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# Regulation of adductor muscle growth by the IGF-1/AKT pathway in the triploid Pacific oyster, *Crassostrea gigas*

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## Abstract

We investigated the insulin-like growth factor 1 (IGF-1)/AKT signaling pathway involved in muscle formation, growth, and movement in the adductor muscle of triploid Pacific oyster, *Crassostrea gigas*. Large and small triploid oysters (LTs and STs) cultured under identical conditions were screened, and the signaling pathways of individuals with superior growth were compared and analyzed. mRNA and protein expression levels of actin, troponin, tropomyosin, and myosin, proteins important in muscle formation, were higher in LTs compared with STs. Expression levels of IGF-1, IGF binding protein (IGFBP), and IGFBP complex acid-labile subunit were also higher in LTs compared with STs. Phosphorylation of the IGF receptor as well as that of AKT was high in LTs. In addition, the expression of phospho-mammalian target of rapamycin and phospho-glycogen synthase kinase 3 $\beta$  was increased and the expression of Forkhead box O3 was decreased in LTs. Therefore, we suggested that the IGF-1/AKT signaling pathway affects the formation, growth, and movement of the adductor muscle in triploid oysters.

**Keywords:** Triploid oyster, Adductor muscle, Muscle growth, Protein synthesis, Protein degradation

## Background

The triploid Pacific oyster *Crassostrea gigas* was first reported by Stanley et al. (1981) and commercialized on the West Coast of America in 1985 (Allen et al. 1989). At present, it is produced by crossing diploid female and tetraploid male hybrids (Guo et al. 1996), and this method is being applied to various oysters. Triploid oysters consume less energy during maturity because they do not develop germ cells. As a result, growth is faster because more energy can be used to grow somatic cells compared with diploid oysters (Allen and Downing 1986; Nell 2002; Nell and Perkins 2005). However, the signaling pathway responsible for this growth has not yet been elucidated.

Activation of the adductor muscle, i.e., muscle activity, is associated with oyster feeding behavior, which directly affects oyster growth (Hopkins 1936). Muscles are divided into striated muscles, such as skeletal and cardiac

muscles, which are regularly arranged, and non-striated muscles, including smooth muscle, which make up the vessels, respiratory system, and stomach. The adductor muscle of the oyster is a typical striated muscle, which contracts and relaxes through the action of actomyosin according to the cytosolic concentration of calcium. Actomyosin is composed of actin and myosin, and troponin (T, C, I) and tropomyosin are required for their binding and formation (Kuo and Ehrlich 2015). Therefore, in this study, the mechanism of the production and activity of the adductor muscle, which is most closely related to oyster growth, was confirmed by evaluating the expression of actin, troponin, and tropomyosin, which affect the formation of actomyosin.

Muscles adapt and change according to functional needs (Rennie et al. 2004). If there is a load, muscle mass increases. However, if there is no load or the muscle is not used, muscle mass will decrease. When muscle growth is active, the roles of growth hormone (GH) and insulin-like growth factor 1 (IGF-1) are important (Velloso 2008). GH binds to the GH receptor (GHR) and regulates various signals related to growth. GHR is ubiquitously expressed in various tissues to mediate the action of GH, and GH

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increases the expression of IGF-1 in most tissues (Frick et al. 1998; D'Ercole et al. 1984; Gosteli-Peter et al. 1994; Jorgensen et al. 2006). Unlike GH and GHR, IGF-1 expression is stable and does not change significantly during the day (Buckway et al. 2001; Velloso 2008). Therefore, it can be used as a factor to measure muscle growth.

Muscle hypertrophy occurs when the rate of protein synthesis exceeds the rate of protein degradation (Schiaffino et al. 2013). The IGF-1/protein kinase B (also known as AKT) signaling pathway, which is activated upon IGF-1 recognition by the IGF receptor (IGF-1R), is the most representative mechanism of muscle mass increase. IGF-1 signaling promotes muscle growth via increased protein synthesis and inhibition of protein degradation (Schiaffino and Mammucari 2011). IGF-1/AKT signaling mediates protein degradation by modulation of muscle atrophy F-box (MAFbx), muscle ring finger 1 (MURF1), and microtubule-associated protein 1 light chain 3 (LC3) via Forkhead box O3 (FoxO) (Manning and Cantley 2007). IGF-1/AKT signaling also promotes protein synthesis via mammalian target of rapamycin (mTOR) and glycogen synthase kinase (GSK)-3 $\beta$ . Confirmation of the expression of each of these factors will confirm that the growth of triploid oysters is dependent on IGF-1/AKT signaling, as has been shown in mammals and other mollusks.

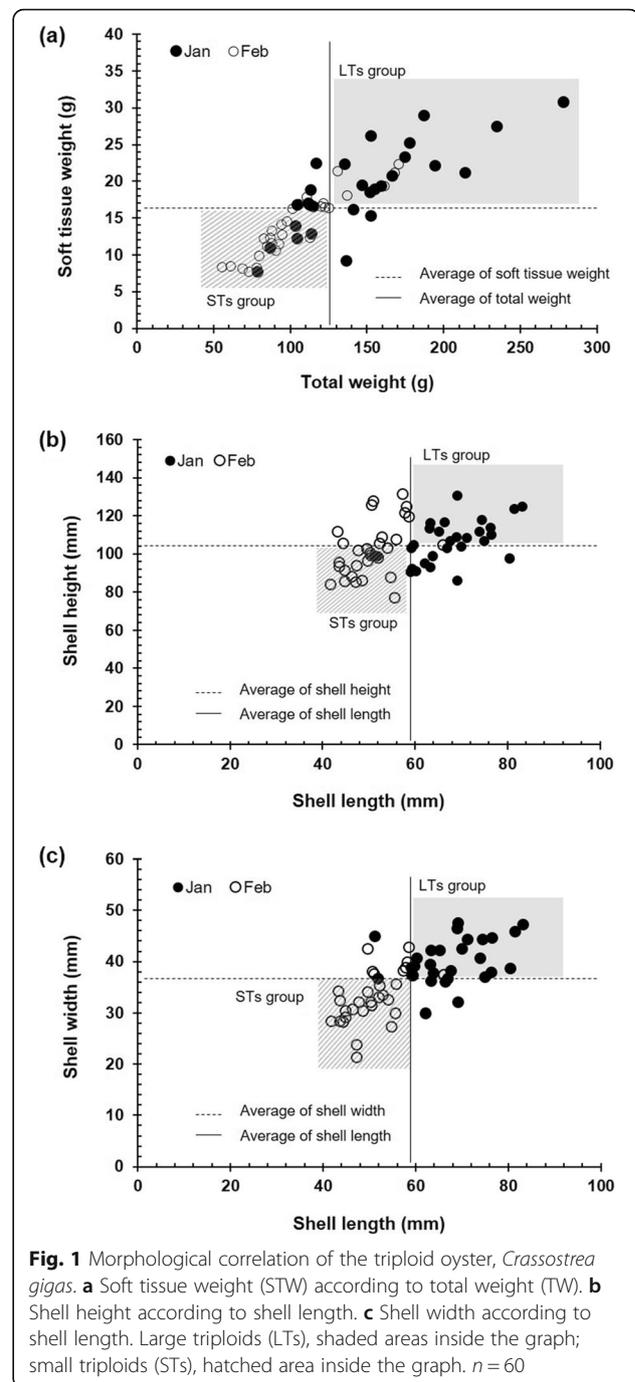
In this study, we analyzed the IGF-1/AKT signaling pathway and its involvement in the growth of triploid oysters and confirmed the relationship between the expression of actin and troponin, which are involved in muscle formation in the triploid oyster.

## Methods

### Sampling and identification of the triploid oyster

Triploid oysters were collected at a farm in *Taeangan, Republic of Korea* (latitude 36.903367, longitude 126.26489), in January and February of 2017. A total of 60 oysters were collected and separated into large and small triploid oysters (LTs and STs, respectively). Since there is no standardized method for classifying oysters, this study divided them according to the following criteria. LTs selected samples that meet the three conditions as shown in Fig. 1. First, both STW and TW of oysters are higher than average. Second, both shell length and shell height of oysters are above average. Third, shell width and shell length of oysters are above average. When the three conditions were satisfied, they were classified as LTs and vice versa as STs. The adductor muscle was isolated, and 0.5 g of each muscle was subdivided, frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ .

Triploidy was confirmed as described previously (Allen 1983) with minor modifications. Plasma was collected from 10 of the collected samples, added to 0.7 mL



ethanol, and stored at  $-20^{\circ}\text{C}$  for more than 4 h. After removing the supernatant by centrifugation (3000 rpm, 10 min), it was washed three times with phosphate-buffered saline. Samples were stained with propidium iodide for 30 min at room temperature and measured using a flow cytometer (BD Accuri C6, BD Biosciences, Franklin Lakes, NJ, USA). Ten diploid oysters were collected from the southern Korean sea for DNA verification compared with the triploid oyster.

### cDNA synthesis and reverse-transcription polymerase chain reaction (RT-PCR)

The adductor muscle (0.5 g, LTs  $n = 3$ ; STs  $n = 3$ ) was pulverized by adding 1 mL Trans-Zol UP (TransGen Biotech, Beijing, China), and total RNA was extracted using Trans-Zol UP according to the manufacturer's instructions. cDNA was synthesized from 2  $\mu$ g total RNA using the PrimeScript first strand cDNA synthesis kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. RT-PCR was performed using Emerald Amp GT PCR Master Mix (TaKaRa Bio). The primers (targeting elongation factor 1 $\alpha$ , IGF-1, IGF-1R, IGF binding protein complex acid-labile subunit [IGF-ALS], actin, myosin, troponin T, troponin I, and tropomyosin) and reaction parameters (denaturation, 95 °C, 30 s; annealing, indicated temperature, 30 s; elongation, 72 °C, 30 s) used for RT-PCR are shown in Table 1. Primers were designed based on the gene sequences of *C. gigas* obtained from NCBI GenBank. The PCR products were confirmed by 1% agarose gel electrophoresis.

### Protein purification and western blotting

Total protein extraction was performed by adding 1 mL radioimmunoprecipitation buffer to 0.5 g adductor muscle (LTs,  $n = 3$ ; STs,  $n = 3$ ). The homogenized tissue was centrifuged (12,000 rpm, 10 min, 4 °C) and the supernatant used. Protein quantification was performed using the bicinchoninic acid assay, and bovine serum albumin (BSA) was used as a standard. Proteins (2  $\mu$ g/ $\mu$ L) were

subjected to gel electrophoresis using a 12% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane for immunoblotting. Membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 1% BSA at room temperature for at least 2 h. The membranes were then incubated with the primary and secondary antibodies at room temperature for at least 1 h, followed by detection with enhanced chemiluminescence western blotting reagents (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Between each step, two washes with TBST were performed. The following primary antibodies and the anti-mouse secondary antibody were obtained from Santa Cruz Biotechnology, Inc., and diluted 1:1000: IGF-1, IGF binding protein (IGF-BP)-3, IGF-1R, phosphorylated (p)-IGF-1R, AKT, p-AKT, mTOR, p-mTOR, FoxO, p-FoxO, GSK3 $\beta$ , p-GSK3 $\beta$ , eukaryotic translation initiation factor 4E binding proteins 1 (4EBP1), Ribosomal protein S6 kinase beta 1 (p70S6K1), MAFbx, MURF1, LC3, eukaryotic translation initiation factor 2B (eIF2B), nebulin, neural Wiskott–Aldrich syndrome protein (N-WASP), peroxisome proliferator-activated receptor  $\gamma$  coactivator (PGC) 1 $\alpha$ , troponin I, troponin T, and F-actin antibodies.

### Statistical analysis

RT-PCR and western blotting results were analyzed using GeneTools software (version 4.03; Syngene, Cambridge, UK). Data are presented as means  $\pm$  standard

**Table 1** Primer sequences for RT-PCR, amplicon size, PCR efficiencies, and GenBank accession numbers of the genes evaluated in this study. Primers were designed based on *Crassostrea gigas* sequences. *F* forward primer, *R* reverse primer, *AT* annealing temperature

Gene	Accession number	Sequence (5'–3')	Amplicon size (bp)	PCR cycle	AT (°C)
EF1 $\alpha$	AB122066.1	(F) CCACTGGCCATCTCATTTAC	393	20	60
		(R) TGTTGACACCAATGATGAGC			
IGF-1	XM_011417420.2	(F) ATGGTTTGCCTGTCTTGAG	336	25	55
		(R) AGATCCTTTCTTCTTGCGGC			
IGF-1R	AJ535669.1	(F) TGAGGAGGGTGATGAGGATA	375	25	55
		(R) ATTGCACTGTAGGGATTGGA			
IGF-ALS	XM_011417921.2	(F) ATGTCCAAAACAATGCGTCT	359	25	55
		(R) TCCAAAACGCGTAACTTTTC			
Actin-2	EKC31894.1	(F) TTTCCGCGGAGATGATGCC	434	20	60
		(R) TGGGGACAGTGTGGGTGACA			
Myosin	EKC37566.1	(F) TTTGGCTGGTGAGGCACAGG	544	20	60
		(R) TTTGCTGAGCTGGCGTTGGA			
Troponin T	XM_020062462.1	(F) AGGAACGCGAGAAAGAACA	375	20	60
		(R) TGATCCTCTGGGACAGGAAG			
Troponin I	XM_011455869.2	(F) CCACCCTGGAGGAAGAAGTC	187	20	60
		(R) AAATTGCCACGGAATCTGA			
Tropomyosin	NM_001308906.1	(F) GCCATGAAAATGGAGAAGGA	381	20	60
		(R) GGCGTTATTGAGGTTTTCCA			

deviation and were analyzed using Statistical Package for Social Sciences, version 10.0 (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using Duncan's multi-range test followed by one-way analysis of variance.  $p < 0.05$  was considered to indicate statistical significance.

## Results

### Growth characteristics and identification of triploid oysters

In total, 60 triploid oysters collected from Taean were classified as LTs and STs depending on their size (shell length, SL; shell height, SH; shell width, SW) and weight (total weight, TW; soft tissue weight, STW). The TW of the triploid oysters ranged from 55.34 to 277.75 g, with a mean of  $125.40 \pm 43.50$  g. The STW ranged from 7.72 to 30.87 g, with a mean of  $16.55 \pm 5.54$  g (Fig. 1a). Fatness ranged from 6.80 to 19.30%, with a mean of  $13.34 \pm 2.19\%$ . The growth rate of SL/SH, ranged from 0.45 to 0.88, while the growth rate of SW/SL, ranged from 2.20 to 4.00 (Fig. 1b, c), with means of  $0.63 \pm 0.09$  and  $2.89 \pm 0.38$ , respectively. In this study, 10 individuals were selected as LTs or STs with a satisfactory combination of STW/TW, SH/SL, and SW/SL and were used to analyze the growth signals of muscles according to their size (Fig. 1, LTs, shaded areas inside the graph; STs, hatched areas inside the graph).

Flow cytometry revealed that the average amount of DNA in diploid oysters, which were used as a control, was  $245 \pm 38.91$ , and the average amount of DNA in triploid oysters was  $368 \pm 23.23$  (Fig. 2). The DNA content ratio of diploid to triploid oysters was 1:1.5, confirming that the oysters used in this study were triploid.

### Expression of muscle proteins according to triploid oyster size

The differences in mRNA and protein expression of actin, myosin, troponin, and tropomyosin, which are integral in muscle formation, were examined. The mRNA expression of actin, troponin, and tropomyosin was 1.43–2.22-fold higher in LTs than in STs (Fig. 3a), while the protein expression of actin, troponin T and I, and tropomyosin was 1.18–3.60-fold higher in LTs than in STs (Fig. 3b). Thus, the expression of mRNAs and proteins responsible for muscle formation, contraction, and relaxation varied depending on the size of the oyster.

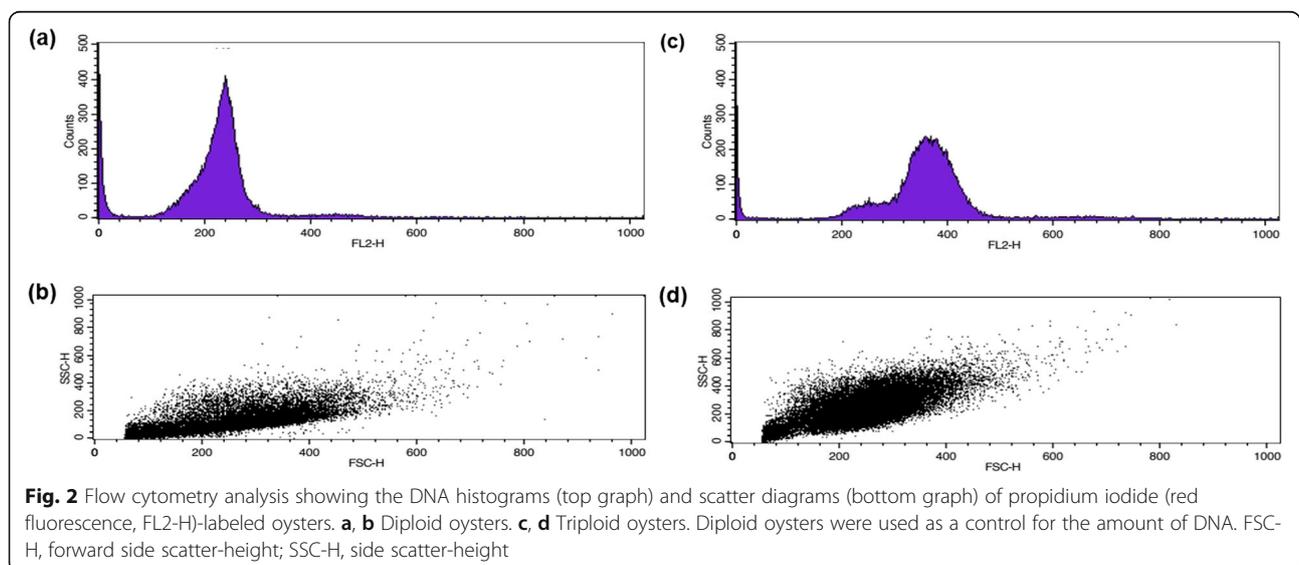
### Expression of IGF-1 according to triploid oyster size

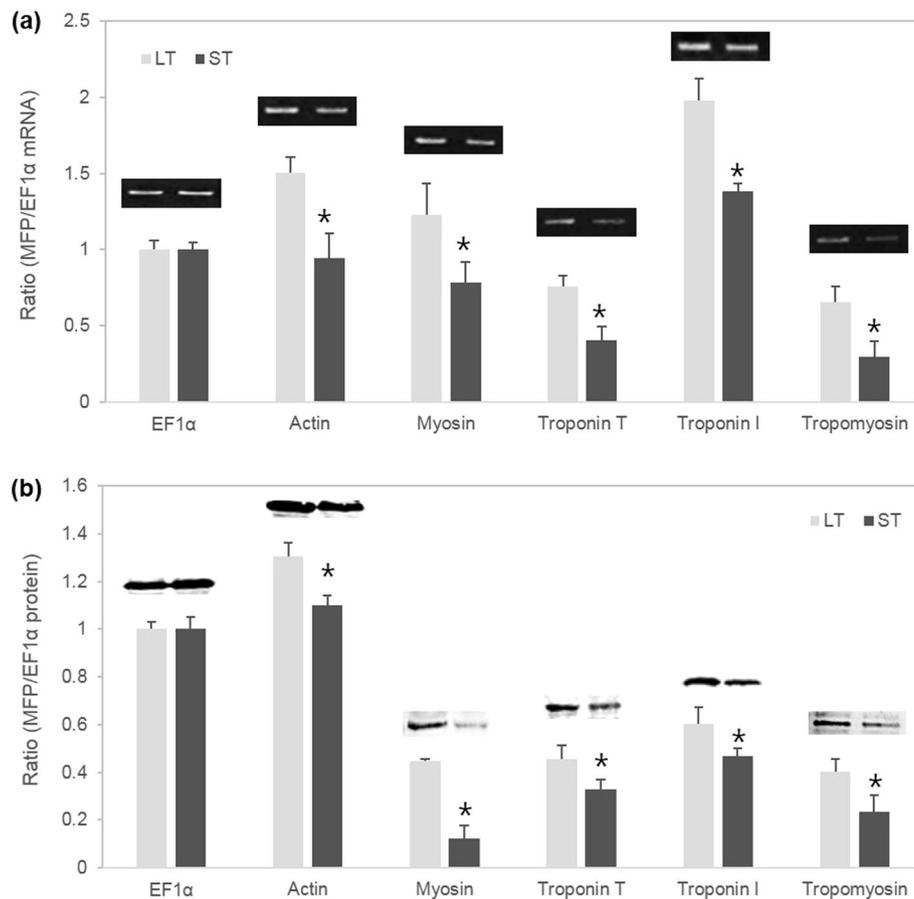
The expression level of IGF-1 mRNA was three times higher in LTs than in STs (Fig. 4a). IGF-ALS, which binds IGF-1 and IGF-IR and initiates IGF-1 signaling, also showed high mRNA expression in LTs (Fig. 4a).

IGF-1 protein expression, as well as that of IGF-BP and IGF-IR, was also higher in LTs compared with STs (Fig. 4b). In particular, the level of p-IGF-IR was significantly increased in LTs compared with STs. To investigate the IGF-1/AKT signaling pathway involved in the synthesis and degradation of muscle proteins, the levels of AKT1 and p-AKT were assessed. The level of p-AKT was 1.29-fold higher in LTs compared with STs.

### Mechanism of protein synthesis

The expression of mTOR, GSK3 $\beta$ , 4EBP1, and p70S6K1, elements downstream of IGF-1/AKT signaling associated with protein synthesis, were confirmed (Fig. 5a). Phosphorylation of mTOR was 1.46-fold higher in LTs compared with STs. Consequently, the expression level of 4EBP1 was decreased and that of p70S6K1 increased. The





**Fig. 3** Expression of muscle-forming proteins according to triploid oyster size. **a** mRNA expression ( $n = 5$ ). **b** Protein expression ( $n = 3$ ). \* $p < 0.05$  vs. LT. LT, large triploid; ST, small triploid; MFP, muscle-forming proteins

expression of p-GSK3 $\beta$  was also 1.37-fold higher in LTs compared with STs. This increased protein synthesis occurred via inhibition of eIF2B expression.

#### Mechanism of protein degradation

The expression of FoxO, an IGF-1/AKT downstream effector related to protein degradation, was increased 2.64-fold in STs compared with LTs (Fig. 5b), in contrast to mTOR and GSK3 $\beta$ . FoxO affects the function of MAFbx, MURF1, and LC3, and the expression of these proteins was decreased in LTs compared with STs. MAFbx and MURF1 are important in the degradation of muscle proteins including myosin via proteasome-dependent ubiquitylation, and LC3 is involved in the autophagy–lysosome pathway.

#### GSK3 $\beta$ and actin polymerization

GSK3 $\beta$  increases the phosphorylation of nebulin. Non-phosphorylated nebulin has been shown to modulate N-WASP to promote elongation and nucleation of actin filaments, thereby enhancing myofibril growth (Takano et al. 2010). Inhibition of GSK3 $\beta$  in the triploid oyster resulted

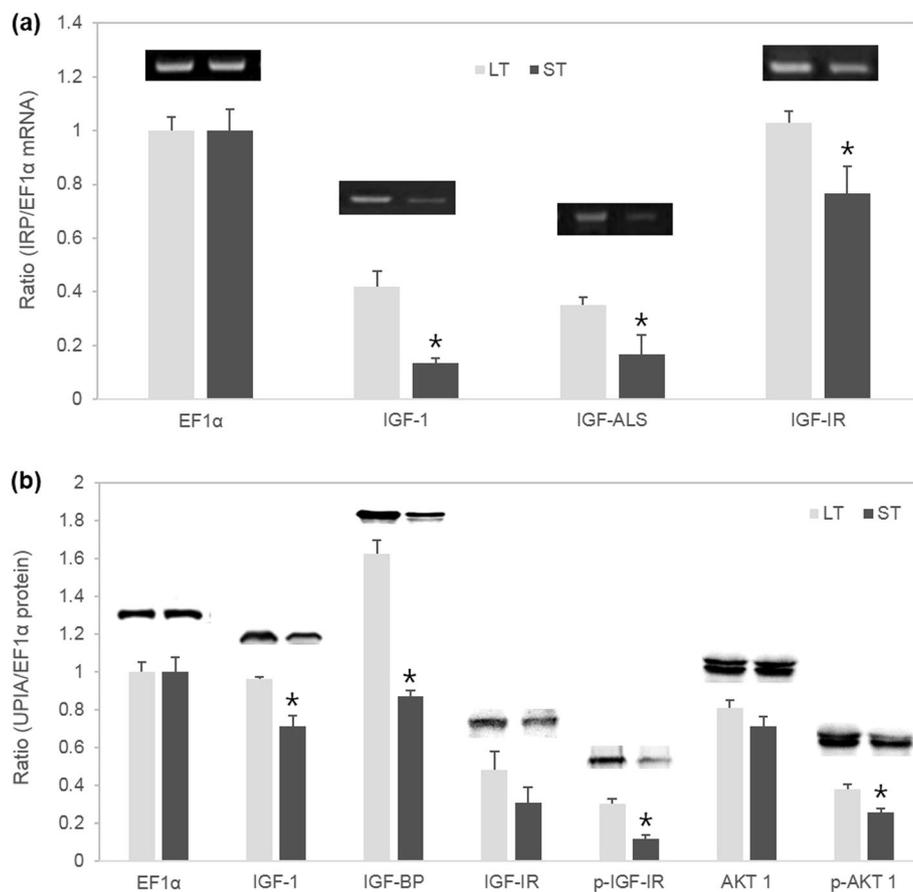
in increased levels of non-phosphorylated nebulin (Fig. 5c). The expression of N-WASP was 1.34-fold higher in LTs compared with STs.

#### PGC1 $\alpha$ and troponin

The expression of troponin T and I and PGC1 $\alpha$  was increased 1.87-, 1.43-, and 1.57-fold in LTs compared with STs, respectively (Figs. 3 and 5c).

#### Discussion

Triploid oysters have been reported to grow faster than diploid oysters because they use the energy required for maturation for flesh obesity (Allen and Downing 1986; Nell and Perkins 2005). However, oyster size differs to some extent even under the same conditions. In this study, we used adductor muscles excised from triploid oysters to analyze this difference. Adductor muscle (AM) plays an important role in the influx of food and communication with the external environment. Oysters limit oxygen exchange through shell closure at low tide. AM plays an important role in regulating the opening and closing of these shells through translucent and white

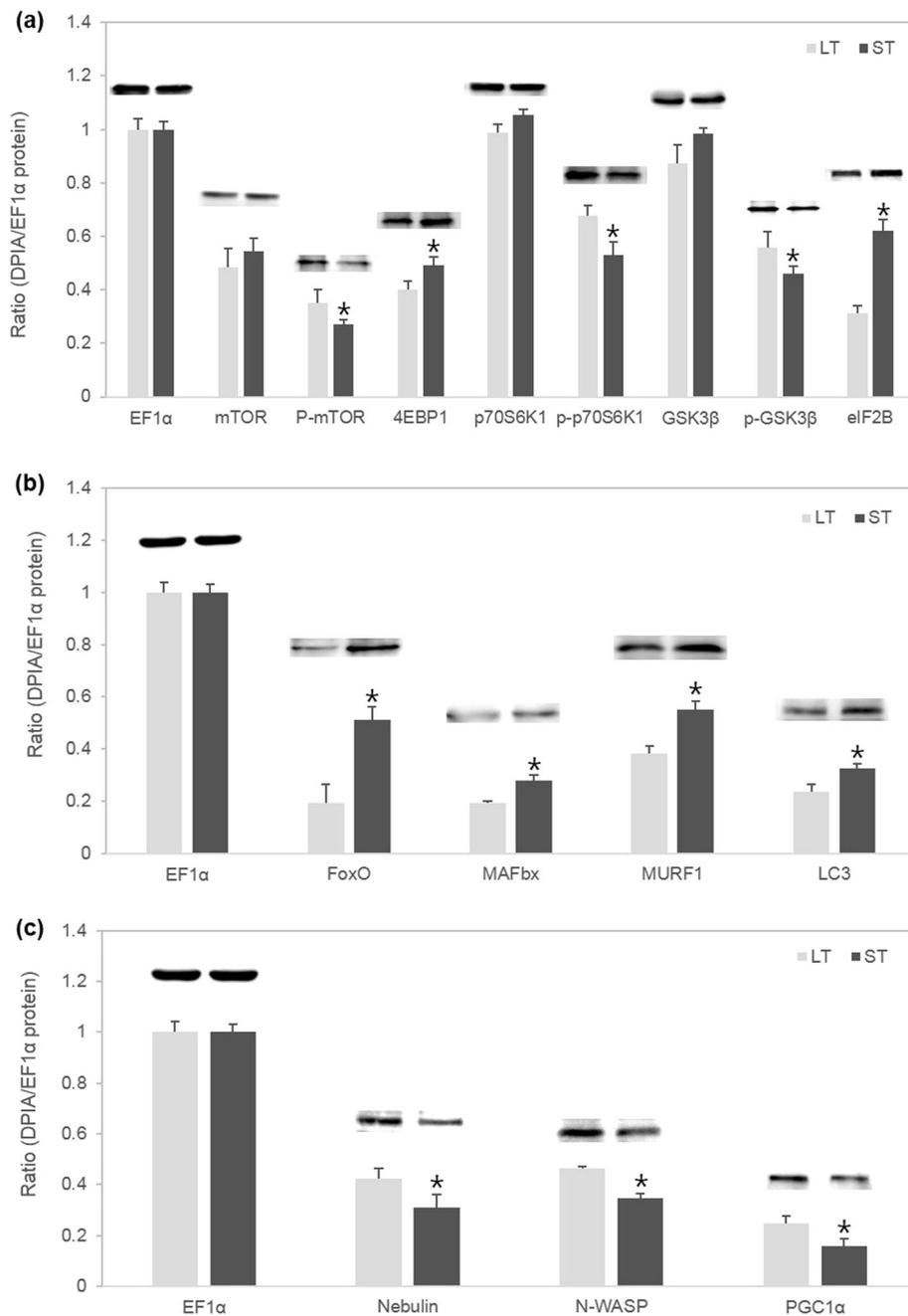


**Fig. 4** Expression of upstream proteins in the IGF-1/AKT signaling pathway according to triploid oyster size. **a** mRNA expression ( $n = 5$ ). **b** Protein expression ( $n = 3$ ). \* $p < 0.05$  vs. LT. LT, large triploid; ST, small triploid; IRP, IGF-1 related proteins

opaque muscles. Oxygen is limited but oysters lower intracellular pH for survival and regulate the expression of carbohydrates, proteins, tRNA, ncRNA, and amino acid metabolism-related genes. Among them, dioxygenase, which corresponds to dietary changes in AM, is drastically reduced and maintains cysteine homeostasis in food-restricted situations (Zhang et al. 2012; Chapman et al. 2011). AM is the main organ of oysters that perform these functions. Therefore, the formation and development of AM has a very important effect on the growth and diet of oysters. On the other hand, AM produces large amounts of melanin, and it is reported that the more melanin in the shell, the higher the dry weight of oysters (Yu et al. 2017; Hao et al. 2015; Xiao yan et al. 2003). Melanin removes free radicals and reduces damage to AM, which allows AM to open larger shells and filter out more algae to speed up growth. We examined the effects of growth factors on muscle formation and activity in the adductor muscle and analyzed related signaling pathways.

IGF-1 plays an important role in muscle growth in triploid oysters. The expression of actin, myosin, troponin,

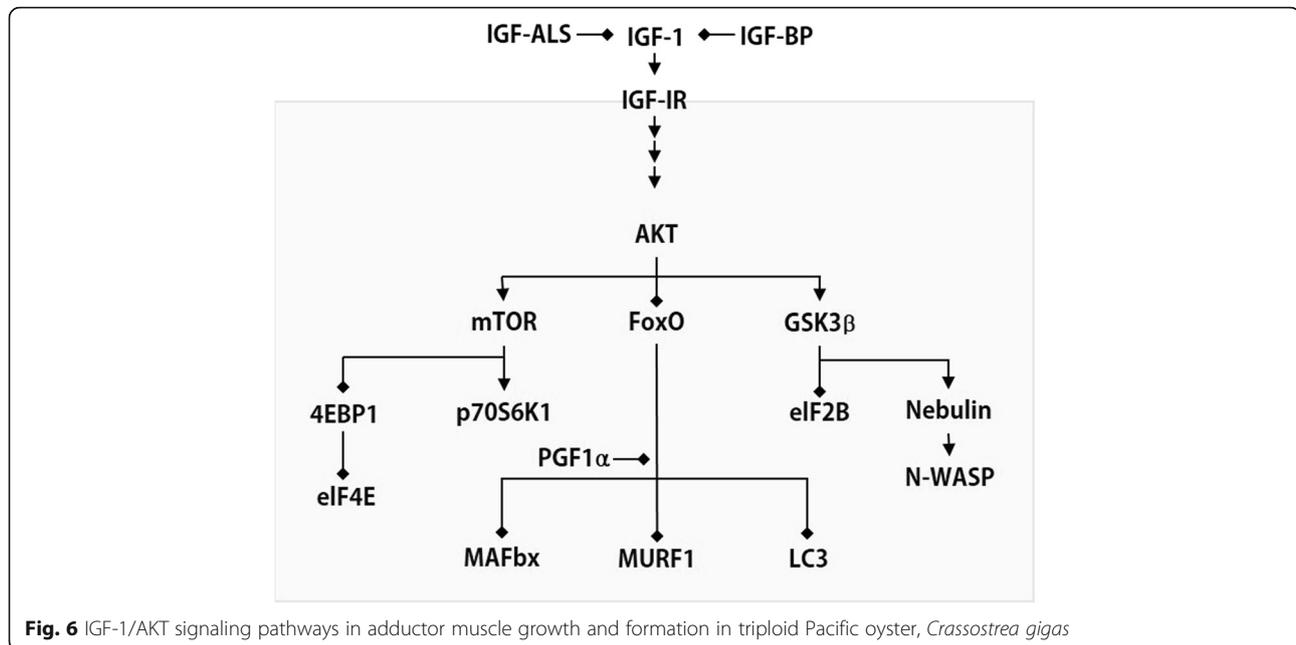
and tropomyosin, which form muscles in LT and ST adductor muscle, as well as the mRNA and protein expression of IGF-1, differed significantly. In addition, the expression of IGF-ALS and IGF-BP, which increases the half-life in combination with IGF-1 (Baxter et al. 1989), was also higher in mRNA and protein. This finding confirmed that IGF-1 affects muscle growth in LTs. In addition, the expression of cofactors required for the activity of IGF-1 in tissues was also increased, and the signaling pathway associated with IGF-1 was activated by increasing phosphorylation of IGF-1R. According to Gricourt et al. (2003, 2006), an insulin-like system functions when *C. gigas* growth is increased; in particular, mRNA and protein levels of IGF-1 and insulin receptor-related receptors are increased. Our results showed that the expression of IGF-1 was high in LTs with a high growth rate, consistent with that previous study. The regression analysis of IGF-1/EF1 $\alpha$  gene expression according to STW confirmed the significance ( $p < 0.05$ ). This is the first report explaining the association between STW and IGF-1 gene expression in triploid oysters.



**Fig. 5** Expression of downstream proteins in the IGF-1/AKT signaling pathway according to triploid oyster size. **a** Expression of signaling factors related to protein synthesis. **b** Expression of signaling factors related to protein degradation. **c** Expression of signaling factors related to myofibrillogenesis. *n* = 3. \**p* < 0.05 vs. LT. LT, large triploid; ST, small triploid

The size-dependent increase in p-AKT suggested the possibility that IGF-1 signaling regulates protein synthesis and degradation of muscle via AKT. AKT activity has been reported to increase the activity of mTOR, which is involved in protein synthesis, and to inhibit the activation of GSK3β, which is involved in muscle formation (Glass 2010; Miyazaki and Esser 2009; Sandri 2008; Manning and Cantley 2007; Sarbassov et al. 2005).

Furthermore, protein degradation is inhibited by inhibition of FoxO expression, which regulates MAFbx, MURF1, and LC3, which induce protein degradation (Stitt et al. 2004; Mammucari et al. 2007). The level of activated mTOR was higher, and the expression of GSK3β and FoxO lower, in LTs than in STs. LTs appear to increase muscle protein content by promoting synthesis and inhibiting degradation of proteins to a greater



degree compared with STs. IGF-1/AKT signaling regulates muscle growth in triploid oysters by promoting protein synthesis and inhibiting degradation. In mammalian cells, PI3K/AKT signaling under hypoxic conditions has been reported to regulate glucose metabolism and apoptosis (Kim et al. 2012; Parcellier et al. 2008; Alvarez-Tejado et al. 2001). Guevelou et al. (2013) also reported increased expression of AKT under hypoxic conditions in the smooth muscle of *C. gigas*; however, AKT expression did not increase under hypoxic conditions in striated muscle. These results suggest that AKT, which is expressed in striated muscle of *C. gigas*, is involved in metabolism related to the synthesis and degradation of muscle protein rather than regulation of glucose metabolism and apoptosis.

GSK3 $\beta$ , which is involved in protein synthesis, increases phosphorylation of nebulin, which results in inhibition of actin polymerization (Takano et al. 2010). In the case of adductor muscle, oyster size affects both muscle formation via protein synthesis and degradation and muscle movement via muscle relaxation. Inhibition of GSK3 $\beta$  by phosphorylation of AKT inhibited the phosphorylation of nebulin, which in turn binds to N-WASP and contributes to muscular movement (Rommel et al. 2001). The expression of nebulin and N-WASP was higher in LTs compared with STs. In particular, the expression of N-WASP was 13-fold higher in LTs than in STs. This result confirms that the IGF-1/AKT/GSK3 $\beta$ /N-WASP signaling pathway influences the formation of adductor muscle and the control of movement in triploid oysters. GSK3 $\beta$  deactivated by IGF-1 increases glycogen synthesis by dephosphorylating glycogen synthase and increases protein synthesis by

dephosphorylating eIF2B (Frame and Cohen 2001). It also increases myofibrillogenesis via the regulation of nebulin. In this study, we confirmed that GSK3 $\beta$  increases protein synthesis in triploid oysters and increases myofibrillogenesis via actin polymerization. In oysters such as *C. angulata* and *C. gigas*, GSK3 $\beta$  gene expression was reported to be high along with glycogen content in the adductor muscle at the time of gonad development (Zeng et al. 2013; Li et al. 2017). This stored energy is used for sexual maturity. However, in the case of triploid oysters, the stored energy of the adductor muscle appears to be important for increasing oyster size through protein synthesis and muscle formation.

On the other hand, the expression of PGC1 $\alpha$ , which regulates the expression of FoxO and promotes protein degradation, was also higher in LTs compared with STs. This influenced the expression of troponin, another factor that acts on PGC1 $\alpha$  (Vescovo et al. 2005). Striated muscle contracts and relaxes by the action of myosin and actomyosin, a complex of actin–troponin–tropomyosin, and Ca<sup>2+</sup> (Clark et al. 2002; Geeves and Holmes 1999; Gordon et al. 2000; Kuo and Ehrlich 2015). Troponin acts as a site for Ca<sup>2+</sup> to bind actomyosin. Therefore, we suggested that the expression of LT was higher than that in ST, as well as muscle formation of muscle protein.

Taken together, these results indicate that growth of the adductor muscle of triploid oysters occurs by promoting the formation of muscle proteins through the IGF-1/AKT signaling pathway and inhibiting degradation. GSK3 $\beta$  and PGC1 $\alpha$  also affect muscle formation and movement (Fig. 6).

In this study, it was examined how various factors affecting muscle growth, formation, and movement of the adductor muscle of triploid oysters vary with oyster size. Our results will improve our understanding of muscle growth, formation, and movement of triploid oysters via IGF-1/AKT signaling. We also confirmed that the adductor muscle of the triploid oyster affects the size of the oyster. The results of this study will be important for further studies investigating muscle growth of triploid oysters and marine mollusks.

## Conclusions

Through the IGF-1/AKT signaling pathway, increased protein synthesis (mTOR/4EBP1 and p70S6K1; GSK3 $\beta$ /eIF2B), inhibition of protein degradation (FoxO/MAFbx, MURF1, LC3), and activation of muscle-forming proteins (PGC1 $\alpha$ /troponin; GSK3 $\beta$ /N-WASP) occur in the adductor muscle of triploid oysters. All of these processes affect the growth of triploid oysters, and activation of IGF-1/AKT signaling results in a larger size of the triploid oyster, *C. gigas*.

## Authors' contributions

YHC contributed to the conceptualization, writing–review and editing, supervision, project administration, and funding acquisition. EYK contributed to the investigation, data analysis, and writing–original draft preparation. Both authors read and approved the final manuscript.

## Funding

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2016R1D1A1B03934914).

## Availability of data and materials

All data sets generated during and/or analyzed during the current study are available from the authors on reasonable request.

## Ethics approval and consent to participate

Not applicable

## Consent for publication

Not applicable

## Competing interests

The authors declare that they have no competing interests.

Received: 21 May 2019 Accepted: 20 August 2019

Published online: 06 September 2019

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