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The global effects of *PmRunt* co-located and co-expressed with a lincRNA *lncRunt* in pearl oyster *Pinctada fucata martensii*

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ABSTRACT

Runt related transcription factors as trans-acting elements play critical roles in the developmental control of cell fate, hematopoiesis, bone formation and cancers. In previous study, the homologue of runt related transcription factor *PmRunt* has been identified from pearl oyster *Pinctada fucata martensii* and considered to play an important role in nacre formation. In this study, we used the same samples to perform RNA-seq to detect the global effects after the decrease of *PmRunt* expression. The transcription levels of several nacre shell matrix protein (NSMP) genes were significantly changed and the potential compensatory effect could happen internal gene families. Downregulation of *PmRunt* could also influence the biosynthesis of NSMPs through affecting amino acid metabolism, translation, protein processing and export. The inhibition of *PmRunt* also possibly affected the expression of caspases, IAPs and C1qs that related to apoptosis and immune. In addition, *PmRunt* highly expressed at 12 h and 12 d after transplantation in hemolymph, which was corresponded to transplantation immunity immune response and the morphology of pearl sac, suggested the cross-talk of biomineralization-immune regulation in hemocytes. Furthermore, a lincRNA (*LncRunt*) that co-located with *PmRunt* was identified and showed a significantly relative expression with *PmRunt*, which suggested the potential regulation. Therefore, these findings provided new idea to find the regulation targets of runt-related transcription factors and offers evidence of lncRNAs in potential biomineralization-immune regulation.

1. Introduction

Biomineralization is a widely ubiquitous biological phenomenon range from unicellular diatom to higher mammals [1]. The diverse products consist with the silica in sponge, the calcium carbonate in coral and shellfish, and the calcium phosphate in vertebrates. Molluscan exoskeleton (shell) plays multiple important roles including structural support, protection from predators and stressors, and physiological homeostasis [2]. Shell formation is a tightly regulated biological process that allows mollusks to build their shells even in environments unfavorable for mineral precipitation [3,4]. The organic framework formed by macromolecular matrix were considered to control the extracellular crystallization process, as the elaborately and coordinated regulation through transcription regulation. Trans-acting elements such as transcription factor and cofactor combine with cis-acting elements including promoter, enhancer and inhibitor, as the ultimately effected switch controlling the complex gene expression regulation network. In vertebrate, transcription factors responding to upstream signaling molecules to regulate cell differentiation of osteoclast/osteoclast and control secretion of organic matrix, directly responsible for the biomineralization processes [5]. In invertebrate, the regulation system of biomineralization, especially in mollusk of which even several transcription factors, such as smad, AP-1 and Sp8/9, had been found to be involved in shell formation, is still unclearly [6–10].

Runt related transcription factors play critical roles in the developmental control of cell fate, and contribute variously as oncoproteins and tumor suppressors to leukemia and other cancers. Vertebrates have three Runx genes, for Runx1, Runx2, and Runx3, that are essential for the development of specific tissues. For example, Runx1 plays critical roles in hematopoiesis [11]. During skeletal development, Runx1 play a possible role in mediating early events of endochondral and intramembranous bone formation, while Runx2 is a potent inducer of late stages of chondrocyte and osteoblast differentiation [12]. In previous study, we had found that the homologue of RUNX1 named PmRunt played critical roles in nacre formation via the expression inhibition mediated by RNA interference, but the regulation mechanism is not

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clear [13]. In addition, runt related transcription factor in scallops played essential roles in regulating hemocyte production in LPS stimulation and hematopoiesis during ontogenesis [14,15]. However, the hemocytes were also reported to participate in the biomineralization processes of shell repair and $CaCO_3$ crystal generation [16]. However, whether biomineralization and immunity regulation correlated was not clear.

Non-coding RNAs accounts for nearly 90% of genome. Long non-coding RNA (lncRNA) is commonly defined as ncRNAs that are at least 200 nucleotides (nt) in length but have little or no protein-coding capacity [17]. They have been as a new class of regulatory transcripts in many biological processes, including transcriptional regulation, post-transcriptional control and epigenetic processes. Some studies evidenced that LncRNAs could participate in development, biomineralization and antiviral immune response through cis- or trans-regulation in mollusk species [18–21]. In this study, we identified a lncRNA (named *lncRunt*) that co-located with *PmRunt* and detected its co-expression with *PmRunt*.

2. Method and materials

2.1. Experimental animals and RNA samples for RNA-seq

The experimental animals were obtained from the coastal area of Liushawan, Zhanjiang (109°57′E, 20°25′N). The pearl oysters used in the experiment had a 5–6 cm shell length and were cultured in circulating seawater for 2 d at 25–27 °C before the RNA interference. Total RNA was extracted by Trizol reagent (Invitrogen, USA). The RNA samples used to performed the RNA-seq were the same as in the reference experiment, two samples were the pearl oysters injected ds-RFP RNA and ds-PmRunt RNA, respectively [13]. And every RNA sample for RNA-seq was a mixture RNA of two individuals. Nine time points of the nucleus grafting operation experiment were 6 h, 12 h, 1d, 2d, 3d, 6d, 12d, 18d and 30d after the operation, with 0 d representing the blank group. At each time point, the hemolymph was collected from at least three pearl oysters as replicates.

2.2. Library construction and illumina sequencing

Library construction and RNA-seq were performed at BGI (Shenzhen, China). RNA degradation and contamination were monitored on 1% agarose gels. RNA quality was checked using NanoDrop ND-1000 microvolume UV/VIS spectrophotometer. A total amount of 200 ng RNA per sample was used as input material for RNA sample preparations. The method of library conduction refer to the publish paper Wang et al. [22]. The raw data were submitted to NCBI, the SRA accession number is PRJNA542487.

2.3. Transcriptome analysis

After removing the adaptor sequences and low-quality sequence reads, raw sequences were transformed into clean reads which were then mapping to the reference genome (*P. f. martensii* genome) [23]. Only reads with a perfect match or one mismatch were further analyzed and annotated based on the reference genome. Cufflinks was then used to splice the mapped reads and then the reads were compared with the annotation of the reference genome to identify new transcripts. The generated new unigenes were used for a BLAST search and annotation against the RefSeq non-redundant proteins (NR), Swiss-Prot, Gene Ontology (Go), co-expressed genes(COG), Clusters of orthologous groups for eukaryotic complete genomes (KOG), Pfam, and Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The edgeR software was used to identify the digital gene expression (DGE) technology with critical thresholds (fold > 2, FDR < 0.001). All differentially expressed genes were mapped to terms in GO and KEGG database, and then we searched for significantly ($P < 0.05$) enriched GO and KEGG terms in DGEs compared with the all transcriptome.

2.4. Sequence analysis of lncRunt

The *P. f. martensii* genome and the same species Pinctada fucata genome Ver 2.00 (http://marinegenomics.oist.jp/) was searched by the BlastN program using full-length *PmRunt* cDNA to obtain the *PmRunt* genomic sequence [13,23]. The *PmRunt* genomic sequences were aligned to the *P. f. martensii* unigenes/transcripts of a transcriptome assembled via high-throughput sequencing by our laboratory (Unpublished) by the local BlastN program. After screening out the lower-identity unigenes, a unigene that was completely consistent with the upstream sequences (~4 kb) of *PmRunt* without coding ability was obtained as a lincRNA.

2.5. Confirmation using RT-PCR

To validate the activated effects of PmRunt, we added the RO5-3335, one of the effective inhibitors of RUNX1 in mammal, when we cultivated the mantle pallial in vitro. 24 h later, all the samples were collected. 6 differentially genes from down/up regulated genes were selected for RT-PCR analysis for the samples. We also analysed the expression pattern of PmRunt at different development stages and in different times after pearl nucleus grafting operation to understand the PmRunt function. The primer sequences used for the qRT-PCR assay are shown in Table S1. The RT-PCR assay was performed on Applied Biosystems 7500/7500 Fast Real-Time System (Applied Biosystems, Foster City, CA, USA) with Thermo Scientific DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific). The cDNA synthesis and qPCR detection of *lncRunt* used the lnRcute lncRNA cDNA synthesis kit and lnRcute SYBR Green qPCR Kit (TIANGEN Biotech, Beijing). The expression of those genes was calculated by the $2^{-\Delta Ct}$ method or $2^{-\Delta\Delta Ct}$, with actin as the reference gene.

3. Results

3.1. Assembly assessment

In this study, the same RNA samples pretreated in previous study were used to perform the RNA-seq analysis. PmRunt in the experiment group with the dsRNA of PmRunt (dsPmRunt) and the negative control (N.C.) with RFP (red fluorescent protein), which had been expounded in our previously published paper [13]. The genome mapping ratio of dsPmRunt and N.C. group is 80.43% and 78.95%, respectively, and the number of identified genes is 21035 and 21462, respectively (Table S2). And the histogram distribution of genes on expression level showed in Fig. S1.

3.2. KEGG and GO enrichment analyses of differential expression genes after RNAi

The edgeR software was used to identify the DGEs with critical thresholds (fold > 2, FDR < 0.001). The results suggested that 1282 genes significantly lowly expressed in dsPmRunt, while 738 genes were highly expressed, compared with that in N.C. (Fig. S2). KEGG metabolic pathway analysis indicated that differentially expressed genes were involved in 39 signaling pathways ($P < 0.05$), which included Amino acid metabolism, protein synthesis and export et al. Among these pathways, Tyrosine metabolism and protein synthesis (including translation, folding, sorting and degradation) were apparently enriched ($Q < 0.05$) (Table S3, Fig. S3). We also used the Gene Ontology Term to perform the function enrichment. The mostly enrichment of aminoacyl-tRNA ligase activity, tRNA aminoacylation and translation (Corrected P-value < 0.05) were in accordance with the KEGG results (Table S4).

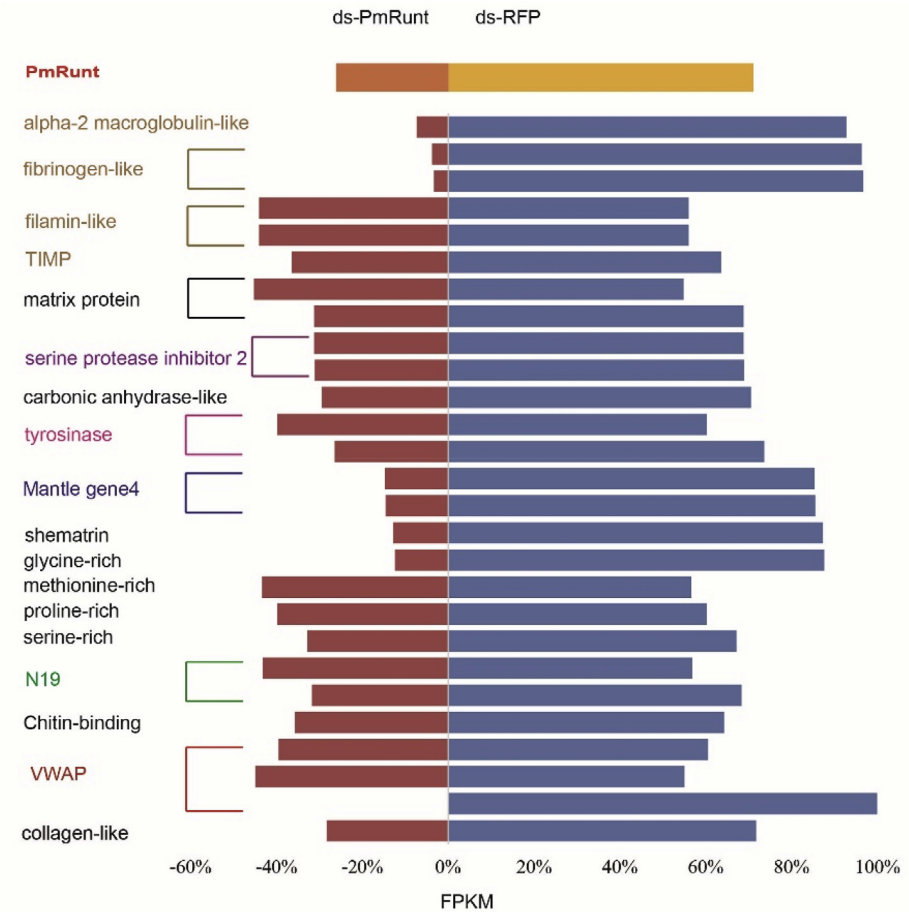

Fig. 1. The known nacre matrix proteins that the expression level significantly decreased after the inhibition of *PmRunt* by RNAi. Left side showed the expression of these genes in ds-PmRunt sample and right side in ds-RFP sample.

3.3. Different expressed nacre-matrix proteins after RNAi

In previous study, we had identified 234 of nacre matrix proteins from the decalcified nacreous layer of *P. f. martensii* with its genome database [23]. RNA-seq analysis exhibited that the transcript of *PmRunt* in dsRunt group was significantly declined to N.C. group, which was in union to the result of RT-PCR [13] (Fig. 1). As the decrease of PmRunt mediated by RNAi apparently affected the formation of nacreous layer in the shell of pearl oyster, the different transcripts of nacre-matrix proteins were filtered from the different expressed genes between dsRunt group and N.C. group. A total of 82 NSMP transcripts were significantly down-regulated in dsPmRunt group, along with 66 were significantly up-regulated (FDR < 0.05). We found that the decreased NSMPs including who contained von Willebrand factor, type A domain (VWAP), Chitin binding domain (CBD), as well as alpha-2-macroglobulin-like protein, tyrosinase (TYR), Complement C1q protein (C1qDC), Laminin, alpha Carbonic anhydrase, Proteinase inhibitor I2 and glycoside hydrolase for chitin, etc. (Fig. 1). In addition, some taxon-specific NSMPs such as N19, Gly-rich, Asp-rich, Ser-rich, Pro-rich and Met-rich protein were obviously downregulated (Fig. 1). These amino acid rich proteins could be the potential regulated genes of PmRunt in nacre formation. While, we then analyzed the interplay of down/up-regulated genes and found that several NSMP exhibited a complementary trend internal gene family memebers, such as VWAPs, C1qDCs, TYRs, CBDs and even in the taxon-specific shematrin families (Fig. 2). Therefore, we proposed that the compensatory effect in NSMP gene families happened when the dynamic balance was broken in the process of nacre formation. In previous study, the mentioned NSMP gene families above expanded in *P. f. martensii* genome, and these proteins also played critical roles in nacre formation such as TYRs and VWAPs. The phenomenon of compensatory effect maybe explain the evolutionary force of their expansions.

3.4. Down-regulation of PmRunt accompanied by reduce of protein biosynthesis

In this study, tyrosine metabolism was the mostly enriched pathway, associating with quinonization and cross-link action catalyticed by tyrosinases. While, the protein synthesis related pathways were relatively abundant, such as protein processing in endoplasmic reticulum, protein export and Aminoacyl-tRNA biosynthesis. Several of -tRNA synthetases transcripts significantly reduced in dsPmRunt, such as Aspartyl, Lysyl, Tyrosyl, Glycyl -tRNA synthetases (Fig. 3). In accordance, the expression level of some Asp-rich, Lys and Gly-rich SMP genes decreased. A certain of tyrosinases that could catalyze the crosslink of tyr-rich proteins were also down-regulated. In addition, a series of genes related to protein processing in endoplasmic reticulum and transporters for protein exports were mostly down-regulated in dsPmRunt group (Fig. S4 and Fig. S5).

3.5. PmRunt related to inflammation and apoptosis

Inflammation is a process that protect cells from pathogen invading. While, apoptotic cell death is normal life process that occurs in growth, development, and maintenance of multicellular organisms. Here, we found that after RNAi of *PmRunt*, the inflammation related elements such as Complement and coagulation cascades and the related main components in apoptosis pathway such as caspase-like and IAP

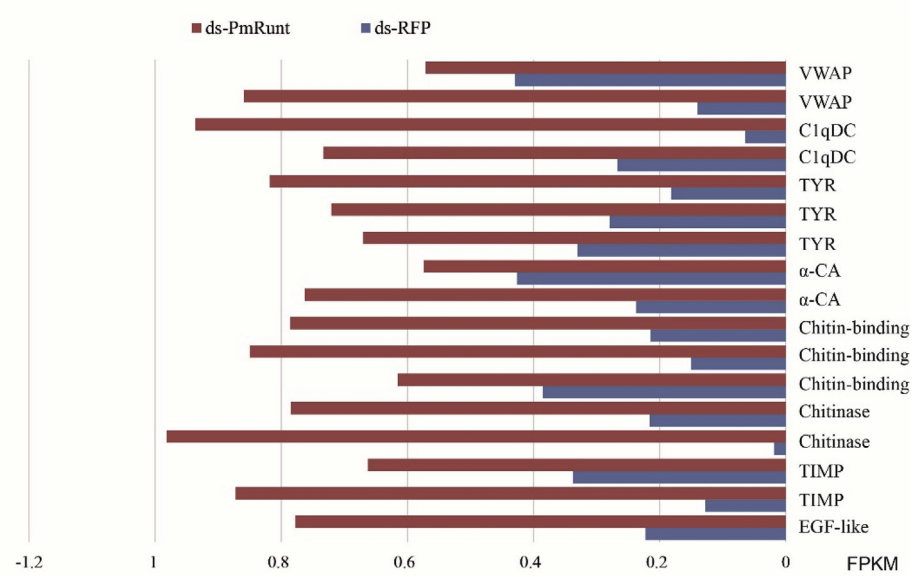

Fig. 2. Nacre matrix proteins that showed the compensatory expression after the inhibition of *PmRunt* by RNAi.

exhibited differential expressions (Fig. S6).

3.6. Inhibitor treatment validated the effects of PmRunt by RT-PCR

In previous study, we have proved that PmRunt and PmCBF could directly interact. To validate the activated effects of PmRunt, we used the RO5-3335, one of the effective inhibitors of it to treat the mantle pallial tissue fragment. Then we selected six genes including *PmRunt* and five affected genes to validate the effect by RT-PCR, as a result with the same expression pattern to RNA-seq (Fig. S7).

3.7. The expression pattern of PmRunt at different times after pearl nucleus grafting operation in hemocyte

As mentioned above, Runt could participate in hemocyte production, and the hemocyte cells were also related to biomineralization and repair of shell damage. To explore the relationship of immune cells to biomineralization, we detected the expression pattern of PmRunt at different development stage and at different times after nucleus grafting operation in hemolymph. The results showed that PmRunt highly expressed at 12 h and 12 d after nucleus grafting operation in hemolymph (Fig. 4).

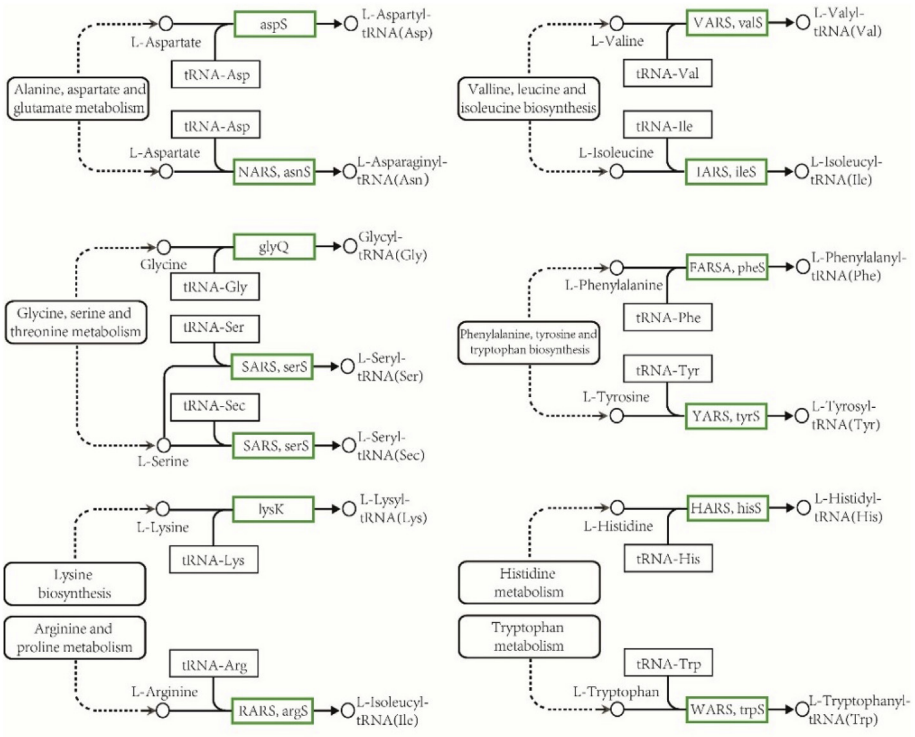

Fig. 3. Different expression genes between ds-PmRunt and ds-RFP group involved in protein processing in aminoacyl-tRNA biosynthesis. The green color represented the downregulation at ds-PmRunt group. aspS: aspartyl-tRNA synthetase; NARS, asnS: asparaginyl-tRNA synthetase; glyQ: glycyl-tRNA synthetase alpha chain; SARS, serS: seryl-tRNA synthetase; lysK: lysyl-tRNA synthetase, class I; RARS, argS: arginyl-tRNA synthetase; VARS, valS: valyl-tRNA synthetase; IARS, ileS: isoleucyl-tRNA synthetase; FARSA, pheS: phenylalanyl-tRNA synthetase alpha chain; YARS, tyrS: tyrosyl-tRNA synthetase; HARS, hisS: histidyl-tRNA synthetase; WARS, trpS: tryptophanyl-tRNA synthetase.. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

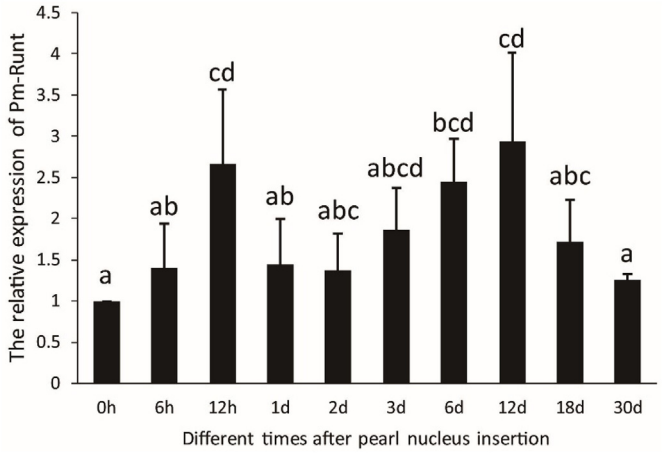

Fig. 4. *PmRunt* expression in the hemolymph after the nucleus grafting operation. The expression levels were determined by qPCR. The expression relative to the internal control is expressed as the mean ± S.D. Significant differences from the control are indicated with different letters ($P < 0.05$).

3.8. Sequence analysis of lncRunt and co-expression detection of PmRunt and LncRunt

By aligning the *PmRunt* genomic sequence to unigenes/transcripts, a lincRNA located at the upstream of *PmRunt* genomic sequence nearly 4 kb was obtained (designated as *lncRunt*), consists of 549 bp with two exons (Fig. 5a), corresponded to scaffold166:543727–545578 with our previously sequenced genome by Du et al. [23], and was verified by the Pinctada fucata genome Ver 2.00 (http://marinegenomics.oist.jp/) (corresponded to scaffold7.1:133220–134891). And the further detection of co-expression trend of *PmRunt* and *LncRunt* exhibited a significant correlation in mantle tissues with sixteen individuals (Fig. 5b). We also tested expression level of *LncRunt* at different times after nucleus grafting operation in hemolymph and found that *LncRunt* showed a highly expression at 12 h (Fig. S8).

4. Discussion

Transcription factors as the trans-regulation elements could multiply regulate down-stream gene expressions via interacting with transcriptional control area or elements. In previous study, we successfully inhibited *PmRunt* gene expression by RNA interference mediated by dsRNA. As a result, the nacre formation become disordered, but the regulating mechanism was not clear [13]. Mostly strategies to detect the functions of transcription factor dependent on the interaction of transcription factor and DNA fragments, means point to point laboriously. Here, we performed the RNA-seq using the same sample of RNA interference in previous study. It could help to overall the complex regulating effects of *PmRunt* in nacre formation.

As mentioned above, the matrix proteins in nacre were considered to directly control the extracellular biomineralization processes [24]. After the inhibition of *PmRunt*, those genes who exhibited the decreased expression were identified. The well-known VWA domain containing proteins, Chitin binding domain proteins, tyrosinase, mantle gene 4, alpha Carbonic anhydrase and glycoside hydrolase for chitin and some taxon-specific NSMPs were affected by *PmRunt*. Hydrophobic Chitin are proposed to provide matrix structure and chitin binding domain proteins has the potential ability to bind to chitin framework and connected with other shell matrix proteins [25]. Glycoside hydrolase for chitin could contribute to the formation and process of chitin framework [23]. Highly content tyrosinases in shell matrix proteins had been considered in pearl oyster, oyster and mussel [26]. And it was proposed as structure proteins and play important roles in protein crosslink. Meanwhile, alpha Carbonic anhydrase and taxon-specific acid proteins such as N19 could control the calcium carbonate crystal formation [27–29]. Previous study had shown that Runt related transcription factor in bivalve species could form the heteropolymer with CBF to improve the interaction with DNA sequence, similar to mammals. To further verify the global effect of *PmRunt* inference in pearl oyster, we treated mantle tissue in vivo using the RO5-3335 that suppressed the transactivation activity of RUNX1/CBF via binding to both subunits of RUNX1 and CBF [30]. The expression level of *PmRunt* significantly decreased and the chosen effect genes also exhibited the same trend to RNA-seq. Therefore, the effects of disordered growth of nacre formation induced by the decrease of *PmRunt* could be integrated effects.

By the genome sequencing and multi-omic studies of pearl oyster, several matrix proteins in shells exhibited as expanded gene families, such as VWAPs, tyrosinases, C1q containing proteins and fibronectin-like proteins [23]. And most of them exist the mantle tissue highly expression clusters. While, how did the same gene family members inter-coordinate was unconsidered. It is well known that exogenous dsRNA mediated RNA interference was not stable and permanent. In this study, we found that after the interference of *PmRunt* expression, several SMP family genes showed a compensatory trend. These findings

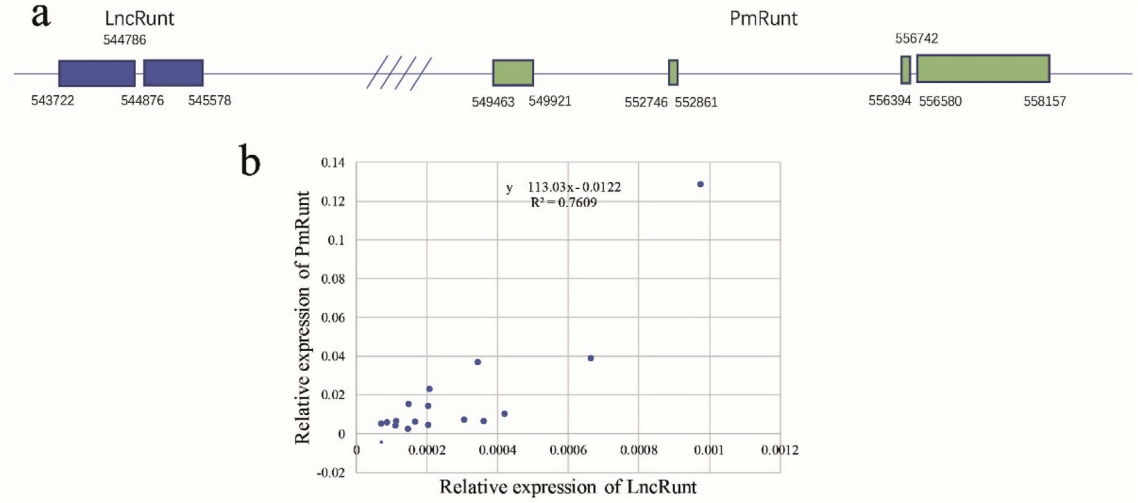

Fig. 5. Genome location and co-expression of *PmRunt* and *LncRunt*. a. *LncRunt* and *PmRunt* located at scaffold166. Blue color represented the exons of *LncRunt*, green color represented the exons of *PmRunt*, and the diagonal represented the gaps. b. the co-expression analysis of *LncRunt* and *PmRunt* at mantle tissues (N = 16). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

indicated that the SMP genes in the multigene family could adjust family members' expression profiles to provide to remedy the unbalanced expression after interference, therefore recovery the normal physiology condition.

In addition, we focus on the significantly enriched pathways and functions between the control group and interference group. The protein synthesis related pathways were relatively abundant, such as protein processing in endoplasmic reticulum, protein export and Aminoacyl-tRNA biosynthesis, which indicated that *PmRunt* regulated bio-calcified processes linking to a series of processes associated to organic matrix secretion. Asp-rich proteins participate in nucleation in nacre tablet formation [31,32]. Lys and Gly-rich proteins always as the components of hydrophobic organic framework offer the support and space the environment of bio-calcification [33–35]. Tyr-rich proteins such as always serve as the substrate of tyrosinase and cross-link to form the matrix framework and could be responsible for chitin binding [23,36]. The decrease of these tRNA synthetases indicated that the biosynthesis of these NSMPs could be affected. In addition, a series of elements related to protein processing in endoplasmic reticulum and transporters for protein exports were mostly down-regulated in dsPmRunt group. That is to say, it could lead to the change of NSMP for the lack of material and the weakness of protein processing and exports in mantle pallial, even the transcripts of these NSMPs were significantly down-regulated or not in dsPmRunt group. However, there was no enough evidences to support that the transcription factor could control the expression of tRNA synthetases. That means, these reductions of tRNA synthetases in dsPmRunt group could be the indirectly effect, but obviously.

As mentioned above, Runt related transcription factors also played essential roles in regulating hemocyte production in LPS stimulation and hematopoiesis during ontogenesis in scallops [15]. Recently, the hemocytes were considered to participate in biomineralization processes including directly involved in shell crystal production, the mineral transport and the formation of the ECMs [16,37]. Since the pearl oyster *P. f. martensii* could form pearl sac to stably secrete nacre after the transplantation and hemocytes also been found to participate in the formation of pearl sac via organizational observation [38,39]. In this study, we detected the expression profile of *PmRunt* in hemocytes after transplantation and found that *PmRunt* highly expressed at 12 h and 12d after transplantation, which was corresponded to transplantation immunity immune response and the morphology of pearl sac, respectively. These findings suggested that hemocytes could be related to nacre formation in pearl sac and PmRunt could be possibly involved in biomineralization-immune regulation in hemocytes. Therefore, deeply studies need to be performed.

Furthermore, we found that the main components of apoptosis signal pathway, including caspase 6/7/8 and IAP exhibited differential expressions after RNAi of PmRunt. In hepatocellular carcinoma cells, the expression of runt-related transcription factor 3 (RUNX3) could induce the increase of apoptotic activity by Bim expression and caspase-3 and caspase-9 activation and Loss of RUNX3 expression leads hepatocellular carcinoma cells to escape apoptosis [40]. This finding hinted that PmRunt may play similar role to vertebrate RUNX3 and control apoptosis processes.

LncRNAs participate in diverse biological processes through multiple regulation mechanism [41]. For example, in mammal, ANCR regulated osteoblast differentiation by targeting EZH2 and regulating Runx2 expression [42]. Cis role is lncRNA acting on neighboring target genes. In Pacific oyster, the potential function of lncRNAs in oyster mantle by analyzing their cis-acting protein-coding gene targets that related to shell and its color formation were identified [20]. Here, we identified a lincRNA (*LncRunt*) that co-located at the uptream of *PmRunt*. According to the co-expression analysis, we found that *LncRunt* and *PmRunt* showed the positive relationship in normal mantle tissue, which indicated a certain regulation relationship, maybe cis-, between *LncRunt* and *PmRunt*. While this relationship needs to be furtherly determined and deeply study in the future.

5. Conclusion

In this study, RNA-seq analyses was used to detect the global effects after the decrease of *PmRunt* expression. The transcription levels of several NSMP genes were significantly changed and the potential compensatory effect could happen from the same family. Downregulation of *PmRunt* could also influence the biosynthesis of NSMPs through affecting amino acid metabolism, translation, protein processing and export. Meanwhile, *PmRunt* maybe affect the apoptosis in mantle. *PmRunt* highly expressed at 12 h and 12d after transplantation in hemolymph, which was corresponded to transplantation immunity immune response and the morphology of pearl sac, suggested the cross-talk of biomineralization-immune regulation in hemocytes. Furthermore, a lincRNA that co-located with *PmRunt* was identified and showed a significantly relative expression with *PmRunt*, which suggested the potential regulation. Therefore, these findings provided new idea to find the regulation targets in transcription factor research of runt-related transcription factors and offers evidence of lncRNAs in potential biomineralization-immune regulation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.037.

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