INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20F–028/2020/24–4–900–908
DOI: 10.17957/IJAB/15.1515
http://www.fspublishers.org

Full Length Article

Comparative Transcriptome Analysis of Body-Color Segregated Progenies Produced by Crossing Albino Sea Cucumbers *Apostichopus japonicus*

Deyou Ma[1,2†], Chunying Wu[1†], Lianlian Yu[1], Chanchan Zhang[1], Weigang Lin[1] and Hongsheng Yang[2*]
[1]*Key Laboratory of Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture and Rural Affairs, Dalian Ocean University, Dalian-116023, China*
[2]*CAS Key Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao-266071, China*
[*]For correspondence: hshyang@qdio.ac.cn
[†]Contributed equally to this work and are co-first author
Received 19 February 2020; Accepted 16 April 2020; Published 16 August 2020

Abstract

Body color is an economic trait of *Apostichopus japonicus* with growing concern. Among the other color variants (green and purple), rare sea cucumbers with completely white body color is greatly appealing to the consumers. In this study, comparative transcriptome analysis of body wall from body-color segregated progenies produced by cross of albino sea cucumber broodstock was performed using RNA-Seq technology in order to profile dynamic characteristics of genome-wide gene expression. Over 4.78 and 4.87 million clean reads were generated from the segregated and albino juveniles crossed by albino parent sea cucumbers. Totally 20,319 assembled unigenes with the average 959.83 bp in length were obtained, including the annotated 7,152. Compared with white juvenile sea cucumbers, 271 genes were validated to be significantly differentially expressed in the body wall of segregated samples. Forty-two differentially expressed genes (DEGs) including 31 upregulated and 11 downregulated were identified presenting 5 and more fold expression changes, 18 of which were involved in growth and development, immunity and respiration. "Cell and cell part", "Cellular process" and "Binding" were the most DEG-enriched GO terms in "Cell component", "Biological process" and "Molecular function", respectively. DEGs were significantly enriched in immunity-related KEGG pathways, such as "Phagosome", "*Staphylococcus aureus* infection", "Complement and coagulation cascades", and "*Vibrio cholerae* infection". These results provided abundant candidate genes and pathways to further reveal differences in growth and immunity between segregated and white progenies crossed from albino *A. japonicus*, for cultivating genetically stable white strain of this commercially important species. © 2020 Friends Science Publishers

Keywords: *Apostichopus japonicus*; Albino progenies; Body color segregation; Transcriptome analysis; RNA-Seq

Introduction

Sea cucumber *Apostichopus japonicus* with high nutritive and medical values is acknowledged one of the most important economic varieties in many regions of Asia (Yuan *et al.* 2006; Okorie *et al.* 2008). This edible invertebrate has been captured for many centuries and wild stocks have been almost as a result of over-exploitation in recent decades. With obviously increasing market demand, the mariculture of *A. japonicus* has been developed quickly in China, and obtained remarkable economic and social benefit in China (Zheng *et al.* 2012; Wu *et al.* 2015; Xing *et al.* 2018b). However, serious germplasm problems have been documented during farming sea cucumbers, causing severe economic losses (Yuan *et al.* 2006; Okorie *et al.* 2008; Zheng *et al.* 2012). Strain selection of *A. japonicus* with excellent traits such as quick growth rate and strong pathogen resistance has been extensively developing. Additionally, body color variation is a noteworthy phenotype of this species, affecting taste and market price (Kang *et al.* 2011; Sun *et al.* 2011; Jiang *et al.* 2013). According to body wall pigment composition and contents, body colors of sea cucumbers have green, purple and white (Fig. 1), in which green is the most common, while entirely white is scarcely found along the wild coast of China (Bai *et al.* 2016; Xing *et al.* 2017). Therefore, individuals with genetically stable white phenotype have been selected as a promising morph with potentially broad market need and high price in China.

Genetic breeding program of albino strains is ongoing due to the broad economic prospect. A fascinating phenomenon body color segregation of progeny was observed in F_1 juvenile sea cucumbers produced by cross of albino parents. The skin colors of offspring crossed by

To cite this paper: Ma D, C Wu, L Yu, C Zhang, W Lin, H Yang (2020). Comparative transcriptome analysis of body-color segregated progenies produced by crossing albino sea cucumbers *Apostichopus japonicus*. *Intl J Agric Biol* 24:900–908

albino sea cucumbers exhibited not only white but green after their pigmentation stage (Xing *et al.* 2018a). Moreover, number and size of albino individuals were visually less than those of the segregated under the common cultivation conditions. These observations implied that white body color was genetically instable and albino offspring could have poor physiological status during cultivation. It is reasonable to speculate that the physiological differences must be reflected in various expression levels (including transcription and translation) of a huge amount of genes involved in extensive biological processes. A series of preliminary studies on albinism of *A. japonicus* have been carried out, including comparison of pigment composition (Xing *et al.* 2017), expression of genes related to melanin production (Zhao *et al.* 2012; Xing *et al.* 2018b), growth and environmental adaptability (Bai *et al.* 2015), and nutritional requirements (Bai *et al.* 2016). However, the molecular mechanism underlying body color segregation of F_1 progenies crossed by parent albino sea cucumbers remains largely unknown.

With high accuracy and low cost, RNA-Seq is popularly used to acquire large amounts of transcript data from various types of biological samples (Birol *et al.* 2009; Trapnell *et al.* 2010), including many non-model organisms (Du *et al.* 2012; Feng *et al.* 2012). Genome-scale transcription profiles can be produced by RNA-Seq to identify differentially expressed novel genes and splice variants under two or more conditions among transcriptomes (Hunt *et al.* 2011; Feng *et al.* 2012; Trapnell *et al.* 2012). RNA-Seq technology has been extensively employed to examine the dynamic changes of transcriptomes in *A. japonicus* during embryonic development (Du *et al.* 2012), intestine regeneration (Sun *et al.* 2013), aestivation (Zhao *et al.* 2014), and immune response to pathogens (Gao *et al.* 2015). It has promoted a sharp increase of genetic resources in echinoderms and identified large-scale candidate genes potentially involved in these above-mentioned processes. Gene expression differences between white and green sea cucumbers have been investigated by RNA-Seq (Ma *et al.* 2014a, b). However, there are scarce published studies on body color segregation of juveniles produced by cross of albino sea cucumbers.

Body color diversity is widespread in animal kingdom, especially insects (Cheng *et al.* 2005; Cui *et al.* 2015) and fish (Ling *et al.* 2016). However, the color segregation of progenies was only documented in Taiwanese red tilapia. The differences in growth and propagation among offspring of this fish were probably owing to hereditary factors (Li and Li 1997). Mixture of several pigments in the epidermis layer of skin presents diverse body colors of animals. Using the Illumina platform, transcriptome data have been obtained from green, black and red *A. japonicus* in South Korea; differentially expressed genes and their corresponding function proteins were discovered by the following comparative analyses to understand the relationships among the three-color variants in taxonomy (Jo *et al.* 2016). Thus, body color segregation of juvenile sea cucumbers crossed by albino brood stock is not clearly understood.

In this study, RNA-Seq based on Illumina HiSeq2000 platform was adopted to evaluate global changes in gene expression between body wall tissues of white and segregated morphs from F_1 progeny of albino *A. japonicus*. Comparative transcriptome analyses between segregated and albino sea cucumbers were performed and hundreds of genes with distinct expression profiles were identified. The transcripts were assembled to unigenes that would be revealed to be involved in multiple metabolism and signaling pathways by subsequent GO and KEGG enrichment analyses. This work provided valuable genomic resources and candidate genes for future studies on elucidating differences of survival and growth between body-color segregated progenies of albino sea cucumbers to facilitate the breeding of stably inherited morphs with whole white body color.

Materials and Methods

Sample collection

F_1 progenies were produced by cross of parent albino sea cucumbers and hatchery-reared in Shandong Oriental Ocean Sci-Tech Co., Ltd. of China until sampling at six-month age after pigmentation stage. Two sample types were recognized by their albino and green body colors, in which green morphs were named as the segregated relative to the white sea cucumbers. Thirty sea cucumbers for each type were randomly collected and mixed into two groups for sequencing. The skin around papillae and papilla were collected for a good reference transcriptome, and finally stored at -80°C for the following RNA extraction after liquid nitrogen quick-freezing.

Transcriptome sequencing

Total RNA was extracted from the mixed skin samples using the RNeasy mini kit (Qiagen) based on the manufacturer's recommendations. The satisfactory RNA was prepared through examination of quality and integrity by 1.2% agarose gel electrophoresis and 2100 Bioanalyzer system (Agilent Technologies), for cDNA construction after concentration quantified by the NanoDrop 1000 (Thermo). Construction of libraries were finished in Beijing Genome Institute (BGI) (Shenzhen, China) Briefly, mRNA with poly(A) tails were enriched by magnetic beads with Oligo(dT) and then interrupted into approximately 200-bp long fragments; the enriched mRNA were used as templates for synthesis of the first-strand cDNA with a random hexamer-primer and reverse transcriptase (Invitrogen); the second-strand cDNA was produced under catalysis of RNase H and DNA polymerase I; sequencing adaptors were ligated to these double-strand cDNA fragments after

purification using the QiaQuick PCR extraction kit, and then washed with EB buffer to achieve end repair and addition of single-nucleotide A (adenine) (Ma *et al.* 2014b); finally, paired-end sequencing of cDNA fragments with length range of 200–700 bp was performed on an Illumina HiSeq 2000 platform.

Transcript assembly

Sequences generated by RNA-Seq were defined as raw reads. Low quality reads (including 10% unknown bases) and adaptors were removed before assembly. After removing their redundancy, the remaining clean reads were *de novo* assembled using the Trinity program (Grabherr *et al.* 2011) to gain unique transcript fragments termed unigenes. In the present study, RNA-Seq quality was evaluated by sequencing saturation and randomness. The former indicator was determined through numbers of identified unique genes. No new unique reads to be found means sequencing is saturated. The latter was estimated by reads distribution on the reference database.

Function annotation

The annotation of unigenes were operated by SOAPaligner/SOAP2 (Li *et al.* 2009) against reference databases inclusive of of NCBI non-redundant (Nr) protein database, the documented RNA-Seq datasets (Sun *et al.* 2011; Du *et al.* 2012) and genome sequences (Zhang *et al.* 2017) of *A. japonicus*. The maximal annotation error was two mismatched bases between reads and reference sequences. Using Blastx hits with the NCBI Nr database, unigene functions were categorized into gene ontology (GO) terms (including "Cellular component", "Molecular function" and "Biological process") and enriched in KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways.

Identification of differentially expressed genes (DEGs)

Transcription levels of genes were checked by RPKM (reads per kilobase per million reads) method (Mortazavi *et al.* 2008). DEGs were identified with the absolute fold change values greater than 1.0 and FDR less than 0.001. Compared to albino F_1 offspring produced from cross of parent albino sea cucumbers, the gene expression fold change of skin tissues of segregated (green) individuals was calculated by the following formula:

$$\text{Fold change} = Log_2 \frac{\text{RPKM}_{\text{segregated}}}{\text{RPKM}_{\text{albino}}}$$

DEGs validation by Real-time PCR

The expression trends of DEGs in RNA-Seq results were verified by Real-time PCR. Primer premier 5.0 was used to design optimal primers according to sequences of chosen DEGs in the RNA-Seq transcriptome database. Double-strand cDNAs were preserved at -80°C for the following qPCR validation after their syntheses based on the manufacturer's instructions (Promega).

Using the SYBR Green real-time PCR assay (Takara), transcription levels of the selected DEGs were examined A total of 25 μL amplification volume contained 12.5 μL SYBR Green Master Mix, 0.5 μL of each gene-specific primer (10 μmol/L), 1 μL of 1:50 diluted cDNA temples, and 10.5 μL of RNase-free water. Thermal cycling was as follows: 95°C for 5 s, 40 cycles at 95°C for 10 s, 60°C for 20 s and 72°C for 30 s. The amplification efficiency of these primers and specificity of the PCR products were confirmed by analyses of melting curve and gel electrophoresis on the amplified products. Three technical replications were performed for each real-time PCR validation. *NDUFA13* reported in previous studies was selected as the reference gene for internal normalization (Ma *et al.* 2014a, b). Expression values of genes calculated by real-time PCR with $2^{-\Delta\Delta CT}$ method were used to distinguish significantly DEGs with *P* less than 0.05 (Schmittgen and Livak 2008).

Enrichment analysis

Genes with similar expression patterns are usually enriched in gene ontology (GO) and involved pathways. With the results of GO and pathway annotation, DEGs were classified on basis of the corresponding official classifications. Terms with ≤ 0.05 *P* values were defined as the significantly enriched in GO enrichment analysis. The threshold of *P* value was estimated by *Q* value in multiple hypergeometric tests. Pathways with less than 0.05 *Q* values were believed to be the significantly enriched KEGG pathways attractive in further study.

Results

Summary of transcriptome sequencing

RNA-Seq samples were the body wall tissues of F_1 progenies with albino and segregated (green) epidermis colors produced by cross of albino parent sea cucumbers. There were 9.66 million raw reads generated on the HiSeq 2000 platform, including over 4.78 and 4.87 million in the segregated and albino samples with SRA accession numbers of SRR11475310 and SRR11475311. After trimming, 47.7 and 48.5 million clean reads were reserved from the two groups, of which 20.1 (42.18%) and 20.6 (42.43%) million were mapped to the reference databases, respectively. Totally, 12.5 (26.28%) and 12.8 (26.44%) million were identified as unique reads in the two transcriptomes used for the subsequent assembly and annotation. Details of sequencing data were summarized in Table 1.

During transcript sequencing process, RNA-Seq efficiency was examined by sequencing saturation and randomness in advance. In this study, the constructed cDNA

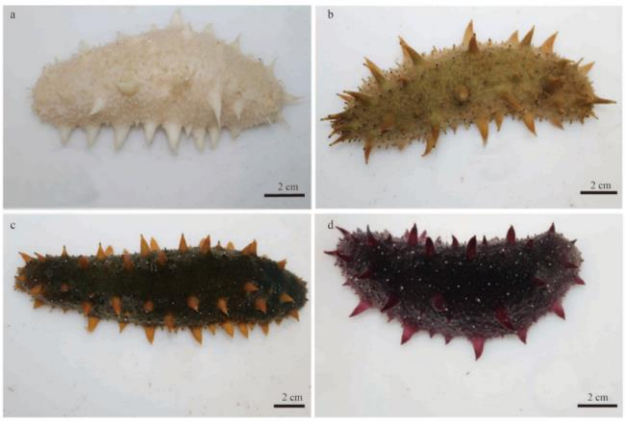

Fig. 1: Common body colors of sea cucumber *Apostichopus japonicus.* **a**. white, **b**. light green, **c**. dark green, **d**. purple (Xing *et al.*, 2017)

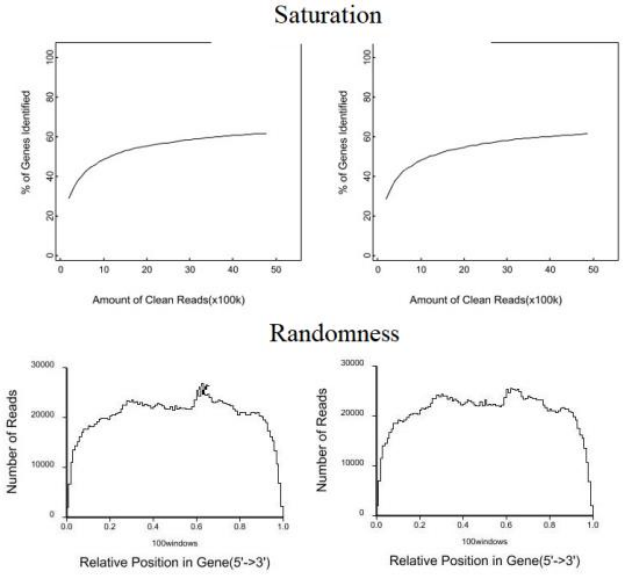

Fig. 2: Saturation and randomness evaluation of RNA-Seq transcriptomes sequenced from segregated (left) and albino (right) F_1 samples crossed by albino parent sea cucumbers

libraries were sequenced to saturation, producing a global profile of transcripts; the reads were evenly distributed at every position, indicating good randomness (Fig. 2). These results showed that high-quality transcriptomes were identified to carry out the following analyses.

Transcript assembly and unigene annotation

Totally 20,319 unigenes with 959.83 bp in average length were present after *de novo* assembly (Table S1), and Fig. 3 showed their length distribution. Of them, 7,152 unigenes (35.19%) were matched with known proteins in the reference databases. On basis of the NCBI-NR database, the Blast top hit species distribution in Fig. 4 showed that unigenes

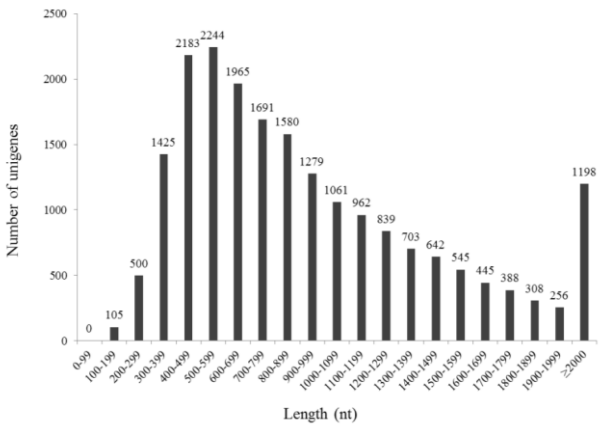

Fig. 3: The length distribution of unigenes *de novo* assembled in the transcriptomes of F_1 offspring crossed by albino parent sea cucumbers (*Apostichopus japonicus*)

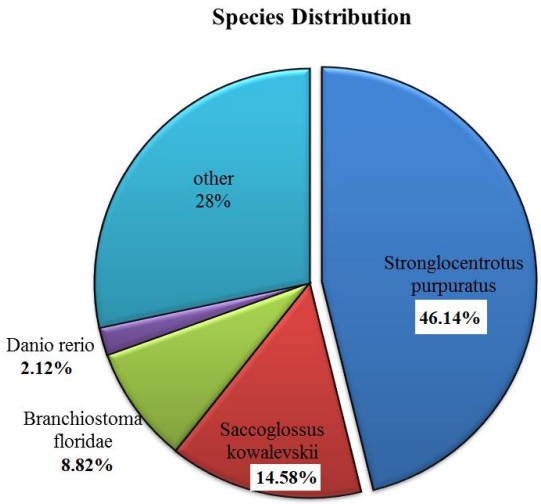

Fig. 4: Distribution graph of species in NCBI-NR database matched to sequences of *de novo* assembled unigenes

matched sequences from numerous species, of which 46.14% unigenes were similar to *Strongylocentrotus purpuratus*, followed by *Saccoglossus kowalevskii* (14.58%), *Branchiostoma floridae* (8.82%), and *Danio rerio* (2.12%).

DEGs screening and validation

DEGs with ≥ 2 fold changes and ≤ 0.001 FDR values were identified using RPKM method in this study. Overall, 271 differentially expressed genes were detected to satisfy this standard (Table S2). There were 271 genes (160 up- and 111 down-regulated) differentially expressed in the body wall of segregated juveniles, compared with the albino that were both albino progenies. DEGs with ≥ 5-fold expression changes should be especially focused on and a total of 42 genes with significantly differential expression pattern

Table 1: List of RNA-Seq sequencing data for segregated and albino progenies produced by cross of albino parent sea cucumbers

Parameters	Segregated	Albino
Total BasePairs	233,812,810	238,021,714
Total reads	4,789,920	4,876,403
Total clean reads	4,771,690	4,857,586
Clean reads ratio (%)	99.62%	99.61%
Total Mapped Reads	2,012,478	2,061,247
Mapped reads (%)	42.18%	42.43%
perfect match	1400701	1442889
perfect match ratio (%)	29.35%	29.70%
unique match	1253804	1284326
unique match ratio (%)	26.28%	26.44%
multi-position match	758674	776921
multi-position match (%)	15.90%	15.99%

Table 2: Detailed information of DEGs with 5 and more fold changes of expression in the segregated transcriptome compared with the albino

Gene ID	BlastX annotation	Fold change	Exp	*P*-value	FDR
isotig09968	angiopoietin-4-like	16.52	↑	1.85E-26	5.44E-24
isotig09381	translationally-controlled tumor protein	14.69	↑	6.68E-14	8.70E-12
isotig05215	selenide, water dikinase-lik	12.36	↑	1.24E-05	0.00055
isotig10757	transcription termination factor, RNA polymerase II	16.09	↑	2.14E-23	5.43E-21
isotig27723	complement factor H-like, partial	15.56	↑	4.40E-08	3.21E-06
isotig08005	60S ribosomal protein L8-like	13.83	↑	6.14E-06	0.00029
isotig05433	cytochrome c oxidase subunit 8A, mitochondrial-like	13.73	↑	8.92E-08	6.25E-06
isotig06081	voltage-dependent anion channel 2-like	13.59	↑	4.40E-08	3.22E-06
isotig05391	cytochrome c1, heme protein, mitochondrial-like	12.92	↑	6.14E-06	0.00029
isotig05240	non-specific serine/threonine protein kinase-like	12.28	↑	1.50E-06	8.20E-05
isotig09541	hypothetical protein BRAFLDRAFT_86061	6.28	↑	8.52E-44	4.81E-41
isotig09542	tenascin R-like, partial	6.1	↑	1.05E-19	2.12E-17
isotig06539	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3-like	-13.97	↓	4.79E-06	0.00023
isotig06616	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 1-like	-5.05	↓	1.62E-09	1.39E-07
isotig11660	hypothetical protein BRAFLDRAFT_83845	-5.42	↓	2.26E-12	2.58E-10
isotig06071	ribophorin II-like	-5.29	↓	3.16E-11	3.19E-09
isotig05633	WD repeat domain 7-like	-14.46	↓	1.59E-07	1.06E-05
isotig05177	conserved hypothetical protein	-11.89	↓	9.46E-06	0.00043

Note: Exp, expression trend; "↑", upregulated expression; "↓", downregulated expression.

Table 3: List of biological processes in which the 18 DEGs were involved

Annotated DEGs	DEGs involved biological process
isotig09968, isotig09381	growth and development
isotig27723, isotig09542,	immunity
isotig05433, isotig05391	respiration
isotig10757, isotig08005	transcription and translation
isotig06081, isotig05240	transport
isotig09541, isotig11660	signal transduction
isotig06071	glycosylation
isotig06539, isotig06616	electron transfer
isotig05633, isotig05177, isotig05215	undiscovered function

including 31 upregulated and 11 downregulated (Table S2) were obtained in the two transcript libraries, of which 18 were Blastx annotated (Table 2). Biological processes in which these annotated DEGs play roles were also investigated for further studies, mainly including growth and development, immunity, and respiration (Table 3).

To validate the expression of DEGs detected from RNA-Seq data, 7 DEGs including upregulated five genes (*ANGPT4*, *TTF2*, *TCTP*, *RPL8* and *CYT1*) and downregulated two (RPN2 and NDUFB1) were randomly selected for real-time PCR detection (primer sequences in Table S3). The RT-PCR results in Fig. 5 and Table 4 indicated that the expression trends of the chosen genes are consistent with the RNA-Seq results, although the change extents determined by RT-PCR were commonly smaller than those of RNA-Seq, probably due to higher sensitivity of RNA-Seq than that of RT-PCR in detection of gene expression (Zhao *et al.* 2014), suggesting the reliability and accuracy of the RNA-Seq method used in this study.

Functional enrichment of DEGs

Functions of DEGs were further investigated in GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analyses. Compared with the albino sample, DEGs in the segregated were classified into 34 GO terms including 20 biological processes, 7 cellular components, and 7 molecular functions (Fig. 6).

Table 4: List of expression changes of the chosen DEGs detected by RNA-Seq for RT-PCR validation in the segregated samples compared with the albino

Gene ID	Gene	Description	Exp	RNA-Seq	RT-PCR	Same Trend
isotig09968	ANGPT4	angiopoietin-4-like (ref|XP_003725072.1)	↑	16.52**	4.19**	Y
isotig10757	TTF2	transcription termination factor 2-like (ref|NP_001084942.1)	↑	16.09**	3.91**	Y
isotig09381	TCTP	translationally-controlled tumor protein (gb|ABC87996.1)	↑	14.69**	9.84**	Y
isotig08005	RPL8	60S ribosomal protein L8-like (ref|XP_796001.1)	↑	13.83**	1.53*	Y
isotig05391	CYT1	cytochrome c1, heme protein, mitochondrial-like (ref|XP_791551.2)	↑	12.92**	1.28*	Y
isotig06071	RPN2	ribophorin II-like (ref|XP_002736461.1)	↓	-5.29**	-2.03*	Y
isotig06616	NDUFB1	NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 1-like (ref|XP_001176933.1)	↓	-5.05**	-1.94*	Y

Note: Genes with the same expression trend (up or down regulation) examined by both RNA-Seq and RT-PCR analyses are indicated by "Y" (yes). *P < 0.05, **P < 0.01. Exp, expression trend; "↑", upregulated expression; "↓", downregulated expression

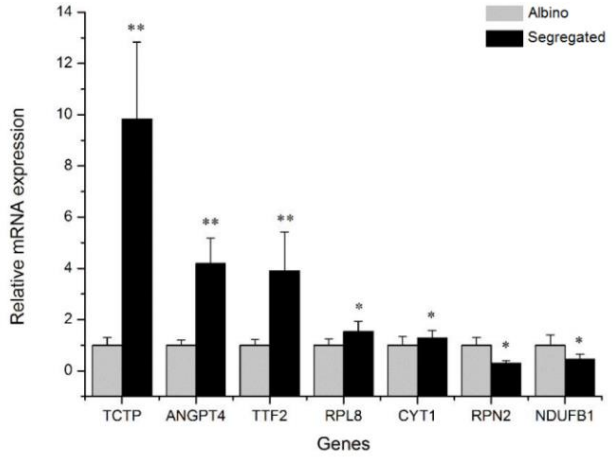

Fig. 5: Comparative expression analysis of seven DEGs identified from RNA-Seq data between segregated (black) and albino (gray) samples using qRT-PCR. RNA-Seq validated the five genes *ANGPT4*, *TTF2*, *TCTP*, *RPL8* and *CYT1* with significantly upregulated expression, and the two significantly downregulated genes RPN2 and NDUFB1. * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively

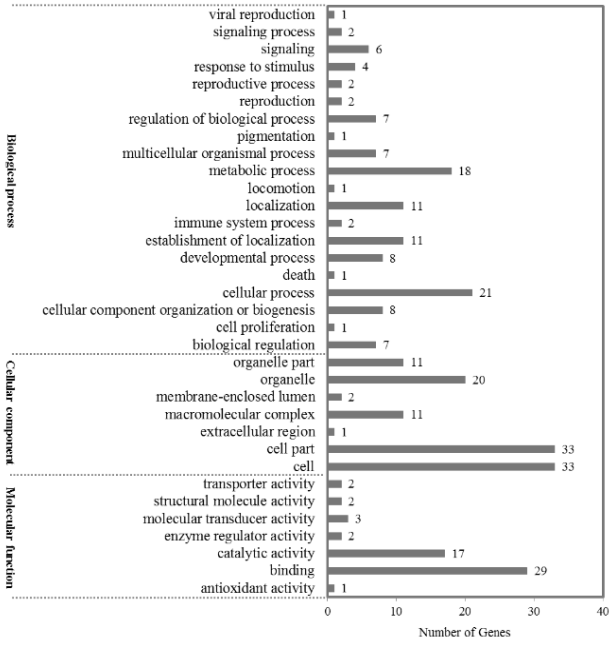

Fig. 6: Function classification of GO terms enriched by DEGs in the segregated juveniles produced by albino parent sea cucumbers compared with the albino morphs

Cellular process and metabolic process were the two most abundant terms in the "Biological process" category. Cell and cell part were the most enriched two categories in "Cellular component". Binding and catalytic activity were the most abundant categories in "Molecular function".

Pathway enrichment results in Table 5 showed that, in comparison with the albino morphs producing from crossing of albino parent sea cucumbers, totally 271 DEGs of body wall transcriptome in the segregated homochronous samples were grouped into 97 pathways in KEGG database, of which 15 pathways were significantly enriched (P value < 0.05). Some pathways related to immune responses, including "Phagosome", "*Staphylococcus aureus* infection", "Complement and coagulation cascades", and "*Vibrio cholerae* infection" were significantly enriched.

Discussion

The body wall tissue of sea cucumbers *A. japonicus* is composed of a thin cuticle over the epidermis and a thick dermis underneath. The outer skin of this species, consisting of cuticle and epidermis, forms the first line of protective barrier against environmental damage for maintaining body homeostasis (Zhou *et al.* 2016), because pigment cells (i.e. melanocytes) and immune cells embed in keratinocytes that are primary contents of the epidermis (Kern *et al.* 2011). Previous studies have focused on roles of *A. japonicus* skin in immune response to pathogens (Liu *et al.* 2010; Zhang *et al.* 2013). It is obviously valid that the intact body wall was chosen as the target sample to investigate segregation of body color and distinguish differences at transcription in *A. japonicus*. In this study, the first genome-scale analysis was conducted between body wall tissues of segregated and albino juvenile sea cucumbers derived from cross of albino parents. Quality estimation of illumina sequencing ensured reliable transcriptome data for the following multiple analyses. Totally 271 DEGs identified between the two color morphs should be placed emphasis on, especially with

Table 5: Enriched pathways involved in DEGs of the segregated juvenile progenies crossed by albino sea cucumber broodstock contrast to the albino homochronous morphs

Pathway ID	Pathway	DEGs with pathway annotation (104)	All genes with pathway annotation (9586)	*P* value
ko04145	Phagosome	12 (11.54%)	276 (2.88%)	4.1699E-05
ko05150	*Staphylococcus aureus* infection	6 (5.77%)	102 (1.06%)	0.0008
ko04610	Complement and coagulation cascades	9 (8.65%)	245 (2.56%)	0.0013
ko05110	*Vibrio cholerae* infection	5 (4.81%)	89 (0.93%)	0.0028
ko05320	Autoimmune thyroid disease	2 (1.92%)	8 (0.08%)	0.0031
ko05152	Tuberculosis	8 (7.69%)	238 (2.48%)	0.0043
ko05144	Malaria	4 (3.85%)	62 (0.65%)	0.0045
ko00510	N-Glycan biosynthesis	3 (2.88%)	39 (0.41%)	0.0085
ko00601	Glycosphingolipid biosynthesis	2 (1.92%)	14 (0.15%)	0.0097
ko05143	African trypanosomiasis	2 (1.92%)	14 (0.15%)	0.0097
ko00190	Oxidative phosphorylation	6 (5.77%)	170 (1.77%)	0.0103
ko05322	Systemic lupus erythematosus	4 (3.85%)	80 (0.83%)	0.0110
ko04510	Focal adhesion	10 (9.62%)	441 (4.6%)	0.0211
ko04640	Hematopoietic cell lineage	3 (2.88%)	58 (0.61%)	0.0249
ko04745	Phototransduction - fly	3 (2.88%)	62 (0.65%)	0.0296

the help of recently documented *A. japonicus* genomic information (Zhang *et al.* 2017; Li *et al.* 2018). This result supported valuable gene resources to elucidate the mechanisms of body color segregation of progenies crossed by albino sea cucumbers.

As previously reported, it is clearly observed that growth rate of albino juvenile sea cucumbers is slower than that od in the green and purple variants (Xing *et al.* 2018b). The same result is also observed in this study. Further research revealed that physiological performances including growth rate, energy allocation, respiration and digestion were lowest among the three-color morphs, although their values had big change range with salinity (Bai *et al.* 2015). Furthermore, a lot of genes associated with growth, development and metabolism were identified to be apparently expressed at lower levels in albino sea cucumbers compared with control (green) morphs, resulting in lower growth rates and reduced physiological activity (Ma *et al.* 2014a, b). Angiopoietin-4 (Ang4) belonging to angiopoietin family of growth factors plays positive roles in regulating endothelial cell growth and angiogenesis (Augustin *et al.* 2009; Thomas and Augustin 2009). Translationally controlled tumor protein (TCTP) plays an important role in motivating mitotic growth by controlling the duration of the cell cycle in eukaryotes (Berkowitz *et al.* 2008; Brioudes *et al.* 2010). Plentiful studies have proved that TCTP is involved in various cellular processes, such as apoptosis, microtubule organization, and ion homeostasis (Bommer and Thiele 2004). The two growth regulatory genes (*Ang4* and *TCTP*) were identified to be extremely downregulated in the juvenile albino sea cucumbers in comparison with the control (Ma *et al.* 2014b). Compared with the segregated F_1 individuals with normally green body color produced by albino sea cucumbers, the same expression trend of *Ang4* and *TCTP* was explicitly detected in the albino progeny.

Besides the growth-related genes as discussed above, some respiration-related genes with highly significant difference of expression were also proved between the two transcriptomes in this study, such as cytochrome C1 and cytochrome C oxidase. As important components of complex III and IV in mitochondrial respiratory chain, they are responsible for electron transport to molecular oxygen during ATP production. Their expression levels were significantly increased with 13.73 and 12.92 folds in the body wall of segregated juvenile sea cucumbers, indicating that their energy requirement was probably higher than that of the albino samples. Moreover, the food conversion efficiency and digestive enzyme activity in albino sea cucumbers are identified to be lowest among juvenile morphs with white, green and purple body colors (Bai *et al.* 2016). Therefore, it is reasonably concluded from these evidences that growth of juvenile albino *A. japonicus* is undoubtedly lower than that of the commonly green morph under the current culture condition.

It is confirmed that excessive deposition caused by abnormal balance of growth factors and cell proliferation can promote local hyperplastic collagen production in skin responding to injury in mammalians (Tuan and Nichter 1998). Tenascin-R improves assembly of the extracellular matrix (ECM) of perineuronal nets, especially excessive deposition of collagen (Morawski *et al.* 2014). ECM remolding has been identified to be closely associated with organ morphogenesis and tissue regeneration in *Holothuria glaberrima* (Quiñones *et al.* 2002) and *A. japonicus* (Sun *et al.* 2011). Expression profiles of this ECM associated gene were overall expressed at a higher level during intestine regenerating process (Sun *et al.* 2013). In the present study, tenascin-R expression was observed to be significantly upregulated in the segregated progeny of albino sea cucumbers, suggesting their higher regeneration capacity in combination with the previously validated roles of tenascin-R.

Enrichment KEGG analysis revealed that immuno-defense related pathways were dominantly highlighted in enriching differentially expressed genes (Table 5). Phagosomes as key organelles of phagocytic cells participate in a variety of innate immunity, including tissue

remodeling, apoptotic cell elimination, and restricting the spread of intracellular pathogens (Garin *et al.* 2001; Griffiths and Mayorga 2007). Proteomic analysis identified at least 140 proteins correlated with latex bead-containing phagosomes. Pathway enrichment result showed that dozens of component proteins were significantly enriched in phagosomes in body wall of the segregated progenies produced from albino sea cucumbers. The enriched phagosome proteins are highly possible to play roles in resistance on infection of *Staphylococcus aureus* and *Vibrio cholera*, and complement and coagulation cascades. The three pathways remarkably enriched DEGs in the transcriptome of the segregated progenies produced from cross of albino sea cucumbers, indicating that the innate immune defense of the segregated progeny might be stronger than that of the albino. Coupled with 15.56-fold upregulated expression of complement factor H in the segregated samples, their ability to resist pathogen invasion is probably better than the albino progeny, although the role of this factor needs to be further understood in the complement system. Consequently, albino juvenile morphs are likely to have poor immune defense to external pathogens and harmful stress.

Nutrition is a crucial factor determining immune defenses, and malnutrition is the most common cause of immunodeficiency in animals (Chandra 1997; Xia *et al.* 2013). Previous studies have proved that the weaker digestive enzyme activity of the albino sea cucumbers *A. japonicus* leads to lower rates of food intake and slower growth rate, and then decreases immunity (Bai *et al.* 2015; Xing *et al.* 2018b). Those factors may be responsible for the low growth and survival rates in white sea cucumbers. Consequently, body color segregation of juvenile progenies of albino cross might be a survival strategy under the current cultivation conditions. The segregation mechanism is advised to be further investigated in epigenetic regulation level. Because of low growth rate and environmental adaptability for albino sea cucumbers *A. japonicus*, as previously documented, maintenance of suitable conditions (such as nutrition, light, water quality and attachment substrate) is necessary for breeding and management of white morphs. Improvements on the purity and genetic stability of white body color are essential to satisfy the market demand for this attractive strain of *A. japonicus*.

Conclusion

In conclusion, the above-mentioned results identified some key genes and pathways for a better understanding of decline in number and size of albino morphs in comparison with the segregated progenies crossed by albino parents. In addition, these data provide credible clues to further investigate the body color segregation in *A. japonicus*. This work will be available to facilitate breeding program in genetically steady strain of albino sea cucumbers.

Acknowledgements

This work was funded by National Key R & D Program of China under contract No. 2019YFD0900405, National Natural Science Foundation of China under contract No. 41706180, and Dalian Supporting High-Level Talent Innovation and Entrepreneurship Program under contract No. 2017RQ031.

References

Augustin HG, GY Koh, G Thurston, K Alitalo (2009). Control of vascular morphogenesis and homeostasis through the angiopoietin–Tie system. *Nat Rev Mol Cell Biol* 10:165–177

Bai YC, LB Zhang, SD Xia, SL Liu, XS Ru, QZ Xu, T Zhang, HS Yang (2016). Effects of dietary protein levels on the growth, energy budget, and physiological and immunological performance of green, white and purple color morphs of sea cucumber, *Apostichopus japonicus*. *Aquaculture* 450:375–382

Bai YC, LB Zhang, SL Liu, XS Ru, LL Xing, XB Cao, T Zhang, HS Yang (2015). The effect of salinity on the growth, energy budget and physiological performance of green, white and purple color morphs of sea cucumber, *Apostichopus japonicus*. *Aquaculture* 437:297–303

Berkowitz O, R Jost, S Pollmann, J Masle (2008). Characterization of TCTP, the translationally controlled tumor protein, from *Arabidopsis thaliana*. *Plant Cell* 20:3430–3447

Birol I, SD Jackman, CB Nielsen, JQ Qian, R Varhol, G Stazyk, RD Morin, Y Zhao, M Hirst, JE Schein (2009). *De novo* transcriptome assembly with ABySS. *Bioinformatics* 25:2872–2877

Bommer UA, BJ Thiele (2004). The translationally controlled tumour protein (TCTP). *Intl J Biochem Cell Biol* 36:379–385

Brioudes F, AM Thierry, P Chambrier, B Mollereau, M Bendahmane (2010). Translationally controlled tumor protein is a conserved mitotic growth integrator in animals and plants. *Proc Natl Acad Sci USA* 107:16384–16389

Chandra R (1997). Nutrition and the immune system: An introduction. *Amer J Clin Nutr* 66:460–463

Cheng MG, QM Qiao, GH Yuan (2005). Progress of the research on body-color diversity in insects. *Chin Bull Entomol* 42:502–505

Cui KR, YE Chuan, W Zhou, CL Chai, 2015. Progress in the research of body color of the silkworm, *Bombyx mori*. *Newslett Seric Sci* 35:21–29

Du H, Z Bao, R Hou, S Wang, H Su, J Yan, M Tian, Y Li, W Wei, W Lu, X Hu, S Wang, J Hu (2012). Transcriptome sequencing and characterization for the sea cucumber *Apostichopus japonicus* (Selenka, 1867). *PLoS One* 7; Article e33311

Feng C, M Chen, CJ Xu, L Bai, XR Yin, X Li, AC Allan, IB Ferguson, KS Chen (2012). Transcriptomic analysis of Chinese bayberry (*Myrica rubra*) fruit development and ripening using RNA-seq. *BMC Genomics* 13; Article 19

Gao Q, MJ Liao, YG Wang, B Li, Z Zhang, XJ Rong, GP Chen, L Wang (2015). Transcriptome analysis and discovery of genes involved in immune pathways from coelomocytes of sea cucumber (*Apostichopus japonicus*) after *Vibrio splendidus* challenge. *Intl J Mol Sci* 16:16347–16377

Garin J, R Diez, S Kieffer, JF Dermine, S Duclos, E Gagnon, R Sadoul, C Rondeau, M Desjardins (2001). The phagosome proteome. *J Cell Biol* 152:165–180

Grabherr MG, BJ Haas, M Yassour, JZ Levin, DA Thompson, I Amit, X Adiconis, L Fan, R Raychowdhury, Q Zeng (2011). Full-length transcriptome assembly from RNA-seq data without a reference genome. *Nat Biotechnol* 29:644

Griffiths G, L Mayorga (2007). Phagosome proteomes open the way to a better understanding of phagosome function. *Genom Biol* 8:207–210

Hunt M, N Kaur, M Stromvik, L Vodkin (2011). Transcript profiling reveals expression differences in wild-type and glabrous soybean lines. *BMC Plant Biol* 11; Article 145

Jiang SH, SL Dong, QF Gao, F Wang, XL Tian (2013). Comparative study on nutrient composition and growth of green and red sea cucumber, *Apostichopus japonicus* (Selenka, 1867), under the same culture conditions. *Aquac Res* 44:317–320

Jo J, J Park, HG Lee, EM Kern, S Cheon, S Jin, JK Park, SJ Cho, C Park (2016). Comparative transcriptome analysis of three color variants of the sea cucumber *Apostichopus japonicus*. *Mar Genomics* 28:21–24

Kang JH, KH Yu, JY Park, CM An, JC Jun, SJ Lee (2011). Allele-specific PCR genotyping of the HSP70 gene polymorphism discriminating the green and red color variants sea cucumber (*Apostichopus japonicus*). *J Genet Genomics* 38:351–355

Kern F, T Niault, M Baccarini (2011). Ras and Raf pathways in epidermis development and carcinogenesis. *Braz J Cancer* 104:229–234

Li JL, SF Li (1997). Primary study on segregation of body colour and its relationship with growth propagation in progeny of Taiwanese red tilapia. *Fish Sci Technol Inform* 24:65–69

Li RQ, C Yu, YR Li, TW Lam, SM Yiu, K Kristiansen, J Wang (2009). Soap2: An improved ultrafast tool for short read alignment. *Bioinformatics* 25:1966–1967

Li YL, RJ Wang, XG Xun, J Wang, LS Bao, R Thimmappa, J Ding, JW Jiang, LH Zhang, TQ Li, J Lv, C Mu, XL Hu, LL Zhang, J Liu, YQ Li, LJ Yao, WQ Jiao, YF Wang, SS Lian, ZL Zhao, YY Zhan, XT Huang, H Liao, J Wang, HZ Sun, X Mi, Y Xia, Q Xing, W Lu, A Osbourn, ZC Zhou, YQ Chang, ZM Bao, S Wang (2018). Sea cucumber genome provides insights into saponin biosynthesis and aestivation regulation. *Cell Discov* 4; Article 29

Ling J, H Jiang, T Yu, GQ Duan (2016). Genetic diversity analysis of three populations of Ricefield eel *Monopterus albus* with different body colors. *Chin J Fish* 29:6–10

Liu HZ, FR Zheng, XQ Sun, XG Hong, SL Dong, B Wang, XX Tang, YQ Wang (2010). Identification of the pathogens associated with skin ulceration and peristome tumescence in cultured sea cucumbers *Apostichopus japonicus* (Selenka). *J Invertebr Pathol* 105:236–242

Ma DY, HS Yang, LN Sun (2014a). Profiling and comparison of color body wall transcriptome of normal juvenile sea cucumber (*Apostichopus japonicus*) and those produced by crossing albino. *J Ocean Univ Chin* 13:1033–1042

Ma DY, HS Yang, LN Sun, MY Chen (2014b). Transcription profiling using RNA-seq demonstrates expression differences in the body walls of juvenile albino and normal sea cucumbers *Apostichopus japonicus*. *J Oceanol Limnol* 32:34–46

Morawski M, A Dityatev, M Hartlage-Rübsamen, M Blosa, M Holzer, K Flach, S Pavlica, G Dityateva, J Grosche, G Brückner, M Schachner (2014). Tenascin-r promotes assembly of the extracellular matrix of perineuronal nets via clustering of aggrecan. *Phil Trans Roy Soc Lond B Biol Sci* 369:1–9

Mortazavi A, BA Williams, K Mccue, L Schaeffer, B Wold (2008). Mapping and quantifying mammalian transcriptomes by RNA-seq. *Nat Meth* 5:621–643

Okorie OE, SH Ko, S Go, S Lee, JY Bae, KM Han, SC Bai (2008). Preliminary study of the optimum dietary ascorbic acid level in sea cucumber, *Apostichopus japonicus* (Selenka). *J World Aquac Soc* 39:758–765

Quiñones JL, R Rosa, DL Ruiz, JE Garcíaarrarás (2002). Extracellular matrix remodeling and metalloproteinase involvement during intestine regeneration in the sea cucumber *Holothuria glaberrima*. *Dev Biol* 250:181–197

Schmittgen TD, KJ Livak (2008). Analyzing real-time PCR data by the comparative C_T method. *Nat Protoc* 3:1101–1108

Sun L, H Yang, M Chen, D Ma, C Lin (2013). RNA-seq reveals dynamic changes of gene expression in key stages of intestine regeneration in the sea cucumber *Apostichopus japonicus*. *PLoS One* 8; Article e69441

Sun L, M Chen, H Yang, T Wang, B Liu, C Shu, D Gardiner (2011). Large scale gene expression profiling during intestine and body wall regeneration in the sea cucumber *Apostichopus japonicus*. *Compar Biochem Physiol D Genomics Proteomics* 6:195–205

Thomas M, HG Augustin (2009). The role of the angiopoietins in vascular morphogenesis. *Angiogenesis* 12:125–137

Trapnell C, A Roberts, L Goff, G Pertea, D Kim, DR Kelley, H Pimentel, SL Salzberg, JL Rinn, L Pachter (2012). Differential gene and transcript expression analysis of RNA-seq experiments with Tophat and Cufflinks. *Nat Protoc*7:562–578

Trapnell C, BA Williams, G Pertea, A Mortazavi, G Kwan, MJV Baren, SL Salzberg, BJ Wold, L Pachter (2010). Transcript assembly and quantification by RNA-seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* 28:511–515

Tuan TL, LS Nichter (1998). The molecular basis of keloid and hypertrophic scar formation. *Mol Med Today* 4:19–24

Wu B, S Xia, MM Rahman, M Rajkumar, Z Fu, J Tan, A Yang (2015). Substituting seaweed with corn leaf in diet of sea cucumber (*Apostichopus japonicus*): Effects on growth, feed conversion ratio and feed digestibility. *Aquaculture* 444:88–92

Xia SD, HS Yang, Y Li, SL Liu, LB Zhang, K Chen, JH Li, AG Zou (2013). Effects of differently processed diets on growth, immunity and water quality of the sea cucumber, *Apostichopus japonicus* (Selenka 1867). *Aquac Nutr* 19:382–389

Xing LL, LN Sun, SL Liu, XN Li, LB Zhang, HS Yang (2018a). Transcriptome analysis provides insights into the mechanism of albinism during different pigmentation stages of the albino sea cucumber *Apostichopus japonicus*. *Aquaculture* 486:148–160

Xing LL, LN Sun, SL Liu, ZX Wan, XN Li, T Miao, LB Zhang, YC Bai, HS Yang (2018b). Growth, histology, ultrastructure and expression of MITF and astacin in the pigmentation stages of green, white and purple morphs of the sea cucumber, *Apostichopus japonicus*. *Aquac Res* 49:177–187

Xing LL, LN Sun, SL Liu, XN Li, T Miao, LB Zhang, HS Yang (2017). Comparison of pigment composition and melanin content among white, light-green, dark-green, and purple morphs of sea cucumber, *Apostichopus japonicus*. *Acta Oceanol Sin* 36:45–51

Yuan XT, HS Yang, Y Zhou, YZ Mao, T Zhang, Y Liu (2006). The influence of diets containing dried bivalve feces and/or powdered algae on growth and energy distribution in sea cucumber *Apostichopus japonicus* (Selenka) (Echinodermata: Holothuroidea). *Aquaculture* 256:457–467

Zhang P, C Li, L Zhu, X Su, Y Li, C Jin, T Li (2013). *De novo* assembly of the sea cucumber *Apostichopus japonicus* hemocytes transcriptome to identify miRNA targets associated with skin ulceration syndrome. *PLoS One* 8; Article e73506

Zhang X, L Sun, J Yuan, Y Sun, Y Gao, L Zhang, S Li, H Dai, JF Hamel, C Liu, Y Yu, S Liu, W Lin, K Guo, S Jin, P Xu, KB Storey, P Huan, T Zhang, Y Zhou, J Zhang, C Lin, X Li, L Xing, D Huo, M Sun, L Wang, A Mercier, F Li, H Yang, J Xiang (2017). The sea cucumber genome provides insights into morphological evolution and visceral regeneration. *PLoS Biol* 15; Article e2003790

Zhao HL, HS Yang, H Zhao, SL Liu, TM Wang (2012). Differences in mitf gene expression and histology between albino and normal sea cucumbers (*Apostichopus japonicus* Selenka). *Chin J Oceanol Limnol* 30:80–91

Zhao Y, HS Yang, KB Storey, MY Chen (2014). Differential gene expression in the respiratory tree of the sea cucumber *Apostichopus japonicus* during aestivation. *Mar Genomics* 18:173–183

Zheng FR, HZ Liu, XQ Sun, LY Qu, SL Dong, JL Liu (2012). Selection, identification and application of antagonistic bacteria associated with skin ulceration and peristome tumescence of cultured sea cucumber *Apostichopus japonicus* (Selenka). *Aquaculture* 334:24–29

Zhou X, J Cui, S Liu, D Kong, H Sun, C Gu, H Wang, X Qiu, Y Chang, Z Liu, X Wang (2016). Comparative transcriptome analysis of papilla and skin in the sea cucumber, *Apostichopus japonicus*. *Peer J* 4; Article e1779