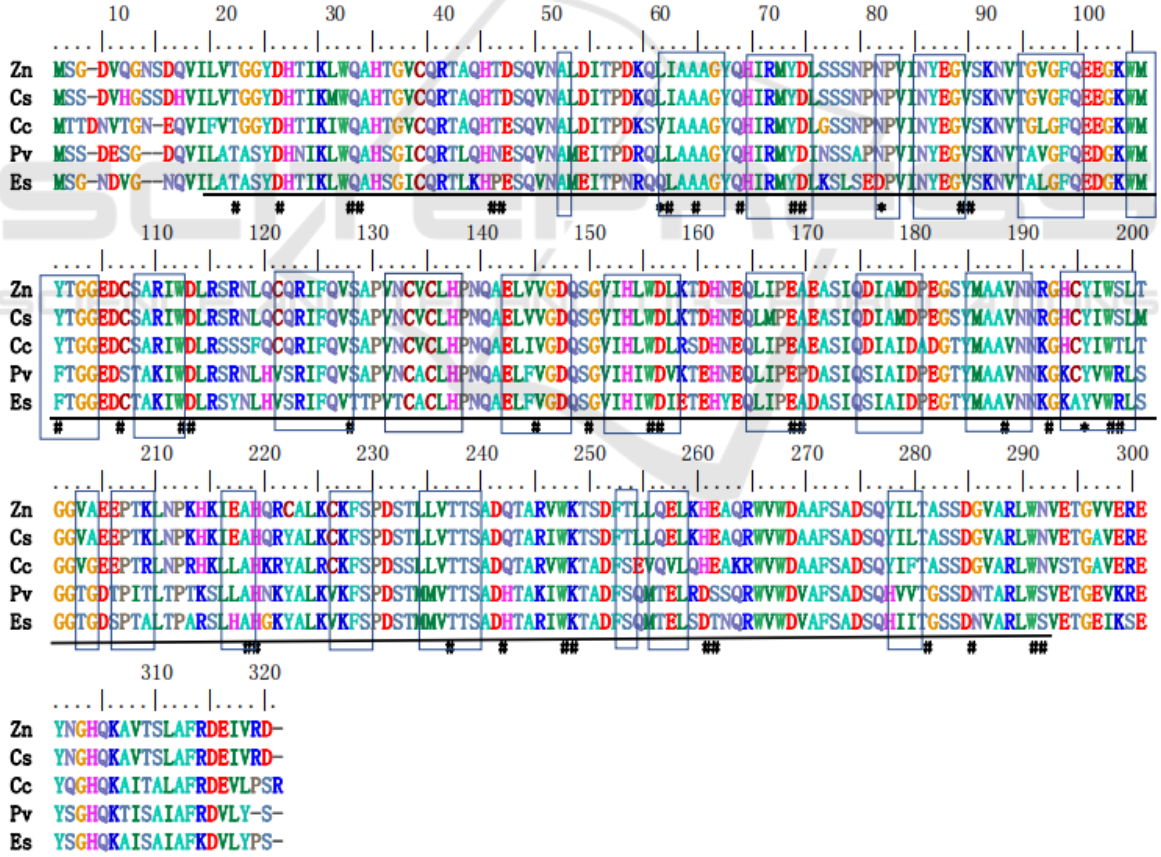

Figure 4: Multiple alignment of EsmLST8. Zn, *Zootermopsis nevadensis*; Cs, *Cryptotermes secundus*; Cc, *Cephus cinctus*; Pv, *Penaeus vannamei*; Es, *Eriocheir sinensis*. Hashtags (#) indicate structural tetrad; Frames indicate W40 repeat motif (structural motif); Underlines indicate WD40 domain; Stars (*) indicate different sites in *Eriocheir sinensis* compared with *Penaeus vannamei* and other 3 insects.

Phylogeny analysis together with homologs from arthropods, vertebrates and a single cell species (*Tetrahymena thermophila*) showed that EsmLST8 and mLST8 of *P. vannamei* clustered together, while did not clustered with that from other crustaceans, i.e. *Daphnia magna*, *Eurytemora affinis*, *Hyalella azteca* and *Armadillidium vulgare*; compared with the 2 decapods, mLST8 in *D. magna*, *E. affinis*, *Hyalella Azteca* and 3 insects (*Cephus cinctus*, *Zootermopsis nevadensis* and *Cryptotermes secundus*) are more close with that in 3 veterbrates (*Homo sapiens*, *Gallus gallus*, and *Xenopus tropicalis*) (Figure 4).

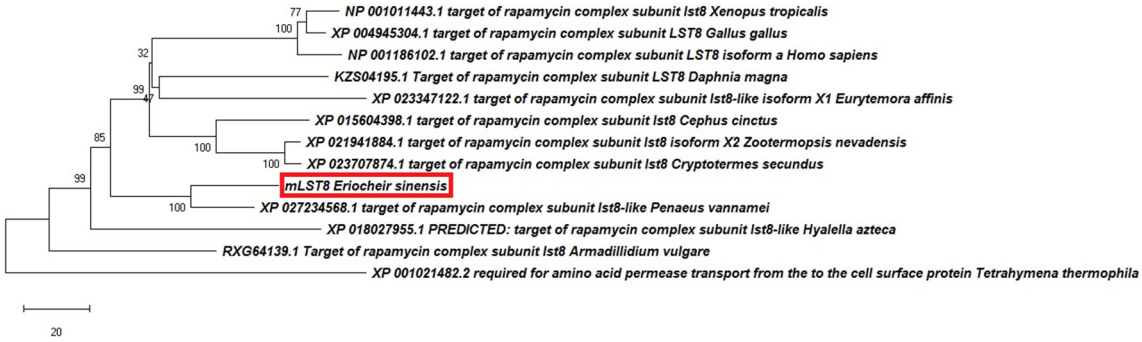

Figure 5: Phylogenetic tree derived from multiple alignments of mLST8 (A) from different organisms. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method and are in the units of the number of amino acid differences per sequence. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.5). This analysis involved 13 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 426 positions in the final dataset.

3.3 Tissue Distribution of *mLST8* in Juvenile *E. sinensis*

The expression pattern of *EsmLST8* was examined among the examined eight tissues (stomach, hepatopancreas, intestines, eyestalk, Y organ, gill, heart and claw muscles) from juvenile *E. sinensis*. It was lowest expressed in eyestalk. The top three tissues with high mRNA levels for the two genes are Y organ, stomach and hepatopancreas (**Figure 6**).

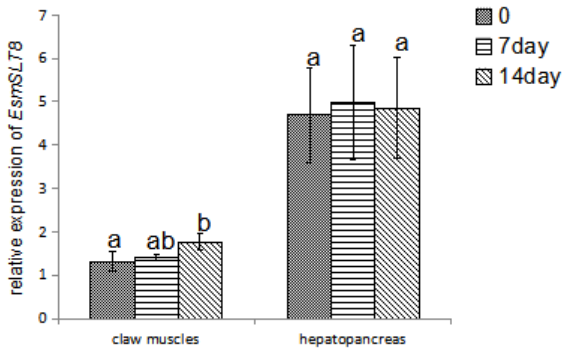

Figure 6: The relative expression of *mLST8* in different tissues of juvenile *E. sinensis*, N=3.

3.4 The Expression of *mLST8* Responded to Starvation in Juvenile *E. sinensis*

Two weeks' starvation had no significant effect on the expression of *EsmLST8* in hepatopancreas. While the expression of EsmLST8 was increased significantly at 14d in claw muscles after food deprivation **(Figure 7)**.

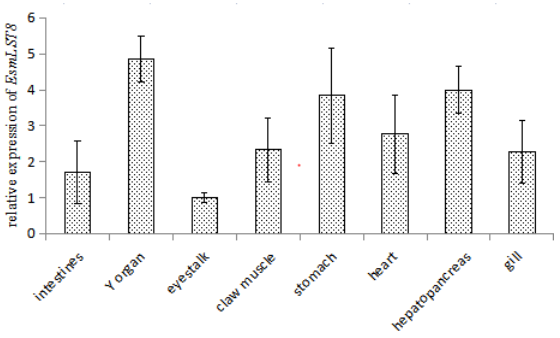

Figure 7: The relative expression of *mLST8* in different tissues of juvenile *E. sinensis*, N=3. Different letters above the errorbars indicate significant differences (P<0.05).

4 DISCUSSION

The mTOR signal pathway is highly conserved among broad range organisms from single-celled yeast to multiple-celled animals and plants. In the yeast *Saccharomyces cerevisiae*, mLST8 homologue negatively regulates amino acid biosynthesis as a component of the mTOR pathway (Chen and Kaiser, 2003). In the plant *Arabidopsis*, mutations in the homolog of mLST8 impaired plant growth, flowering, and metabolic adaptation to long days (Moreau et al., 2012). It is reported that mLST8 was upregulated in human colon and prostate cancer cell lines and tissues, and knocking down of its expression suppressed formation of mTORC1/C2 complex, at the same time inhibit tumor growth and invasiveness in different human cancer cells (Kakumoto et al., 2015). Over expression of mLST8 promoted normal epithelial cells growth, while knockdown had no effect on their growth (Kakumoto et al., 2015). We hypothesize that in crustaceans the mLST8 is also important components of the pathway and have important functions involving in metabolic and growth controlling. So, we cloned and sequenced the cDNA encoding the proteins in *E. sinensis* and its expression of transcription was investigated in this study.

The EsmLST8 contains the WD40 superfamily domain including 7 WD40 repeats. The WD40 repeat is a short structural motif of around 40 amino acids, often terminating in a tryptophan-aspartic acid (W-D) dipeptide (Neer et al., 1994). WD40 domain-containing proteins have 4 to 16 repeating units, all of which form a circularised beta-propeller structure (Villanueva et al., 2016). The predicted structure of EsmLST8 is consistent with this knowledge, and it is highly conserved with and almost perfectly superimposed to the human mLST8. The exception to this is the region Asp257-Trp262 of EsmLST8, which did not form the longer loop in the corresponding region as human mLST8. What's more, there are 3 sites with unique amino acids in regions of WD repeats of EsmLST8 compared with mLST8 from *P. vannamei*, *C. cinctus*, *C. secundus*, and *Z. nevadensis*. The meaning of these differences on its function are need to be investigated further in the future research. The cDNA we cloned encoding an EsmLST8 was also supported by the phylogenic analysis with mLST8 homologues from other organisms.

The *EsmLST8* was expressed in all the examined tissues. For crustaceans, the biosynthesis of ecdysteroids in Y organ depends on mTOR pathway (Abuhagr et al., 2016; Shyamal et al., 2018). However, it was inhibited by MIH during the inter-molt stage of the molting cycle. Accordingly, the transcripts abundance of *EsmLST8* was highest in Y organ and lowest in eyestalk. Moreover, some much higher expressions of *EsmLST8* was also identified in digestive organs, such as stomach and hepatopancreas, indicating a strong digestion in juvenile crabs contribute to their rapid growth is connected with the mTOR signaling pathway.

Crustaceans often encounter starvation for a short or long time in their livelihood due to different reasons: molting, environmental changes, or pollution (Hu et al., 2012). Hepatopancreas is a vital organ for animal in growth and metabolism and store much of lipids and glycogen in crustaceans (Tian et al., 2012). During the inter-molt stage of decapods, food deprivation causes the energy resources to be re-allocated for tissue maintenance and survival (Morales et al., 2012). Some decapods adopt an adaptive strategy to avoid the usage of high costly macromolecules instead preferentially utilize energy from glycogen or lipid catabolism during food deprivation period (Sacristan et al., 2017). This contributes to maintain the stability of the proteins and the intracellular pool of amino acids, which can account for the stability of mTORC1 signaling, and the transcriptions of *EsmLST8* was kept around the same level during the 14 days' period of food deprivation in hepatopancreas identified in these studies.

However, the expression of *EsmLST8* increased in claw muscles under the conditions of 14 days' food deprivation in juvenile *E. sinensi*. This result is consistent with the research report in species of juvenile pacu, *Piaractus mesopotamicus*, in which a short period of starvation induced the expression increasing of anabolic genes, such as *PI3K*, *mTOR*, *mLST8* and *RAPTOR* in skeletal muscle (Paula et al., 2017). In mouse and human cells, the activation of mTORC1 was observed to promote an increase of protein degradation as well as protein synthesis (Zhang et al., 2014). Our results indicate that maybe as well as in mammals, amino acids could be derived from protein degradation in crustacean skeletal muscles, which was triggered by food deprivation, and maintained the intracellular pool of amino acids for essential proteins synthesis via increasing the expression of mTORC1 pathway components.

5 CONCLUSION

We cloned and sequenced the cDNA sequences encoding an important mTOR pathway component EsmLST8 in *E. sinensis.* EsmLST8 contains WD40 superfamily region comprising of WD40 repeats

domains, which make a circularized beta-propeller structure. Y organ, stomach and hepatopancreas were the top three tissues with high levels of *EsmLST8* transcripts while the lowest expression of the gene is in eyestalk. The transcriptional expression of *EsmLST8* was increased significantly at 14d in claw muscles after two weeks' food deprivation. The results of this study provide basic data for studying the roles of mTOR signaling pathway in the regulation of crustacean growth and nutritional metabolism.

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