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The regulation mechanism of IncRNAs and mRNAs in sea cucumbers under global climate changes: Defense against thermal and hypoxic stresses

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HIGHLIGHTS

• Sea cucumbers under three types of stresses at two molecular levels were examined.
• A total of 389, 873 and 1142 DE genes were identified under three stresses, respectively.
• The IncRNA- mRNA co-regulation networks were constructed and validated.
• “HIF/Aja-miR-2013-3p/MSTRG.34610 and MSTRG.10941” axis may be important in defending environmental stresses.
• Environmental stress altered immunity, energy and cell cycle processes in Apostichopus japonicus.

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ABSTRACT

The aquatic environment can be greatly impacted by thermal and hypoxic stresses, particularly caused by intensified global warming. Hence, there is an urgency to understand the response mechanisms of marine organisms to adverse environment. Although long non-coding RNAs (IncRNAs) are involved in many biological processes, their roles in stress responses still remain unclear. Here, differentially expressed (DE) IncRNAs and mRNAs were identified as responses to environmental stresses in the economically important sea cucumber, Apostichopus japonicus, and their potential roles were explored. Based on a total of 159, 355 and 495 significantly upregulated genes and 230, 518 and 647 significantly downregulated genes identified in the thermal, hypoxic and combination thermal + hypoxic stress treatments, respectively, we constructed DE-IncRNA-mRNA coexpression networks. Among the networks, eight shared pairs were identified from the three treatments, and based on the connectivity degree, MSTRG.27265, MSTRG.10941 and MSTRG.95524 were shown to be crucial.

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1. Introduction

Due to global climate change, the natural water temperature has significantly increased, resulting in decreased dissolved oxygen levels in the past few decades (Dong et al., 2020). Thus, in aquaculture ponds, high temperature (HT) and low dissolved oxygen (LO) conditions in aquatic systems may co-occur more frequently. Extremely high temperature and low dissolved oxygen would cause thermal stress and hypoxic stress, respectively. These environmental stresses could lead to yield loss and reduce the germplasm quality in the aquaculture industry (Huo et al., 2018). The sea cucumber Apostichopus japonicus is a deposit feeder that participates in carbon cycling in marine ecosystems (Huo et al., 2019b). In China, it is an economically important food species raised extensively in the mariculture; for example, the area, yield and quantity of seedlings in the country were 2.2 × 10^5 hm^2, 2.1 × 10^8 kg and 52.8 billion by 2017, respectively (Ministry of Agriculture, 2018). Thermal and hypoxic stresses have been identified as the main causes of massive death of sea cucumbers over the summer in China in recent years, with these stresses negatively impacting of these stresses on the behavioral, physiological, histomorphological and molecular characteristics of A. japonicus (Huo et al., 2019a). To reduce the damage caused by environmental stress, sophisticated adaptive response mechanisms have been developed by sea cucumbers. The regulation of gene expression occurs at the transcriptional, posttranscriptional and posttranslational levels (Shukla et al., 2008).

Long non-coding RNAs (lncRNAs) are RNA molecules without protein-coding abilities and lengths over 200 nucleotides (Kowalczyz et al., 2012). LncRNAs can function at the site of transcription in cis (cis-regulation) or trans (trans-regulation) modes and, as such, participate in multiple networks regulating gene expression through diverse mechanisms including alternative splicing (Gonzalez et al., 2015), translation (Carrieri et al., 2012; Yoon et al., 2012), and regulation of epigenetic and posttranscriptional gene (Ibeagha-Awemu et al., 2018; Mercer and Mattick, 2013; Yoon et al., 2013). Although lncRNAs have been reported to play roles in the regulation of the innate immunity (Heward and Lindsay, 2014), chromatin reprogramming (Gupta et al., 2010), cellular differentiation and development (Fatica and Bozzoni, 2013), as well as cardiac development and aging (The Cardiolinc et al., 2015) in model organisms, the biological functions of lncRNAs remain largely unknown in aquatic animals, especially for those involved in stress-responsive mechanisms.

Understanding dysregulated lncRNAs and miRNAs is important for revealing the molecular mechanisms occurring in sea cucumber coping with stress. Due to the recent availability of high-quality genomic information for A. japonicus (Zhang et al., 2017), research on non-coding RNAs in sea cucumber can now be conducted. Therefore, we obtained lncRNA and messenger RNA (mRNA) expression profiles from respiratory tree of sea cucumbers under thermal stress, hypoxic stress, and combined thermal + hypoxic stress (HL) as well as control treatment conditions (NC). Respiratory tree was selected as the target tissue in the present study, because it is the major organ responsible for respiratory metabolism in the sea cucumber and is very sensitive to environmental stress (Huo et al., 2017). The integrated analysis of the differentially expressed (DE) lncRNAs and miRNAs may provide clues to find genes with active roles in stress adaption in sea cucumbers under environmental stress in the context of global climate change. Our results represent the first construction of lncRNA-miRNA coexpression networks and lncRNA-miRNA-mRNA networks identified by the dual-luciferase reporter system in sea cucumbers. The data reported herein provides important information about stress resistance in echinoderm with potential applications to other species in aquaculture.
NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, USA), and the details are as follows: first, ribosomal RNA was removed utilizing Epicentre Ribo-Zero™ Gold Kits (Epicentre, USA), and then NEBNext First-Strand Synthesis Reaction Buffer under raised temperature was used to fracture RNA into short RNA strands. Subsequently, random hexamer primers were used to synthesize the first-strand cDNA by utilizing RNA fragments as a template. Afterwards, second-strand cDNA synthesis was performed using a buffer, dNTPs, DNA polymerase I and RNase H. Then the library fragments were purified with QIAQuick PCR kits, and eluted with elution buffer (EB). Later on, terminal repair, poly(A) tailing and adapter ligation were implemented. The library fragments were purified with agarose gel electrophoresis to referentially select cDNA fragments of 300 bp in length and the UNG enzyme was used to digest second strand cDNA. After performing PCR, aimed products were retrieved by agarose gel electrophoresis. After measuring the RNA concentration of each library by a Qubit® RNA Assay Kit with a Qubit® 2.0 instrument, the RNA was diluted to 1 ng/μL. The insert size was assessed using an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), and the Taqman fluorescence probe of the AB Step One Plus Real-Time PCR system (library valid concentration ≥ 10 nM) was used to quantify the qualified insert size. Clustering of the index-coded samples was performed on a cBot cluster generation system using a TruSeq PE Cluster Kit v4-cBot-HS (Illumina). After generating cluster, the libraries were sequenced on an Illumina HiSeq 4000 platform and 150 bp paired-end reads were generated.

2.3. Quality control, alignment and identification

Raw data were processed with Perl scripts to ensure the quality of data to be used in subsequent analyses. Adapter-polluted reads (reads containing >5 adapter-polluted bases), low-quality reads (reads with low-quality bases (phred quality value <19) accounting for >15% of the total bases) and the reads with N bases accounting for >5% of total bases were removed. For the paired-end sequencing data, both reads were filtered out if any read of the paired-end reads was adapter-polluted. The filtered clean data was subjected to statistical analyses for quantity and quality.

The reference genomes and the annotation file of A. japonicus were used (Zhang et al., 2017). Bowtie2 (v2.2.3) was used for building the genome index, and the clean data were mapped to the reference genome using TopHat (v2.0.12). Moreover, TopHat could call Bowtie2 for mapping, increasing speed and accuracy. Cufflinks was used to predict the new transcripts and those with lengths were ≥200 bp, exons ≥2 and cover degree ≥3 were selected. Coding Potential Calculator (CPC), Coding-Non-Coding-Index (CNCI), Coding Potential Assessment Tool (CPAT) and Pfam were used to identify IncRNAs, and those IncRNAs that were identified in only one sample were removed. Venn diagrams were presented using Venny 2.1 (Oliveros, 2007).

2.4. Quantitation of gene expression levels and differential gene expression analysis

Fragments per kilobase million (FPKM) were then calculated to estimate the expression level of genes in each sample. The formula is as follows:

$$\text{FPKM} = \frac{10^3 \times F}{N \times L / 10^6}$$

where N is the total number of mapped fragments in the given sample, L is the length of the certain gene, and F is the number of fragments in a given sample that is assigned to a certain gene. FPKM can eliminate the effect of sequencing depth and gene length on the gene expression level, and the data can be compared between each other directly.

DESeq (v1.16) was used for differential gene expression analysis between two samples with biological replicates using a model based on the negative binomial distribution. A P-value was assigned to each gene and adjusted by Benjamini and Hochberg’s approach for controlling the false discovery rate as a Q-value. Genes with q < 0.05 and \( \log2\text{FoldChange} \geq 1 \) were identified as differentially expressed genes.

2.5. GO and KEGG enrichment analyses of DE-genes (DEGs)

The GO (http://www.geneontology.org/) and KEGG (http://www.genome.jp/kegg/) databases were used to classify and group the identified mRNAs and lncRNAs. GO and KEGG pathway enrichment analyses were based on significantly up- and down-regulated genes, respectively. The ratio of the number of differentially regulated genes annotated in a pathway term to the number of all genes annotated in the same pathway term was defined as rich factor. A greater rich factor indicates greater intensiveness. The hypergeometric test was used to identify the significantly enriched GO terms and pathways of differentially regulated genes, and those with P-value < 0.05 were considered significant (Huo et al., 2019b). The GO terms with top 5 lowest P-values for the ontologies biological process, molecular function and cellular component in the three treatments showing in Fig. 3 were made by ggplot2 (Wickham, 2016). The most significantly enriched GO terms and most significant KEGG pathway were identified with the lowest P-value among all the significantly enriched terms.

2.6. Real-time quantitative polymerase chain reaction (RT-qPCR) validation

Ten DE-mRNAs and ten DE-lncRNAs with high-fold-change values in the three group comparisons were selected for expression detection. Total RNA was extracted from the respiratory tree, the same tissue used for the construction of the RNA-seq profile, by using a MiniBEST Universal RNA Extraction Kit (Takara, Shiga, Japan). A NanoDrop 1000 (Thermo Fisher Scientific, USA) was used for measuring the quality and concentration of RNA before using reverse transcriptase (Takara) Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) were used for examining the mRNA expression levels, and β-actin was used as a reference gene for internal standardization (Yang et al., 2010b).

Primer3 was used for designing primers according to the sequence information in the transcriptome database. All primer sequences are shown in Table S1. In the present study, three biological replicates × three technical replicates were used for the detection of gene expression in each group. The total volume of the amplification mix was 20 μL, which contained 10 μL of SYBR Green Master Mix (Takara), 8 μL of RNase-free water, 0.5 μL (each) of the forward and reverse primers (10 mM), and 1 μL of diluted cDNA. The thermal cycling was performed according to the following procedure: 95 °C for 5 s, followed by 40 cycles at 95 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s. Melting curve analysis was used for illustrating the specificity of the amplification products, and the 2−ΔΔCT method was used to analyze the comparative mRNA expression levels of the selected genes (Schmittgen and Livak, 2008). All data were analyzed by SPSS19 software (IBM Corp., Armonk, NY, USA), and presented as mean ± SD. The threshold of statistical significance was a P-value < 0.05 analyzed by one-way ANOVA with Tukey’s test. Among the RT-qPCR results of the three treatments compared with the control, genes showing the same tendency as the sequencing data for all the three stress treatments relative to the control treatment were defined as completely matched, and those showing the same tendency in at least one comparison were defined as partially matched.
2.7. Trans-target prediction and validation

To identify the trans-targets of IncRNAs, the FASTA sequences of IncRNAs and mRNAs were extracted based on the genome and annotation file, and they were used to calculate the free energy of binding using RNAhybrid, a software specially designed to quickly find possible hybridization sites for a query RNA in large RNA databases and to predict the RNA-RNA interaction (Rehmsmeier et al., 2004). The IncRNA-mRNA co-expression network was built by Cytoscape software (Cytoscape Consortium, San Diego, CA, USA) (Shannon et al., 2003) based on the correlation between the DE-IncRNAs and DE-mRNAs. IncRNAs and coding genes were identified based on Pearson’s correlation coefficients (equal to or >0.8) for trans-targets or based on the genomic positional relation in 50 kb regions (upstream and downstream) for cis-targets. Based on our previous study, Aja-miR-2013-3p was taken as a key stress-responsive-miRNA connected with IncRNAs and mRNAs in *A. japonicus* (Huo et al., 2017). Hypoxia-inducible factor (HIF-1α), a key hypoxia responder, was predicted by RNAhybrid to be one of the targets of Aja-miR-2013-3p. Thus, these two were used for IncRNA binding possibility screening and IncRNA-miRNA-mRNA network prediction. All the plasmids used in the present study were custom synthesized by GeneChem Co., Ltd. (Shanghai, China) (Table S2) and cloned into a GV272 firefly luciferase plasmid (GeneChem). The GV272 empty vector was utilized as a negative control (3′ UTR-NC) (GeneChem), and the multiple cloning sites of it were digested by XbaI/XbaI restriction enzymes. The 3′ untranslated regions (UTR) segment of mRNA (HIF-1α) and IncRNA (MSTRG.93731, MSTRG.10941, MSTRG.34610 and MSTRG.81281) or their mutant was inserted into the vector to yield the 3′ UTR or 3′ UTR-MU. The recombinant products were verified by DNA sequencing, and the group details can be found in Table S2. Hsa-miR-146b and its target gene TRAF6 were utilized as positive controls (Park et al., 2015). The pGL3 control vector (Promega) and pRL-TK were used as a firefly luciferase reporter vector and a Renilla luciferase control reporter vector, respectively. PCR amplification of the RNA sequences was performed using a PrimerSTAR Max DNA Polymerase Mix (Takara Bio Inc., Japan). The top 10 competent bacterial cells were transformed with the ligation product of the luciferase reporter vector and the digested PCR fragment. Transformed cells were

![Fig. 1. Characteristic of identified IncRNAs vs mRNAs in the sea cucumber.](image)
incubated at 37 °C on LB agar plates with antibiotics overnight, and isolated clones were inoculated in LB medium with antibiotics at 37 °C with shaking overnight. The ZR Plasmid Miniprep Kit (Zymo Research, Orange, CA, USA) was used for the plasmid DNA purify. All constructs were verified by sequencing (Table S2). The transfection and detection of the luciferase activities were performed using Li's method (Li et al., 2018). The human embryonic kidney cell line, HEK 293 T at 90% confluence in 24-well plates was transfected with a 3’ UTR luciferase plasmid (0.1 μg), a microRNA plasmid (0.4 μg) and a Renilla plasmid (0.02 μg) using the X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland). The firefly luciferase plasmid and the Aja-miR-2013-3p expression plasmid were co-transfected with a Renilla luciferase vector (Promega, Madison, WI, USA) for normalization. Luciferase activity was measured using a microplate reader (M2000PR, Tecan infinite) after 48 h following the manufacturer’s instructions. All experiments were performed in triplicate, and the original data and the normalization method are shown in Table S2. The analysis methods were the same as those described for RT-qPCR, including software, statistical test and figure construction.

3. Results

3.1. RNA-seq analysis and identification of expressed mRNA and IncRNA genes in the respiratory tree of A. japonicus

3.1.1. Basic property comparison of mRNAs and IncRNAs

In the present study, RNA-seq of A. japonicus samples from control (NC), high temperature exposure (HT), low oxygen exposure (LO), and high temperature + low oxygen (HL) were performed, and the data were submitted to the GEO database [GSE131676]: 118 to 159 million raw reads and 105 to 143 million clean reads were obtained per sample. Moreover, 28,586 mRNAs were identified. Every potential IncRNA in all samples was assembled by Stringtie. Finally, 59,967 IncRNAs were retained at the intersection of the CPC, CCM, CPAT and Pfam scans (Fig. S1). Because of the lack of IncRNA information for invertebrates, especially for sea cucumbers, all identified IncRNAs were novel, and the names of novel IncRNAs begin with “MSTRG.” Fig. S2 shows the hierarchical clustering of DE-IncRNAs (Fig. S2a) and DE-mRNAs (Fig. S2b) of sea cucumbers under environmental stress and normal conditions, detailed information of DEGs could be found in Table S3. Key features of DE-IncRNAs, such as exon number and sequence length, were characterized by comparison with identified DE-mRNAs under the same conditions (Fig. 1). The results showed that the exon number of IncRNAs was less than that of mRNAs. Moreover, most IncRNAs had fewer than 7 exons, but several mRNAs had more than thirty exons. In addition, the exon numbers of IncRNAs were distributed over a wider range than mRNAs. The lengths of most IncRNAs were shorter than mRNAs.

3.1.2. Differential expression of mRNAs and IncRNAs in sea cucumbers under environmental stress

A total of 389, 873 and 1142 representative DEGs (mRNAs and IncRNAs) were identified in sea cucumbers under thermal, hypoxic and thermal + hypoxic stress compared with controls based on the criteria of |log2FoldChange| ≥ 1 and q < 0.05. When A. japonicus was exposed to thermal stress, 88 mRNAs and 71 IncRNAs were significantly upregulated and 230 genes were significantly downregulated, including 107 mRNAs and 123 IncRNAs. When A. japonicus was exposed to hypoxic stress, 355 genes were significantly upregulated including 247 mRNAs and 108 IncRNAs, whereas 518 were significantly downregulated, including 233 mRNAs and 285 IncRNAs. When A. japonicus was exposed to both thermal and hypoxic stress, more significantly changed genes were observed: 495 significantly upregulated genes were identified including 277 mRNAs and 218 IncRNAs, as well as 647 significantly downregulated genes including 346 mRNAs and 301 IncRNAs. Volcano plots illustrating these data are shown in Fig. S3. The top 10 DE- IncRNAs and DE-mRNAs with highest fold change value are shown in Tables 1 and 2.

3.2. Validation of the transcription levels of IncRNAs and mRNAs

To confirm the expression of IncRNAs and mRNAs, RT-qPCR analysis was applied to verify the RNAseq results (Fig. 2). Among the 10 selected IncRNAs and 10 mRNAs (Tables 1 and 2), the expression levels of six IncRNAs and seven mRNAs completely matched those of the high-throughput sequencing data; these were MSTRG.10941, MSTRG.2005, MSTRG.83990, MSTRG.34610, MSTRG.93731, MSTRG.72169, complement factor B (A2AP22103), egg coat matrix protein (A2AP9945), serine/threonine-protein kinase Nek4 isoform X5 (A2AP24609), G2/mitotic-specific cyclin-B-like (A2AP03163), fatty acid-binding protein 2 (A2AP18992), topoisomerase II-associated protein PAT1 (A2AP03092) and twin BRCT domain (A2AP10472). The expression levels of three other IncRNAs and one mRNA partially matched those of high-throughput sequencing data (MSTRG.79659, MSTRG.39061, MSTRG.26009, and heat shock protein 70). The expression levels of one IncRNA and two mRNAs did not achieve significant levels. Validation of key DE-IncRNA and DE-mRNA transcripts by RT-qPCR yielded results consistent with the RNA-seq analysis in most cases, thus confirming the reliability of the RNA-seq technique and the high quality of the identified IncRNAs and mRNAs.

3.3. Gene ontology (GO) and pathway analysis of DE-mRNAs and DE- IncRNAs

GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted to explore the function of the DE-IncRNAs and DE-mRNAs (Fig. 3 and Table 3). From the perspective of DE-mRNAs in the HT, LO and HL groups, the most significantly enriched GO terms in biological processes category were the cell cycle process, glucosamine-containing compound biosynthetic process and the mitotic cell cycle; the most significantly enriched GO terms in cellular components category based on DE-mRNAs were sarcoplasmic reticulum lumen, extracellular region part and extracellular region; finally, the most significantly enriched GO terms in molecular functions category based on DE-mRNAs were metalloendopeptidase activity, monosaccharide binding and peptidase regulator activity.

Based on the results of significant DE-IncRNA analysis in the HT, LO and HL groups, the most significantly enriched GO terms in biological processes category were the regulation of Fc receptor mediated stimulatory signaling pathway, ribosomal large subunit biogenesis and microtubule-based process; the most significantly enriched GO terms in cellular components category based on DE-IncRNAs were ribosome, prerinosome and intracellular non-membrane-bounded organelle. The most significantly enriched GO terms in molecular functions category based on DE-mRNAs were metalloendopeptidase activity, monosaccharide binding and peptidase regulator activity.

Regarding KEGG pathways, based on the DE-mRNAs, olfactory transduction was the most significant pathway in the HT group, and thyroid hormone synthesis was the most significant pathway in LO and HL groups. Based on the DE-IncRNAs, ribosome was the most significant pathway in the HT and HL groups, and ribosome biosynthesis in eukaryotes was the most significant pathway in the LO group (Table 3).

3.4. Integrated analysis of mRNAs and IncRNAs

3.4.1. IncRNA target gene prediction and IncRNA-mRNA co-expression network construction

In the present study, the cis- and trans-targets of the DE-IncRNAs were predicted to address the function of IncRNAs in
concert with their target genes transcripts (mRNAs) to adapt to adverse environments (Table S4). Among the trans-target gene predictions in the three groups, the number of positive correlations between the lncRNA-mRNA pairs was higher than the number of negative correlations. DE-lncRNAs and their corresponding interactions between the lncRNA-mRNA pairs was higher than the number of DE pairs found in the HT, LO and HL groups was 87, 1326 and 2430, respectively (Table S5). The greatest number of DE pairs in the HL group might be a result of the highest number of DEGs identified in the HL group. These results also indicated that the combined stress (HL) caused more extensive responses at the molecular level in sea cucumbers than the single stresses alone (HT and LO). Moreover, eight co-identified DE lncRNA-mRNA pairs were found in all three treatments (Fig. 4d). Based on the connectivity degree, the most important lncRNAs in the interaction networks in the HT, LO and HL groups were
The top 10 significantly up- and down-regulated mRNAs in sea cucumber under the three treatment groups.

| Gene ID | Gene name | Pfam name | Pfam description | Fold change | P-value |
|---------|------------|-----------|------------------|-------------|---------|
| HT Up   | AJAP20068  | Unknown   |                 |             |         |
|         | AJAP9379   | RNA-directed DNA polymerase from mobile element jockey-like |                 |             |         |
|         | AJAP27125  | Characterized protein LOC5393642 isomorf X2 | CAP | Cysteine-rich secretory protein family | 146.4026 | 4.83E-05 |
|         | AJAP6685   | Unknown   |                 |             |         |
|         | AJAP27351  | Heat shock 2-O-sulfotransferase 1-like | DUF221 | Domain of unknown function (DUF221) | 123.6578 | 0.000229 |
|         | AJAP10341  | Puteative characterised transposon-derived protein F5293.6 | RVT_1 | Reverse transcriptase (RNA-dependent DNA polymerase) | 114.1713 | 0.000249 |
|         | AJAP22103  | Complement factor B | HlyIII | Haemolysin-III related | 97.26164 | 2.02E-17 |
|         | AJAP10857  | Galactosylceramide sulfotransferase isomorf X1 | Gal-3-O-sulfotransferase | Galactose-3-O-sulfotransferase | 89.54334 | 0.00011 |
|         | AJAP20812  | Uncharacterized protein LOC105442795 | 7tm_7 | 7tm Chemosensory receptor | 78.97268 | 1.61E-07 |
|         | AJAP13651  | Heat shock protein 70 | HSP70 | Hsp70 protein | 60.91213 | 0.07137 |
|         | AJAP181992 | Fatty acid-binding protein 2 | Lipocalin_7 | Lipocalin/cytosolic fatty-acid binding protein family | 0.006061 | 1.93E-22 |
|         | AJAP06654  | Fibrillin-1 | NIDO | Nudogen-like | 0.002527 | 0.000208 |
|         | AJAP18275  | Orthodenticle | TF_Box | Ox1 transcription factor | 0.003162 | 9.33E-09 |
|         | AJAP05915  | RING finger protein 207 | zf_B_box | B-box zinc finger | 0.003856 | 1.01E-06 |
|         | AJAP23548  | Unknown | L6_membrane | L6 membrane protein | 0.004067 | 2.12E-07 |
|         | AJAP25354  | Egg matrix protein | F5_F8_type_C | F5/8 type C domain | 0.004226 | 2.03E-06 |
|         | AJAP03163  | G2/mitotic-specific cyclin-B-like | Cyclin_N | Cyclin, N-terminal domain | 0.004395 | 2.06E-05 |
|         | AJAP24669  | Serine/threonine-protein kinase Nek4 isomorf X5 | Nek4 kinase | Nek4 protein kinase domain | 0.004731 | 2.01E-06 |
|         | AJAP28660  | Unknown | Cythadesin_P30 | Cythadesin P30/P32 | 0.004934 | 1.51E-06 |
|         | AJAP18483  | Hypothetical protein NEMVEDRAFT_v1g77984 | DFU946 | Plant protein of unknown function (DFU946) | 0.00715 | 3.48E-06 |
|         | AJAP27125  | Uncharacterized protein LOC5393642 isomorf X2 | CAP | Cysteine-rich secretory protein family | 297.2703 | 5.08E-08 |
|         | AJAP16209  | Fatty acid-binding protein 2 | Lipocalin_7 | Lipocalin/cytosolic fatty-acid binding protein family | 173.9777 | 1.14E-05 |
|         | AJAP20068  | Unknown | Corona_S2 | Coronavirus S2 glycoprotein | 132.2862 | 0.00052 |
|         | AJAP18483  | Hypothetical protein NEMVEDRAFT_v1g77984 | DFU946 | Plant protein of unknown function (DFU946) | 352.4156 | 1.45E-08 |
|         | AJAP27125  | Uncharacterized protein LOC5393642 isomorf X2 | CAP | Cysteine-rich secretory protein family | 297.2703 | 5.08E-08 |
|         | AJAP16209  | Fatty acid-binding protein 2 | Lipocalin_7 | Lipocalin/cytosolic fatty-acid binding protein family | 173.9777 | 1.14E-05 |
|         | AJAP20068  | Unknown | Corona_S2 | Coronavirus S2 glycoprotein | 132.2862 | 0.00052 |
|         | AJAP05319  | Unknown | Methyltransfer_15 | RNA cap guanine-N2 methyltransferase | 131.738 | 1.22E-06 |
|         | AJAP23198  | Uncharacterized protein LOC105439756 | THAP | THAP domain | 106.7227 | 6.50E-08 |
|         | AJAP24721  | Glutamate receptor 1 | Lig_chan | Ligand-gated ion channel | 97.99706 | 6.50E-05 |
|         | AJAP22103  | Complement factor B | HlyIII | Haemolysin-III related | 87.21897 | 5.02E-19 |
|         | AJAP01630  | 5-Hydroxysteroid oxidase-1 | 7tm_1 | 7 transmembrane receptor (rhodopsin family) | 85.54646 | 0.000793 |
|         | AJAP03092  | Unknown | Pkinase | Protein tyrosine kinase | 0.001134 | 2.17E-16 |
|         | AJAP25354  | Egg matrix protein | F5_F8_type_C | F5/8 type C domain | 0.001258 | 4.74E-31 |
|         | AJAP12569  | Uncharacterized protein LOC100377183 | CUB | CUB domain | 0.0015 | 7.12E-05 |
|         | AJAP20119  | Transf10b | Death | Death domain | 0.002005 | 4.04E-13 |
|         | AJAP13651  | G2/mitotic-specific cyclin-B-like | Cyclin_N | Cyclin, N-terminal domain | 0.005044 | 7.17E-10 |
|         | AJAP27549  | Uncharacterized protein LOC105442568 | HYR | HYR domain | 0.005891 | 4.36E-06 |
|         | AJAP10472  | Unknown | PTCB-BRCT | twin BRCT domain | 0.005997 | 1.83E-11 |
|         | AJAP18483  | Hypothetical protein NEMVEDRAFT_v1g77984 | DFU946 | Plant protein of unknown function (DFU946) | 0.006168 | 1.60E-04 |
|         | AJAP15987  | RNA-directed DNA polymerase from mobile element jockey | RVT_1 | Reverse transcriptase (RNA-dependent DNA polymerase) | 0.007862 | 1.43E-05 |

**MSTRG.27265, MSTRG.19729 and MSTRG.95524, respectively. The most important mRNAs in the HT, LO and HL groups were interferon-induced very large GTPase 1 (AJAP10355), 5'-AMP-activated protein kinase subunit gamma-1 (AJAP18467), and scavenger receptor cysteine-rich domain superfamily protein (AJAP11160), respectively.**

### 3.4.2. Validation of lncRNA-miRNA-mRNA networks

In the present study, a dual-luciferase reporter assay system (DLRAS) was used for validation, and the results are shown in Fig. 5. The group interactions without practical meanings or significance were not shown in the figure. Our previous study found several environmental-stress-responsive miRNAs that include Aja-miRNA-
Fig. 2. Validation of high throughput sequencing results using RT-qPCR.
2013-3p (Huo et al., 2017), and its predicted binding structure and mature sequence were shown in Fig. 6c. We analyzed the possible targeted mRNAs and lncRNAs, and found that hypoxia-inducible factor 1α (HIF-1α) was a potential target gene, which is a key responder in hypoxia defense. Moreover, binding sites for Aja-miRNA-2013-3p were also found in four lncRNAs (MSTRG.93731, MSTRG.10941, MSTRG.34610 and MSTRG.81281) (Fig. 6b). Details of information of DLRAS, such as group content, sequences and primary data can be found in Table S2.

Compared with that in the positive control (PC)-miRNA-NC group, the PC-miRNA was significantly downregulated. Thus, the transfection system used in the current study proved to be effective. Compared with the mmi2 group, the mmi4 group showed a significant decrease of over 20% in expression. Thus, Aja-miRNA-2013-3p was proven to suppress the expression of HIF-1α. Compared with the lmi2 group, the lmi4 and lmi16 groups showed significant decreases of over 20% in expression. Moreover, the lmi12 group showed significantly decreased expression levels compared with the expression levels in the lmi2 group, but the difference was only approximately 17%. Thus, Aja-miRNA-2013-3p was proven to bind lncRNAs such as MSTRG.93731, MSTRG.10941 and MSTRG.34610. Under hypoxic stress, the HIF-1α gene and MSTRG.10941 showed increased expression levels and could both bind Aja-miR-2013-3p; under thermal stress and combined stress, the HIF-1α gene and MSTRG.34610 showed decreased expression levels and could both bind Aja-miR-2013-3p. Therefore, the "HIF-1α gene/Aja-miR-2013-3p/MSTRG.10941" network might play important roles in sea cucumbers under environmental stress (Fig. 6). The relative expression levels of these lncRNAs, miRNAs and mRNAs were compared for the three treatment groups and the NC group, and the results are shown in Fig. S4 (unpublished data).

4. Discussion

Long noncoding RNAs (lncRNAs) could act as competing endogenous RNAs (ceRNAs) that compete with mRNAs for binding to miRNAs by sharing at least one miRNA response element (MRE), and thus affecting gene expression (Salmena et al., 2011). Emerging evidence indicates that lncRNAs play crucial roles in many biological processes and contribute to stress resistance. However, the regulatory mechanisms remain unclear, especially in sea cucumber. To date, many lncRNAs have been identified in model animals including human (Wapinski and Chang, 2011), rat (Wang et al., 2014), zebrafish (Pauli et al., 2012), arabidopsis (Liu et al., 2012), fruit fly (Nyberg and Machado, 2016), and elegans (Nam and Bartel, 2012); they have also been identified in other vertebrates, such as pig (Wang et al., 2016) and chicken (Li et al., 2012); and other marine animals, such as oyster (Feng et al., 2018) and salmon (Valenzuela-Miranda and Gallardo-Escárate, 2016). However, information concerning the systematic identification and function of lncRNAs in marine invertebrates remains lacking, especially for echinoderms. A. japonicus is a good model holothurian species, because of its economic and ecological value. LncRNAs of A. japonicus have fewer exons and shorter transcripts in length compared with mRNAs, and these results are in agreement with those of previous studies examining pig (Wang et al., 2016), rat (Wang et al., 2014) and oyster (Feng et al., 2018). With the absence of lncRNA data for other holothurians, the conservation of lncRNAs is difficult to properly evaluate in sea cucumbers. Delightfully, recent advances in sea cucumber genome sequencing have resulted in the identification of novel lncRNAs in A. japonicus and the exploration of novel regulatory pathways in different situations (Zhang et al., 2017).

In the current study, based on sequencing data, we investigated transcriptomic changes and systematically identified the lncRNAs associated with the responses to environmental stress, specially, thermal, hypoxic and combined stresses. Although the functions of most lncRNAs in A. japonicus are not yet clear, evidence suggests that lncRNAs can regulate protein-coding gene expression in organisms through cis-acting mechanisms or trans-acting mechanisms (Han et al., 2012). Thus, we constructed a co-expression network joint by lncRNAs and their target mRNAs and explored potential lncRNA-miRNA-mRNA networks, which may play crucial roles in A. japonicus exposed to adverse environmental stress. As a key modulator of most oxygen-limitation...
responses, HIF-1α has received extensive attention in hypoxia-related studies. Aja-miRNA-2013-3p was reported to be important in sea cucumbers coping with hypoxic stress (Huo et al., 2017). The luciferase assay results showed that Aja-miRNA-2013-3p was able to bind to the HIF-1α gene, and so were lncRNAs, such as MSTRG.93731, MSTRG.10941 and MSTRG.34610. For example, under hypoxic conditions, Aja-miRNA-2013-3p was downregulated, but the HIF-1α gene and MSTRG.10941 were upregulated under hypoxic stress (Huo et al., 2017). This might be explained by a competent relationship between the HIF-1α gene and MSTRG.10941 binding to Aja-miRNA-2013-3p. However, no reported function was found for the novel lncRNAs mentioned above. Future studies of the regulatory mechanisms of coding and non-coding genes are needed.

Thermal stress and hypoxic stress have been shown to impact the immune response of organisms (Baze et al., 2011; Jin et al., 2011). In vertebrates, emerging evidence suggests that lncRNAs are expressed in a highly lineage-specific manner and play an important role in immune response. The immune system responds to both thermal and hypoxic stressors in a way that helps the organism cope with these environmental challenges. The immune response involves a complex network of interactions between different immune cells and molecules, and lncRNAs can modulate these responses by regulating the expression of genes involved in immune cell function. Understanding the role of lncRNAs in immune response is crucial for developing strategies to mitigate the impacts of thermal and hypoxic stress on ecosystems.

### Table 3

| KEGG pathways analysis of differentially expressed mRNAs and lncRNAs in sea cucumbers under environmental stresses. |
|----------------------------------------------------------|
| **HT** | **HL** | **LO** |
| Cell adhesion molecules (CAMs) | Staphylococcus aureus infection | Taste transduction |
| Amyotrophic lateral sclerosis (ALS) | Amino sugar and nucleotide sugar metabolism | |
| Drug metabolism - other enzymes | Bile secretion | |
| Gastric acid secretion | Fat digestion and absorption | |
| Mineral absorption | Offactory transduction | |
| Progesterone-mediated oocyte maturation | Renin-angiotensin system | p53 signaling pathway |
| | | Prion signaling pathway |
| | | Salivary secretion |

| Jak-STAT signaling pathway |
|---------------------------|
| Protein processing in endoplasmic reticulum | Legionellosis |
| Prion diseases | Insulin signaling pathway |
| Influenza A | Fructose and mannose metabolism |
| Vitamin digestion and absorption | Estrogen signaling pathway |
| Caffeine metabolism | Hepatitis B |
| Progesterone-mediated oocyte maturation | Adipocytokine signaling pathway |
| | Glycolysis / Gluconeogenesis |
| | Staphylococcus aureus infection |
| | Cocaine addiction |

| Prostate cancer |
|----------------|
| Chronically myeloid leukemia |
| Endometrial cancer |
| Measles |
| Neurotrophin signaling pathway |
| Peroxisome |

| Base excision repair |
|---------------------|
| Adipocytokine signaling pathway | Acute myeloid leukemia |
| amoebiasis | Alcoholism |
| cell cycle | Apoptosis |
| Fanconi anemia pathway | Caffeine metabolism |
| Fructose and mannose metabolism | Glycine, serine and threonine metabolism |
| Herpes simplex infection | Hippo signaling pathway |
| Insulin signaling pathway | Influenza A |
| MAPK signaling pathway | Mucin type O-glycan biosynthesis |
| Oocyte meiosis | Propanoate metabolism |
| Pathways in cancer | Protein export |
| PI3K-Akt signaling pathway | Protein processing in endoplasmic reticulum |
| Renal cell carcinoma | Pyruvate metabolism |
| Small cell lung cancer | Ribosome biogenesis in eukaryotes |
| Tight junction | Thyroid cancer |
| Toxoplasmosis | Tuberculosis |
| Transcriptional misregulation in cancer | Valine, leucine and isoleucine biosynthesis |
| | Valine, leucine and isoleucine degradation |
Fig. 4. LncRNA-mRNA interactive network for comparison of (a) HT vs. NC; (b) LO vs. NC; (c) HL vs. NC and (d) 8 co-identified DE lncRNA-mRNA pairs. Up-regulated mRNAs are displayed as red squares, down-regulated mRNAs are displayed as blue squares. Up-regulated lncRNAs are displayed as red triangles, and down-regulated lncRNAs are displayed as blue triangles.
immune regulation (Chen et al., 2017). The complement system is an essential immune response in invertebrates (Mastellos and Lambris, 2002). In the present study, thermal stress and hypoxic stress lead to the up-regulation of complement factor B, indicating mediation of the alternative pathway. In addition, the complement component C3 was also found to be induced in sea cucumbers under environmental stress in our previous study (Huo et al., 2019b). Heat shock proteins (HSPs) are widely viewed as a bioindicator of thermal stress in A. japonicus (Huo et al., 2019b). Heat shock proteins (HSPs) can stimulate the immune system (Yang et al., 2010a) and were reported to be related to apoptosis (Roberts et al., 2010) and protein degradation (Nelson et al., 1992). The expression of HSPs was more sensitive to thermal stress than to hypoxic stress, possibly because it is easier to induce protein denaturation during thermal stress than during hypoxic stress.

Thermal and hypoxic stress alters the body's energy metabolism because of trade-off effects. In the current study, many energy metabolism-related pathways, such as “amino sugar and nucleotide sugar metabolism”, “fructose and mannose metabolism” and “fat digestion and absorption” were significantly changed under stress conditions (Table 3). Fatty acid-binding protein 2 (FABP2) is a lipid-binding protein that mediates fatty acid absorption, transport and intracellular metabolism (Lowe et al., 1987; Nakanishi et al., 2004). Additionally, it is an important regulator of insulin resistance (Sipilainen et al., 1997). Its significantly decreased expression under environmental stress (Fig. 2) also indicated the depression of fatty acid metabolism and may also indicate a potential changes of energy supply.

The cell cycle pathway was significantly impacted as indicated by KEGG analysis based on DE-mRNAs in the HT group and DE-IncRNAs in the HL group (Fig. 3 and Table 3). Moreover, the cell cycle process was the most significantly enriched GO term of the biological processes category, as indicated by the DE-mRNAs in the HT, LO and HL groups. In the current study, topoisomerase II-associated protein (PAT1), a gene that accurately facilitates chromosome separation during cell division (Alzan et al., 2016), showed significantly decreased expression under the three different environmental stresses relative its expression under control conditions (Fig. 2). In addition, thermal stress and the combined stress treatment significantly changed serine/threonine-protein kinase Nek4 isoform X5 (Nek4) expression, which potentially affects threonine residues, and is required for normal cell cycle arrest in response to double-stranded DNA damage (Nguyen et al., 2012). All the evidence showed that the cell cycle process was extensively changed when sea cucumbers were exposed to environmental stress.

![Fig. 5. Dual-luciferase assay reporter system results of mRNA-miRNA (mmi) and IncRNA and miRNA (lmi). (mmi2: 3’ UTR-NC + miRNA; mmi4: 3’ UTR + miRNA; lmi2: 3’ UTR-NC + miRNA; lmi4; MSTRG10941.1(Aja-miR-2013-3p) + miRNA; lmi6: MSTRG10941.1(Aja-miR-2013-3p)-mut + miRNA; lmi8: MSTRG81281.1(Aja-miR-2013-3p) + miRNA; lmi12: MSTRG93731.1(Aja-miR-2013-3p) + miRNA; lmi16: MSTRG34610.1(Aja-miR-2013-3p) + miRNA; PC-miRNA NC: 3’ UTR positive control + miRNA-NC; PC-miRNA: 3’ UTR positive control + miRNA positive control; * p < 0.05, ** p < 0.01, *** p < 0.001).](image-url)
5. Conclusion

Although IncRNAs appear increasingly important in molecular mechanism studies, an understanding of IncRNAs in the echinoderm phylum remains lacking at present. To our knowledge, the present study represents the first comprehensive analysis of the IncRNA-mRNA co-expression networks and the validation of IncRNA-miRNA-mRNA networks in the response of marine organisms towards individual and combined influences of heat and hypoxia. Although this study was still limited by the absence of a IncRNA database for marine organisms, the identification and annotation of the putative IncRNAs provide a basis for the further clarification of the mechanisms underlying the regulation of mRNAs expression by IncRNAs in the environmental stress response of invertebrates. In future research, overexpression, CRISPR-Cas9 and RNA interference gene silencing strategies in sea cucumbers are expected to be useful for elucidating the specific roles of IncRNAs.

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List of abbreviations

| Acronym | Definition |
|---------|------------|
| HT      | high temperature |
| LO      | low dissolved oxygen |
| HL      | high temperature and low dissolved oxygen |
| NC      | (normal) control |
| HIF-1α  | hypoxia-inducible factor |
| GO      | gene ontology |
| KEGG    | Kyoto Encyclopedia of Genes and Genomes |
| IncRNAs | long non-coding RNAs |
| mRNA    | messenger RNA |
| CPC     | Coding Potential Calculator |
| CNCI    | Coding-Non-Coding-Index |
| CPAT    | Coding Potential Assessment Tool |
| DE      | differentially expressed |
| DEGs    | differentially expressed genes |
| DLRAS   | dual-luciferase reporter assay system |
| PC      | positive control |
| MRE     | miRNA response element |
| ceRNAs  | competing endogenous RNAs |
| HSPs    | heat shock proteins |
| FABP2   | fatty acid-binding protein 2 |
| Nek4    | serine/threonine-protein kinase Nek4 isoform X5 |
| RIN     | RNA integrity number |

Authors’ contributions

Da Huo designed the study, carried out the experiment, analyzed data and drafted the paper.
Lina Sun revised the draft and offered the foundation.
Kenneth B. Storey contributed to examination of data and revision of the manuscript draft.
Libin Zhang was responsible for the source of sea cucumber and offered the foundation.
Shilin Liu contributed to the source of sea cucumber and sample preparation.
Jingchun Sun helped with sequencing work and data curation.
Hongsheng Yang designed the study and supervised the project.
All authors contributed to manuscript revision, and read and approved the submitted version.
Declaration of competing interest

The authors declare no conflict of interest.

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Ethics approval and consent to participate

The sea cucumber (A. japonicus) is not an endangered or a protected species and no permission was needed for sea cucumber collection.

Availability of data and materials

The data generated during the current study were submitted to the GEO database [GSE131676].

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