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*Short Communication*

# Identification of Small Non-coding RNAs Responsive to $ZnSO_4$ Stress in Gonad of Sea Urchin (*Strongylocentrotus nudus*)

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## Abstract

In this research, a large number of putative siRNAs and piRNAs were identified in male gonad of sea urchin and subjected to microarray assay to compare their expressional profile between normal and 0.1mM Zins treated gonad. The results showed 24 up- and 57 down-expressed siRNAs and piRNAs in stressed gonad. Subsequently, functional annotation showed 5 down-expressed ncRNAs (3 siRNAs and 2 piRNAs) have 2 types of retrotransposons, 2 types of simple repeat motifs and a few mRNAs and ESTs as their putative targets. More so, the majority putative siRNA and piRNAs that differently expressed under Zinc stress, was neither mapped to selfish elements nor transcribed from repeat sequences, which were similar to their mammal counterparties. Results revealed the biological significance of small non-coding RNAs in responding to heavy metal stress. © 2014 Friends Science Publishers

**Keywords:** Small ncRNAs; Solexa sequencing; Microarray assay; Zinc stress; Sea urchin

## Introduction

Metal-working industries are developed with a surprising progress, as the problem of heavy metal pollution on sea environment has become increasingly attractive. There are growing studies focus on the heavy metal pollutants in the marine environment and on the accumulation in tissues of various organisms (Zehra *et al.*, 2003). Exposure For decades, sea urchins have been used to sensor seawater pollutant and to elucidate the mechanism of heavy metal toxicity (Wei *et al.*, 2011), because urchin is a convenient system since many aspects of metal homeostasis and stress resistance are conserved between urchins and humans.

It has been documented that environmental pollutants have been shown to interfere with reproductive function in animals. Currently, there is great interest in characterizing the functions of the different classes of ncRNAs and their relevance to heavy metal treatments,since small non-coding RNAs were uncovered as key regulators controlling various stress responses (Yao *et al.*, 2010). Although sea urchin is widely used for evaluating biological effects of contaminants in marine environment, there are only few studies investigating the effects of chemicals on gonad of sea urchins.

Recently, we carried out a deep sequencing applied to sea urchin *Strongylocentrotus nudus* in a small RNA library isolated from male and female gonad, and found 36 miRNAs significantly varied between control and $ZnSO_4$ stressed samples (Wei *et al.*, 2011), providing the information about the roles of small non-coding RNAs in resistance to abiotic stress. However, the potential functions of other classes of ncRNAs such as siRNAs and piRNAs in sea urchin on responding to heavy metal stress is absent yet, although the siRNAs have been verified to participate in stress adaptation (Simmons *et al.*, 2009). Whether the siRNAs and piRNAs participated in cellular response to heavy metal in sea urchin gonad remain concerning.

## Materials and Methods

### Sample Collection and ZnSO4 Treatment

Sea urchin (*Strongylocentrotus nudus*) with about 5 cm in diameters was collected in the coast of Dalian, liaoning province, China. The animals were acclimated in laboratory with filters natural seawater for a week prior to RNA extraction prior to treatment with $ZnSO_4$ at 0.1 mM for 12 h.

### RNA Isolation, Solexa Sequencing and Microarray Assay

Total RNA of *S. nudus* was extracted from male gonad using Trizol reagent (Invitrogen, CA, USA), and approximately 20 μg of total RNA was used for solexa sequencing (Wei *et al.*,2011). After masking of adaptor sequences and removal of contaminated reads, the clean reads were filtered for siRNA and piRNA identification with ACGT101-miR-v3.5 package (LC Sciences, Houston, USA). Total 202 siRNAs and 153 piRNAs were selected to design probes (in triplicate) and subject to expressional profile analysis between normal and $ZnSO_4$ treated male gonad using μParaflo$^{(TM)}$ small ncRNA microarray (LC Sciences, Houston, USA) assay, and data analyzed (Gao *et al.*, 2004).

### Target Prediction and Annotation

The siRNA and piRNA sequences were used as query to search *S. purpuratus* mRNA and repeat sequences to perform targets prediction using BLASTn with -E as 1*e*-0.0001

(Wang *et al.*, 2011). The targets were then subjected to BLASTx and KEGG searching.

## Results

### Identification of siRNA and piRNA Using Solexa Sequencing

Sequencing of small RNAs generated 4,966,572 reads, then the reads matched to rRNA, tRNA, sno/snRNA, mRNA and miRNAs were discarded. From remaining 11,433,100 reads, total putative 4968 siRNAs were identified according to the criteria that these sequences do not derived from hairpin structures, and are not related to other ncRNAs deposited in Rfam and NT databases. Of these siRNAs, 2,584 sequences complementary to *S. purpuratus* mRNA, ESTs and repeat elements were recognized as endo-siRNAs. Majority (62%) of the small RNAs were 16 to 26 nt in size, appearing typical feature for Dicer-derived products. The length distribution and 5' end bias is illustrated in Fig. 1C. The average copy number is 184, while total copy number is 1,274,728.

Furthermore, two groups of putative piRNA like RNAs were identified based on their base composition and genome mapping information. The first group comprised 12,201 sequences with "U" at the first position of 5'end (termed as 5U RNAs), while the second group comprised 851 sequences with "A" at the position 10 from 5'end and with 10 bp overlapped to the sequences in the first group (termed as 10A RNAs), similar to that observed by Aravin *et al* (2007).

### Microarray Analysis of siRNAs and piRNAs in Normal and Zinc-stressed Male Gonad

Results of microarray assay showed drastically decreasing of expressions of siRNA and piRNA under Zinc stress, resulting in losing signals of 77 small RNAs including 70 piRNAs and 7 siRNAs under stress. Out of 81 ncRNAs varied significantly between control and Zinc stressed male gonad, 57 of them were down-regulated, up to 4.09 times that of control (Fig. 1A). Interestingly, among the top 20 abundant small RNAs with signals from 24380 to 16500 (control), 14 ncRNAs showed significant increasing of signal under Zinc stress, and siRNA92 give the maximum increasing from 20090 to 89362. In contrast, among 56 small RNAs with array signal from 12000 to 3000, 45 showed obvious decreasing of signals (Fig. 1A), implying that small ncRNAs appeared different expressional profiles.

### Functional Annotation of Putative Targets

Recent findings show that siRNA could target either repeat elements or mRNAs, while piRNAs have shown a role in the silencing of DNA transposons and retrotransposons in several animal models (Aravin *et al.*, 2007), these allow us to search their complementary RNAs. According to the methods described by Wang *et al.* (2011), BLASTn was used to find potential targets of putative siRNAs and piRNAs. The results revealed 3 siRNAs and 2 piRNAs highly complementary to sequences of 2 types of simple repeat

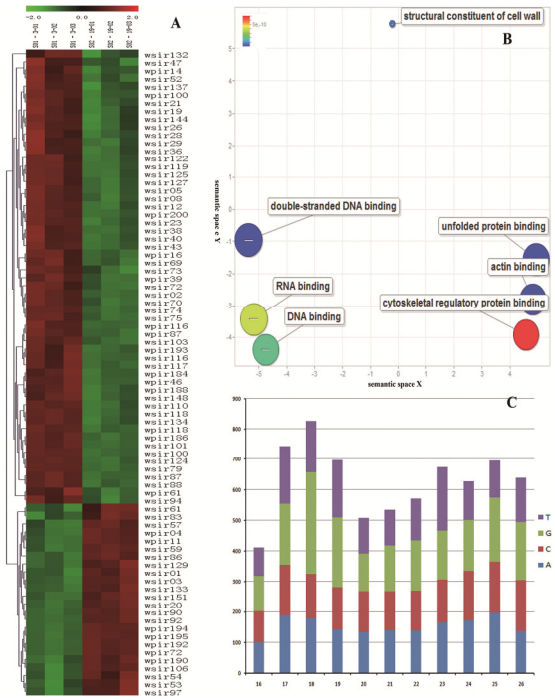


**Fig. 1:** Expressional profile, length distribution result and GO similarity of targeted ESTs of siRNAs

A:microarray assay results; B:Two-dimensional plots GO terms of targeted ESTs of siRNAs, the pairwise semantic similarity of GO terms are represented by discs, it denoted that that more semantically similar GO terms are also closer in the plot, size of bobble indicates the frequency of the GO term in the underlying GOA database; C:siRNA length distribution

**Table 1:** siRNAs complement to sea urchin mRNAs

| ncRNA ID | Expressional variation | Complemented sequence | Complemented regions | Functional annotation |
|---|---|---|---|---|
| wsir21 | -1.15 | M20117 | 61-85 | CHK1 |
| wsir28 | -1.09 | M20117 | 186-210 | CHK1 |
| wsir124 | -2.26 | M20117 | 83-106 | CHK1 |
| wsir23 | -1.98 | M20118 | 270-292 | Similar to DAPPUDDRAFT_67127 |
| wsir52 | -1.65 | M20117 | 63-83 | CHK1 |
| wsir40 | -1.13 | M20118 | 30-47 | Similar to DAPPUDDRAFT_67127 |
| wsir83 | 1.63 | AY465426 | 633-650 | PLCδ |

motifs and two types of transposons including SINE2-1_SP and SPRP1. These 5 ncRNAs were down-regulated under Zn stress, suggesting an attenuated silencing for these TEs.

Moreover, BLASTn against sea urchin mRNA database showed 2 mRNA sequences were complemented by these them, phospholipase C delta (PLCδ) was targeted by siRNA83, which up expressed in Zinc stressed gonad. PLCδ was functional mapped onto KEGG 00562, 04020 and 04070, indicating a key role in IP3-dependent $Ca^{2+}$ signaling system. Inhibition of PLC has been proved as one of toxic effects of $Hg^{2+}$ and $Cu^{2+}$ on *Mytilus galloprovincialis* Lam (Panfoli *et al.*, 2000). In other instance, Checkpoints kinase 1 (CHK1) was intensively targeted by 4 siRNAs that all down expressed under stress conditions (Table 1), indicating a decreased expressional controlling of chk1. Taking into account the CHK1 is one of the key proteins responding to

stress, this result maybe uncover a novel relationship between Zinc stress and small ncRNAs regulations.

Furthermore, these siRNAs were BLASTn against EST database of sea urchin, which showed 272 ESTs to be putative targets of siRNAs. Then these ESTs were assembled and functionally annotated. Of these 13 ESTs were mapped to GO database with cutoff lower than $1e^{-10}$. In the context of semantic similarity of GO terms (Fig. 1B), the functions of targeted ESTs fell into two clusters, one comprised protein binding (GO:0051082, GO:0003779 and GO:0005519), another included nucleoid acid binding activities (GO:0003690, GO:0003677 and GO:0003723). These data provided new idea on how can small non-coding RNAs participate in responding to heavy metal stress in sea urchin.

## Discussion

Previous research has evidenced that sea urchin evolved a conserved pathway contributing to biogenesis of small non-coding RNAs such like miRNAs, siRNAs and piRNAs (Wei *et al.*, 2012). In addition to discovering of miRNAs and piRNAs (Wei *et al.*, 2011; Wei *et al.*, 2012), this research identified 2584 siRNAs in the male gonad of sea urchin, thereafter conduce to elucidate the biological roles of these small RNAs both in development and stress resistance.

Zinc treatment remarkably changed the expressional profiles of tested siRNAs and piRNAs, indicating a putative roles of these RNAs in responding to stress, resulting in either up-expression or down expression for each ncRNA. Furthermore, BLASTn searching revealed 2 types of transposons as putative targets of 5 down regulated ncRNAs, suggesting that these repeat sequences would be more activity under Zinc stress. Typically, increasing transposons activity is one reason for gonadal dysgenesis (Kale *et al.*, 2005), therefore it is likely that attenuated silencing of TEs is served as a novel inducer for developmental abnormal of sea urchin under heavy metal stress (Kale *et al.*, 2005). In consist with this hypothesis, Farkash *et al.* (2006) found that abiotic stress increased endonuclease-dependent L1 retrotransposition. Therefore, this results indicated that small ncRNAs are newly emerged regulators concerning to heavy stress by means of controlling chromosome remodeling in sea urchin gonad similar to other animal model (Mizutani *et al.*, 2012).

Similar to previous research, the 5U piRNAs were rare complemented to TEs, well consisting with the notion that small RNAs with function to silence gene expression by multiple mechanisms (Yao *et al.*, 2010). In adult testis of mammals, the piRNAs have no known function, and in mouse testes, the promoter regions of particular retrotransposons are methylated through cooperating by piRNA and several proteins (Cowley and Oakey, 2013). Considering the observations that urchin piRNAs were less come from repeat-rich regions and the identification of cognate of DNMT3B in urchin genome, (Sp-Dnmt3A and SPU_024347), we suggested that the piRNA identified in this research are functionally similar to their mammal counterparties. On other hand, only 355 small ncRNAs were tested in this research, while thousands of small ncRNAs were remained for analysis in further research. Therefore, it is likely that the small ncRNAs identified in this research have more complex functions responding to Zn stress, such like methylating promoter regions of particular retrotransposons (Shpiz *et al.,* 2011).

In crux, out of 355 siRNAs and piRNAs, 81 siRNAs and piRNAs expressed significatively different under Zinc stress. Subsequently, bioinformatical analysis showed these 81 ncRNAs were targeted to 2 types of repeat sequences and to a few number of mRNAs and ESTs. Data revealed the biological significance of small ncRNAs on heavy metal stress in male gonad of sea urchin.

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