

Gene Expression Profiles of Long-Chain Acyl-Coenzyme A Dehydrogenase, Nuclear Distribution C-Containing Protein 3, and Receptor Tyrosine Kinase Tie-1 in Swimming Larva of Sea Cucumber *Apostichopus japonicus*

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Conflict of interests

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Abstract

The sea cucumber, *Apostichopus japonicus*, is one of the most valuable aquatic species. The color of body wall and appearance are important for the value of sea cucumbers. To examine expression pattern of long-chain acyl-coenzyme A dehydrogenase (*LCAD*), nuclear distribution C-containing protein 3 (*NUDCD3*), and receptor tyrosine kinase *Tie-1* (*TIE1*), previously reported as differently expressed genes during the pigmentation of sea cucumber, we analyzed the temporal profiles of *LCAD*, *NUDCD3*, and *TIE1* mRNAs in LED-exposed and light-shielded *A. japonicus*. Real-time quantitative PCR revealed that the *LCAD*, *NUDCD3*, and *TIE1* mRNAs from the juveniles at 40–60 days post-fertilization (dpf) exhibited increasing patterns as compared to those of an early developmental larva (6-dpf). At 60-dpf juveniles, the *LCAD* and *TIE1* mRNA levels of LED-exposed individuals were higher than those of light-shielded ones, whereas at 40-dpf and 50-dpf juveniles, the *NUDCD3* mRNA expression was higher in the light-shielded condition ($p < 0.05$). In the pigmented juveniles (90-dpf), the *LCAD* and *TIE1* mRNA levels tended to show higher levels in red individuals than those in green ones, but there was a conversely higher level of *NUDCD3* mRNA in green larva. *In situ* examination of *LCAD* and *NUDCD3* mRNAs in light-shielded 6-dpf larva revealed that both genes are mainly expressed in the internal organs compared to the body surface. Together, these results may provide insights into the differential gene expression of *LCAD*, *NUDCD3*, and *TIE1* during pigmentation process of the sea cucumber.

Keywords: Swimming larva, Body color, Gene expression, Light emitting diode, *Apostichopus japonicus*

INTRODUCTION

Sea cucumbers are valuable marine resources supporting coastal livelihoods around the world. Of

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Authors' contributions

Conceptualization: Kim S, Sohn YC.
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Validation: Kim S, Sohn YC.
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Ethics approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

these, the sea cucumber, *Apostichopus japonicus* (Echinodermata: Holothuroidea), is one of the most economically important species inhabiting the shallow shelf of coastal regions around the Korean Peninsula, China, Japan (main island), and the Russian Far East (Liu, 2015). Color variation is an important trait in the sea cucumber aquaculture industry, which affects the market price of holothurian products (Jiang et al., 2013; Ru et al., 2019). Environmental factors, such as food and habitat, were found to determine the body color of sea cucumbers (MOF, 2004; Jiang et al., 2013). Interestingly, the red type sea cucumber showed definite genetic differentiation from the green and black types (Kan-No & Kijima, 2003). A report also suggested that the body color is affected by genetic factors in the parental generation (Park, 2006), partially supported by significantly different single-nucleotide polymorphism genotypes in allele-specific *HSP70* gene between color variants (Kang et al., 2011). However, several studies have shown that there is no reproductive isolation and significant species-specific nucleotide polymorphism between color variants (Hu & Li, 2009; Jo et al., 2017). As the genetic distance between color variants (red, black, and green) of *A. japonicus* was 0.76%, irrespective of their body color, sea cucumbers can be classified as the same species (Sun et al., 2010). Recently, several transcriptomic and metabolomic analyses provided information for the potential molecular mechanisms underlying pigmentation, and color-determining genes and metabolites (Xing et al., 2017, 2018, 2021).

Light is an environmental factor that affects the feeding behavior, growth, and survival of aquatic animals directly or indirectly (Zhou et al., 1999). In addition, appropriate light intensities induced better growth and development of larval and juvenile sea cucumbers, and the photoperiod has diverse effects on sea cucumbers, for example, emerging at night and finding shelter during daytime (Zhang et al., 2006; Dong et al., 2010, 2011; Li et al., 2019). A comparison of juvenile sea cucumbers at different photoperiods showed the highest growth level at 12 h light and 12 h dark condition (Chen et al., 2007). However, further elucidation of the environmental and endogenous factors is required to fully understand the mechanism underlying pigmentation of juvenile sea cucumber. Recently, *long-chain acyl-coenzyme A dehydrogenase (LCAD)*, *nuclear distribution C-containing protein 3 (NUDCD3)*, and *receptor tyrosine kinase Tie-1 (TIE1)*, whose expression levels increase at each pigmentation stage, were suggested to be involved in body color related proteins (Xing et al., 2017). In this study, we investigated the transcript levels of *LCAD*, *NUDCD3*, and *TIE1* genes in swimming larva and juveniles of *A. japonicus* to obtain basic information for the relationship between expression of the body color related genes and pigmentation.

MATERIALS AND METHODS

1. Sea cucumber larva, light exposure, maintenance, and sampling

In June 2021, the larva and juvenile sea cucumbers used in the experiments were produced by 4 h heat-shock stimulation (24°C from 20°C) of mature female and male sea cucumbers (n=200, mean body weight 200 g) that were separated by body colors (brown and black; Wondeok Fishermen's Union, Samcheok, Korea). The fertilized eggs, developing embryos, larva, and juveniles were maintained at 21°C with filtered seawater and air supplied using an air blower. Commercial feed of spirulina powder (Shandong, China) and bakers' yeast (AngelYeast, Hubei, China) was supplied once a day to the swimming larva (until 20 days post-fertilization, dpf) and increased to twice a day (90-dpf) after the juveniles attached. Seawater was changed twice a day using a siphon and overflow during the experimental period.

To examine effect of light on the body color, the larval sea cucumbers were divided into two groups (LED-exposed/ light-shielded) and further reared at 200 L plastic tanks from 30-dpf to 90-dpf. One group was exposed to LED lights (3,000 lx; MB-600S, Mobydick, China) for 10 h in a

day (LED-exposed condition, light phase (L): dark phase (D)=10/14 h), and the other group was covered with a PVC board during the experimental period, except for feeding time (light-shielded condition, L:D=0/24 h) (Table 1). The light exposure time was based on the average sunrise/sunset time at Samcheok, Gangwon-do, Korea, June 2021. This study did not require approval by the institution and ethics committee because there are no vertebrate participants.

2. cDNA cloning and sequence analysis

Nucleotide sequences for *LCAD*, *NUDCD3*, and *TIE1* were retrieved using the Chinese Sea Cucumber Database (http://www.genedatabase.cn/aja_genome_20161129.html). According to the sequence information in the database, the primers were designed using the ApE program (A plasmid Editor Version 2.0.36. by M. Wayne Davis) and synthesized by Bioneer (Daejeon, Korea; Table 2). Total RNAs were extracted from the intestines and the respiratory trees of mature sea cucumbers as previously described (Maeng et al., 2019). The *LCAD*, *NUDCD3*, and *TIE1* cDNAs were amplified by PCR methods using primer sets (Table 2) and LA Taq (Takara, Osaka, Japan) and inserted into pGEM-T Easy vector (Promega, Madison, WI, USA). The PCR cycling conditions for *LCAD* and *TIE1* were as follows: 1 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 35 s at 72°C; 10 min at 72°C. For *NUDCD3*, the PCR conditions were as follows: 1 min at 94°C; 32 cycles of 10 s at 98°C and 30 s at 68°C; 10 min at 72°C. PCR products were purified using PCR and/or Gel Purification Kit (Bioneer) and ligated with pGEM-T Easy vector

Table 1. Sampling schedule, sampled larva and juvenile numbers of sea cucumber, *Apostichopus japonicus*

Sampled date	Sampled larva and juvenile number	Cumulative LED-exposed days	
		10L:14D	0L:24D
6-dpf	>10,000	NA	NA
40-dpf	100–300	10 days	NA
50-dpf	100–300	20 days	NA
60-dpf	100–300	30 days	NA
90-dpf	10–20	60 days	NA

dpf, days post-fertilization; NA, not applicable.

Table 2. Oligo primer sequences for PCR and real-time qPCR

Target	Direction	Sequence (5'-3')	Application
<i>LCAD</i>	Forward	ACGAGTTAGCTCCACATCAGG	cDNA cloning
	Reverse	CTTTGAGGATGTACGCCTCCCT	
	Forward	GGTTGAAGCTGGTACTCCTGG	qPCR
	Reverse	CTTTGAGGATGTACGCCTCCC	
<i>NUDCD3</i>	Forward	GAGTTGATGCTTTCCTGGATGTC	cDNA cloning
	Reverse	CATCCATAGTCAACGTCAGCACA	
	Forward	ACTCACATAAAGTTGCCCTCC	qPCR
	Reverse	CGGAGCTGAGTGTAAGACTAC	
<i>TIE1</i>	Forward	CAAGGTGGATGGTAGTAGTGATG	cDNA cloning
	Reverse	CACCTTCATGGTTCCCTTACA	
	Forward	ATTAATGGAGAGCCTGGGATG	qPCR
	Reverse	CTGCCRTAGATGCTTCACTGAG	
<i>β-actin</i>	Forward	TCCTTCGTCTTGATCTTGCTG	qPCR
	Reverse	CTACGTTGCTCTCGACTTTGA	

LCAD, long-chain acyl-coenzyme A dehydrogenase; *NUDCD3*, nuclear distribution C-containing protein 3; *TIE1*, tyrosine kinase Tie-1.

for 24 h at 16°C using T4 DNA ligase (Promega). The pGEM-T Easy vectors containing *LCAD*, *NUDCD3*, and *TIE1* cDNAs were transformed into DH5 α competent cells and cultured in Luria-Bertani ampicillin medium for 24 h at 37°C. The pGEM-T easy vectors were extracted using a plasmid mini extraction kit (Qiagen, Valencia, CA, USA) and the inserted cDNAs were sequenced by Sanger method (Solgent, Daejeon, Korea). The nucleotide sequences were analyzed using the MultAlin multiple sequence alignment site (<http://multalin.toulouse.inra.fr/multalin/>) and the ApE program.

3. Real-time qPCR

Total RNAs were extracted from juvenile sea cucumbers sampled at 40-, 50-, and 60-dpf (10, 20, and 30 days after LED exposure) using the RNeasy Mini Kit (Invitrogen, Waltham, MA, USA). To synthesize cDNAs, PrimeScript RT reagent Kit (Takara) was used along with total RNA (1 μ g each), 5 \times gDNA Eraser Buffer, 2 μ L of gDNA Eraser (1 μ L), and the PCR mixtures including 5 \times PrimeScript Buffer II, 4 μ L PrimeScript RT Enzyme Mix I, 1 μ L RT Primer Mix, 1 μ L RNase-Free distilled water were reacted for 2 min at 42°C. Reverse transcription was performed for 15 min at 37°C and incubated at 85°C for 5 s to inactivate reverse transcriptase to synthesize cDNAs. qPCR was performed using the QuantStudio™ 7 Flex Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The primers used in qPCR were designed using Primer Express v3.0 software (Applied Biosystems) and synthesized by Bioneer (Table 2). Using 10 ng of the synthesized cDNA as a template, primers (10 μ M), TB Green™ Premix EX Taq II (Takara), and ROX Reference Dye II (Takara) were used, and the final qPCR mixture was a two-step qPCR with a volume of 20 μ L. qPCR conditions were 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 34 s; 95°C for 15 min and 60°C for 1 min.

4. Whole-mount in situ hybridization (WMISH)

Larval specimens (1.5–9 dpf) were hybridized *in situ* using a digoxigenin-labeled *LCAD* and *NUDCD3* antisense probes. Detection of mRNA was carried out essentially according to the protocol of Arenas-Mena et al. (2000) and Maeng et al. (2012).

5. Statistical analysis

Statistical significance was evaluated by one-way ANOVA using SPSS software (version 23.0; IBM, Armonk, NY, USA) and Tukey's post hoc test. In addition, the Student's *t* tests were used to compare the expression levels data between the LED-exposed and light-shielded conditions. Differences between the groups were considered significant at $p < 0.05$.

RESULTS

1. Spatial distribution of *LCAD* and *NUDCD3* transcripts in swimming larva

WMISH shows that sea cucumber *LCAD* and *NUDCD3* transcripts are mainly localized to a population of cells in the internal organs of the auricularia larva, such as intestine, although the mRNAs could not be observed clearly (Fig. 1B and C). In addition, the gene expression levels showed increasing tendencies during each development stages of the auricularia larva (Fig. 1D and E).

2. Effect of LED-exposure on *LCAD*, *NUDCD3*, and *TIE1* expression during larval development

In general, the examined *LCAD*, *NUDCD3*, and *TIE1* transcript levels showed increasing

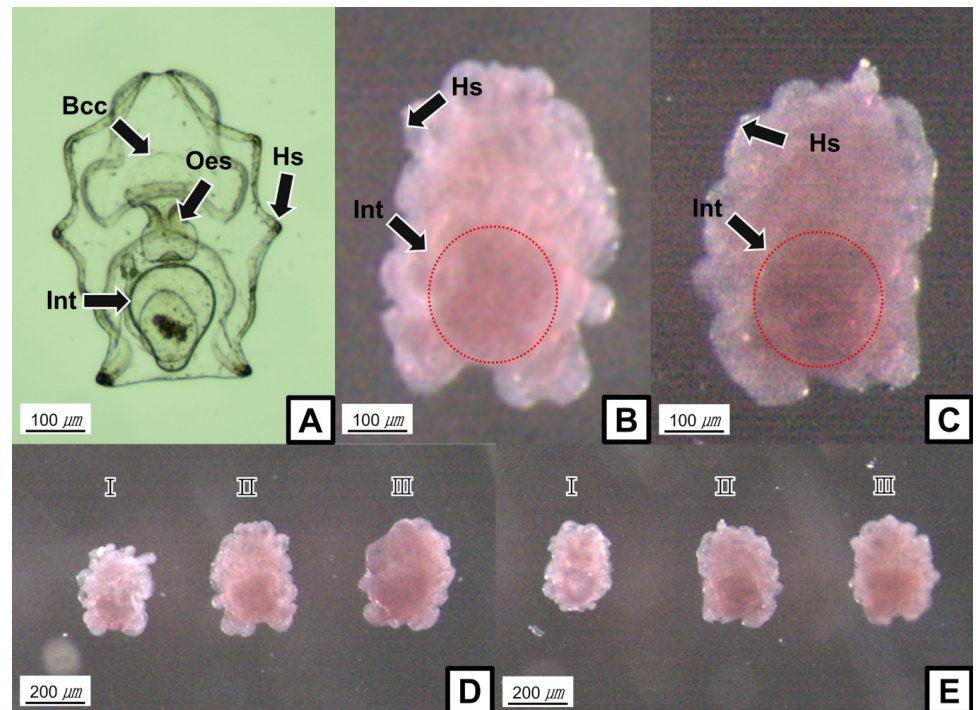


Fig. 1. Whole-mount *in situ* hybridization of *LCAD* and *NUDCD3* transcripts in larval sea cucumber, *Apostichopus japonicus*. (A) Anatomy of auricularia larva (6 days post-fertilization, dpf). (B, D) Expression of the *LCAD* and (C, E) *NUDCD3* mRNAs. Bcc, buccal ciliated cavity; Hs, hyaline sphere; Int, intestine; Oes, Oesophagus; I, 1.5–3 dpf larvae; II, 4–6 dpf larvae; III, 7–9 dpf larvae. *LCAD*, long-chain acyl-coenzyme A dehydrogenase; *NUDCD3*, nuclear distribution C-containing protein 3.

tendencies during larval development stages (6-dpf to 60-dpf) of sea cucumbers. In light-shielded individuals, however, the *LCAD*, *NUDCD3*, and *TIE1* mRNA were not significantly changed during 40-dpf and 60-dpf stages. At 60-dpf juveniles, the *LCAD* and *TIE1* mRNA levels of LED-exposed individuals were significantly higher than those of light-shielded individuals ($p < 0.05$; Fig. 2A and C). At 40-dpf and 50-dpf juveniles, however, the *NUDCD3* mRNA expression levels were significantly higher in light-shielded condition than LED-exposed condition ($p < 0.05$; Fig. 2B).

3. Effect of LED-exposure on body color and relationship with *LCAD*, *NUDCD3*, and *TIE1* expression

In the pigmented juveniles (90-dpf), the *LCAD* and *TIE1* mRNA levels showed higher tendencies in the red individuals than those in green ones, whereas the *NUDCD3* mRNA was higher level in the green juveniles (Fig. 2D). In addition, the *LCAD* mRNA levels were significantly higher in the LED-exposed red individuals compared to the light-shielded ones and the *NUDCD3* mRNA level was slightly but significantly higher in the LED-exposed red juveniles ($p < 0.05$).

DISCUSSION

More than 50 years ago, the body color of sea cucumbers was known to be determined by pigment cells in the dermis layer of the body wall in granule forms (Menton & Eisen, 1970). Among the different color morphs, red, black, and albino are rare and have great appeal to consumers as high-quality traits (Ru et al., 2019). In this study, we examined spatiotemporal expression profiles of the body color related mRNAs, *LCAD*, *NUDCD3*, and *TIE1* at several

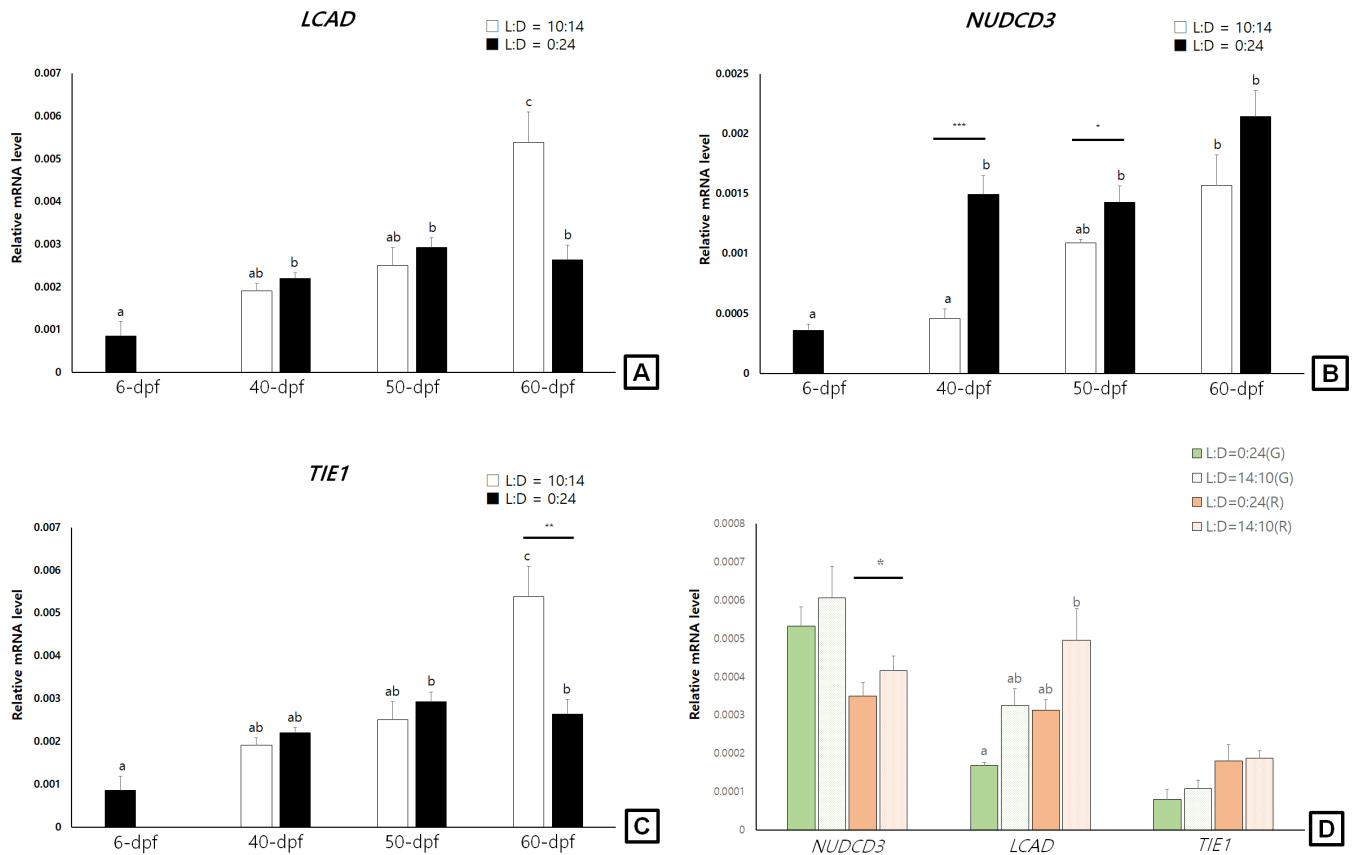


Fig. 2. Effect of LED-exposure on body color related gene transcripts in sea cucumber, *Apostichopus japonicus*, larva and juveniles. (A) *LCAD*, (B) *NUDCC3*, and (C) *TIE1* transcript levels during 6–60 days post-fertilization (dpf) larval stages. (D) *LCAD*, *NUDCC3*, and *TIE1* transcript levels at 90-dpf juvenile stage which were separated by body colors (G, green; R, red). The *LCAD*, *NUDCC3*, and *TIE1* transcript levels were analyzed by qPCR as described in Materials and Methods. Different lowercase letters on the bars indicate significantly different values among the groups ($p < 0.05$). Asterisks mean significantly different values between LED-exposed and light-shielded individuals (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). L:D=10:14 and L:D=0:24 mean LED-exposed and light-shielded conditions, respectively. *LCAD*, long-chain acyl-coenzyme a dehydrogenase; *NUDCC3*, nuclear distribution C-containing protein 3.

developmental and pigmented stages of *A. japonicus*. In addition, LED-exposed and light-shielded juveniles were applied to the qPCR analysis to better understand the relationships among body color, LED-exposure, and the gene expression levels *LCAD*, *NUDCC3*, and *TIE1*.

In various mammalian melanocytes, tyrosine kinase receptors (*RTKs*), such as growth factor receptors and *TIEs*, produce a variety of signals associated with stimulation of proliferation, differentiation, migration, and survival (Zhou et al., 2019). In fact, activation of *RTKs* by a ligand induced transcription of tyrosinase and melanin synthesis in differentiated melanocytes, and expression of a mutant *RTK* was associated with lesser pigmentation of melanocytes, suggesting the downregulation of tyrosinases (Luo et al., 1995; Alexeev & Yoon, 2006). In accordance with the mammalian *RTKs*, we showed that the sea cucumber *TIE1* transcript levels were gradually increased at juvenile stage nearby pigmentation period compared to those of the earlier developmental stages, irrespective of LED-exposed condition. Interestingly, the *TIE1* gene expression level of LED-exposed sea cucumber juveniles was significantly higher than that of light-shielded juveniles, implying the possible involvement of light in *TIE1*-mediated pigmentation process.

Melanin is considered as the major pigment widely present in diverse animal phyla, and the

variations in melanin formation result in a wide range of body colors including echinoderms (Calestani & Wessel, 2018). In the sea cucumber body, the concentration of melanin pigment was gradually increased with deepening color (Xing et al., 2021). In this study, *LCAD* expression increased at juvenile stage nearby pigmentation period, suggesting the potential role of *LCAD* in the synthesis and accumulation of melanin pigments. In 60-dpf juveniles, LED-exposure increased transcript level of *LCAD* than that of light-shielded sea cucumber, which are similar results for both protein and mRNA levels of *LCAD* during development of purple sea cucumber (Xing et al., 2017) and upregulation of *LCAD* protein level in human keratinocytes under radiation condition (Amar et al., 2019). In future studies, fine-tuned examination for diverse light sources and photoperiods are required to better understand the relationship between higher expression level of *LCAD* and body color of sea cucumber.

In differentially expressed proteins during pigmentation stages of purple sea cucumber, *NUDCD3* was classified in two functional groups of melanogenesis and Wnt signaling pathway (Xing et al., 2017). Activation of the Wnt signaling pathway enhanced melanocyte specification at early stages of zebrafish development (Vibert et al., 2017), suggesting a pivotal role of Wnt signaling in regulation of melanocyte differentiation in vertebrates. Interestingly, we observed an increasing pattern of *NUDCD3* expression levels in light-shielded 40- and 50-dpf juveniles compared to those of LED-exposed ones, whereas LED-exposure increased the *NUDCD3* expression levels at later pigmentation stages. Considering the promoting effect of Wnt signaling in melanocyte development and pigmentation (Schepsky et al., 2006; Wang et al., 2017), it is noteworthy that the Wnt signaling pathway associated with melanin synthesis and regulation in sea cucumbers are likely dependent to light-exposure and developmental stages.

Spatial distribution of *LCAD* and *NUDCD3* transcripts in swimming larva stage was expected to be high in the body surface. Unexpectedly, *LCAD* and *NUDCD3* were shown to be highly expressed in internal organs such as intestine. The spatial gene expression related to the skeletogenic mesenchyme lineage in sea cucumber larvae also showed similar patterns with the ingression of skeletogenic pigment cells in sea urchin embryos (McCauley et al., 2012). Thus, it is presumed that genes distributed in internal organs through various metamorphic processes, such as impregnation and rotation, migrate to the body surface and participate in the formation of the skeleton and body color. However, the detailed involvement of *LCAD* and *NUDCD3* genes in pigmentation process of sea cucumbers remains to be further studied. To select and produce high-quality sea cucumbers, further studies are essentially required, such as evaluation of the body color related genes and characterization of pigmentation process in the melanocytes.

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