The Roles of Two miRNAs in Regulating the Immune Response of Sea Cucumber

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ABSTRACT MicroRNAs (miRNAs) have emerged as key regulators in many pathological processes by suppressing the transcriptional and post-transcriptional expression of target genes. MiR-2008 was previously found to be significantly up-regulated in diseased sea cucumber *Apostichopus japonicus* by high-through sequencing, whereas the reads of miR-137, a well-documented tumor repressor, displayed no significant change. In the present study, we found that miR-137 expression was slightly attenuated and miR-2008 was significantly enhanced after *Vibrio splendidus* infection or Lipopolysaccharides application. Further target screening and dual-luciferase reporter assay revealed that the two important miRNAs shared a common target gene of betaine-homocysteine S-methyltransferase (*AjBHMT*), which exhibited noncorrelated messenger RNA and protein expression patterns after bacterial challenge. In order to fully understand their regulatory mechanisms, we conducted the functional experiments *in vitro* and *in vivo*. The overexpression of miR-137 in sea cucumber or primary coelomocytes significantly decreased, whereas the inhibition of miR-137 increased the mRNA and protein expression levels of *AjBHMT*. In contrast, miR-2008 overexpression and inhibition showed no effect on *AjBHMT* mRNA levels, but the concentration of *AjBHMT* protein displayed significant changes both *in vitro* and *in vivo*. Consistently, the homocysteine (Hcy) contents were also accordingly altered in the aberrant expression analysis of both miRNAs, consistent with the results of the *AjBHMT* silencing assay *in vitro* and *in vivo*. More importantly, small interfering RNA mediated *AjBHMT* knockdown and Hcy exposure analyses both significantly increased reactive oxygen species (ROS) production and decreased the number of surviving invasive pathogen in sea cucumber coelomocytes. Taken together, these findings confirmed the differential roles of sea cucumber miR-137 and miR-2008 in regulating the common target *AjBHMT* to promote ROS production and the clearance of pathogenic microorganisms through Hcy accumulation.

KEYWORDS *Apostichopus japonicus*; miR-137; miR-2008; betaine-homocysteine S-methyltransferase; homocysteine; ROS, genetics of immunity

MICRORNAS (miRNAs) are a class of endogenous small noncoding RNAs of ~22 nucleotides, which typically function as repressors of target genes, resulting in direct messenger RNA (mRNA) degradation or translation inhibition (Bartel 2004). These RNAs play crucial roles in many cellular processes, such as proliferation, differentiation, apoptosis, and survival (Lai 2002; He and Hannon 2004; Miska 2005). The expression of up to 60% of all protein-coding

genes is estimated to be under the control of miRNAs (Friedman 2009), and combinatorial regulation has been identified as a prominent feature of miRNA regulation. Thus, a given miRNA might have multiple different mRNA targets, and a given mRNA might similarly be targeted by multiple miRNAs (Krek *et al.* 2005; Rajewsky 2006). Consistent with the crucial roles of miRNA in the normal functioning of eukaryotic cells, the aberrant expression of miRNAs has also been observed in various diseases (Calin and Croce 2006; Wu *et al.* 2007). Notably, miRNAs have also emerged as pivotal regulators of the host immune response and key players in host-pathogen interactions (Scaria *et al.* 2006; Asgari 2011).

Sea cucumber (*Apostichopus japonicus*) belongs to the phylum Echinodermata, class Holothuroidea. As a representative economic species of Echinoderms, the aquaculture of

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Figure 1 Relative expression of miR-137 and miR-2008 *in vitro* and *in vivo*. (A) Relative miR-137 expression in *V. splendidus*-challenged *A. japonicus*. (B) Relative miR-137 expression in LPS-treated coelomocytes. (C) Relative miR-2008 expression in *V. splendidus*-challenged *A. japonicus*. (D) Relative miR-2008 expression in LPS-treated coelomocytes.

A. japonicus has become an important sector of the marine industry and has grown rapidly over recent years. The rapid expansion and intensification of farming have also led to a series of problems, including the occurrence of skin ulceration syndrome (SUS), considered as one of the limiting factors in the sustainable development of this industry (Deng *et al.* 2009). Many reports have demonstrated that the pathogens responsible for the outbreak of SUS include spherical virus (Wang *et al.* 2005), *Vibrio splendidus*, and *Pseudomonas* spp. (Ma *et al.* 2006; Zhang *et al.* 2006). Although extensive research concerning the identification of SUS-related pathogens has been conducted, the understanding of the etiology and pathogenesis of this disease remain unclear. In a previous study, we showed abnormal expression level of miR-2008 in diseased *A. japonicus* by high-throughput sequencing, whereas the reads of miR-137, a well-documented tumor repressor, displayed no change (Li *et al.* 2012). In human, miR-137 has been extensively investigated and shown to play important roles in various cancers, cell cycle signaling, and embryonic stem cell development. Studies have demonstrated that miR-137 targets cell division control protein 42 (Cdc42), a Rho GTPase family protein up-regulated in many

human cancer types, such as colorectal, lung, and breast cancers (Arias-Romero and Chernoff 2013). Through the inhibition of the Cdc42/PAK signaling pathway, miR-137 decreases the proliferation, invasion, and G0/G1 cell cycle progression of colorectal cancer cells (Liu *et al.* 2011; Zhu *et al.* 2013). In addition, miR-137 also targets mind bomb-1 (Mib1), a ubiquitin ligase involved in regulating neurogenesis and neurodevelopment (Smrt *et al.* 2010). Another notable target of miR-137 is microphthalmia-associated transcription factor (MITF), a master regulator of melanocyte development and function, frequently misregulated in human melanomas (Bemis *et al.* 2008). Recent studies also show that miR-137 is involved in the doxorubicin resistance of neuroblastoma cells by targeting the constitutive androstane receptor (CAR) (Takwi *et al.* 2013; Chen *et al.* 2014). Moreover, the epigenetic silencing of miR-137 has been associated with the hypermethylation of the miR-137 promoter (Balaguer *et al.* 2010; Vrba *et al.* 2013); however, compared with miR-137, the function of miR-2008 has been rarely studied.

Although the function of several miRNAs in vertebrates and model organisms has been elucidated, scarce miRNA

Figure 2 *AjBHMT* mRNA and protein expression levels in *V. splendidus*- or LPS-challenged samples. (A) *AjBHMT* mRNA transcripts in *V. splendidus*-challenged *A. japonicus*. (B) *AjBHMT* mRNA transcripts in LPS-treated coelomocytes. (C) *AjBHMT* protein expression in *V. splendidus*-challenged *A. japonicus* by iTRAQ analysis.

targets have been identified and characterized in nonmodel marine organisms, such as sea cucumber. In the present study, *V. splendidus*, a well-documented pathogen in sea cucumber SUS, was used to explore the potential roles of miR-137 and miR-2008 in host–pathogen reactions *in vitro* and *in vivo*. The decreased expression profiles of miR-137 were detected both in *V. splendidus*-challenged sea cucumber or LPS-exposed coelomocytes, which slightly differed from the results from diseased samples. More importantly, we observed that both miR-137 and miR-2008 targeted the 3'-UTR of betaine homocysteine S-methyltransferase (*AjBHMT*) and modulated the transcription and translation of this gene, respectively. Moreover, *AjBHMT* affects Hcy accumulation in coelomocytes and induces respiratory bursts to kill invasive microorganisms in nonmodel invertebrate pathogen infections.

Materials and Methods

Ethics statement

Sea cucumbers (*A. japonicus*) and BALB/c mice are commercially cultured animals. All experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocol was approved by the Experimental Animal Ethics Committee of Ningbo University, China.

Target prediction of miRNAs through RNA-seq

RNA-seq and miRNA target prediction were based on the results of a previous study (Zhang *et al.* 2013). In brief, the potential targets for miR-137 and miR-2008 were predicted using the miRanda v3.01 toolbox based on the complementary region between miRNAs and mRNAs, as well as the thermodynamic stability of the miRNA–mRNA duplex. All of the mRNAs used for target prediction came from the differentially expressed unigenes obtained above, in which the candidate targets with scores less than the threshold parameter of $S > 90$ (single-residue pair scores) and a minimum free energy lower than -17 kcal/mol were selected as potential targets.

Experimental animals, bacterial strains, and cell culture

A total of 120 healthy adult sea cucumbers (*A. japonicus*) of $\sim 60 \pm 10$ g were obtained from the Bowang Aquaculture

Company (Ningbo, China), randomly assigned to six tanks, and acclimatized in aerated natural seawater [28 practical salinity units at $16^\circ \pm 1^\circ$] for 3 days before conducting experiments. *V. splendidus* was initially isolated from the skin ulcerations of diseased sea cucumbers and cultured in liquid 2216E broth (5 g·liter⁻¹ tryptone, 1 g·liter⁻¹ yeast extract, pH 7.6) at 28° , with shaking at 140 rpm. HEK-293T cells were purchased from ATCC and maintained in DMEM supplemented with 10% FCS at 37° and 5% CO₂.

Full-length cDNA clone and analysis of *AjBHMT* from *A. japonicus*

Total RNA was extracted using RNAiso plus reagent (Takara), and reverse transcription was performed with 1 μ g of RNA using the Primescript First cDNA Synthesis Kit II (Takara). Gene-specific primers (Supporting Information, Table S1) were designed based on the sequences obtained from a previous study (Zhang *et al.* 2013), and the PCR products were cloned into pMD18-T simple vector (Takara) and bidirectionally sequenced using M13-47 and RV-M primers (Sangon). Subsequently, the cDNA sequence of *AjBHMT* was analyzed using the BLAST algorithm at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast>), and the deduced amino acid sequence was analyzed using the Expert Protein Analysis System (<http://www.expasy.org/>). The percentage of similarity and identity of the full-length amino acid sequences between each gene and its counterparts in other organisms was determined using the Identity and Similarity Analysis program (<http://www.biosoft.net/sms/index.html>). Multiple alignments of each protein were performed using the ClustalW Multiple Alignment program (<http://www.ebi.ac.uk/clustalw2/>) and the Multiple Align Show program (<http://www.biosoft.net/sms/index.html>). Domains in the *AjBHMT* amino acid sequence were detected using the simple modular architecture research tool (SMART) program (<http://www.smart.embl-heidelberg.de/>).

3'-UTR luciferase reporter assay

The 3'-UTR of *AjBHMT* mRNA, containing the putative target site of miR-137 and miR-2008, was cloned into the pMIR-REPORT luciferase plasmid (Promega) between the *Mlu*I and *Hind*III restriction sites and subsequently subjected to DNA

Figure 3 Identification and characterization of the miR-137 and miR-2008 binding sites in the 3'-UTR of *AjBHMT* and cooperative functional effect of miR-137 and miR-2008 on *AjBHMT*. (A) Schematic representation of the two putative miR-137 and miR-2008 targeting sites in *AjBHMT* mRNA and the respective mutant sites. (B) HEK-293T cells were cotransfected with the pMIR-REPORT-BHMT-WT vector, carrying the wild-type BHMT 3'-UTR, pRL-CMV-Renilla-luciferase, and control or miR-137 or miR-2008 mimics (final concentration: 100 nM) as indicated. After 24 hr, firefly luciferase activity was measured, and the values were normalized to Renilla luciferase activity. (C) HEK-293T cells were cotransfected with the pMIR-REPORT-BHMT-MT vector, carrying a mutation in the BHMT 3'-UTR, pRL-CMV-Renilla-luciferase, and control or miR-137 or miR-2008 mimics (final concentration: 100 nM) as indicated. (D) HEK-293T cells were transfected with different concentrations of miR-137 and miR-2008, or cotransfected with mixed miR-137 and miR-2008 mimics as indicated.

sequencing for confirmation. The primers used to clone the *AjBHMT* 3'-UTR are shown in Table S1. The complete 3'-UTR of wild-type *AjBHMT* was amplified by gene-specific primers with restricted endonuclease sites of *Mlu*I and *Hind*III. Puta-

tive miRNA binding sites were mutated using a PCR approach and served as the mutant type. All purified fragments were digested by double enzymes and ligated into the digested pMIR-REPORT vectors, respectively. These clones were

Figure 4 Functional validation of miR-137 and miR-2008 targeting *AjBHMT* mRNA *in vitro*. (A) Relative expression of miR-137 in primary cultured cells at 24 hr after miR-137 mimics transfection. (B) *AjBHMT* mRNA expression in primary cultured cells at 24 hr after transfection with miR-137 mimics. (C) Relative expression of miR-137 in primary cultured cells at 24 hr after miR-137 inhibitor transfection. (D) *AjBHMT* mRNA expression in primary cultured cells at 24 hr after miR-137 inhibitor transfection. (E) Relative expression of miR-2008 in primary cultured cells at 24 hr after miR-2008 mimics transfection. (F) *AjBHMT* mRNA expression in primary cultured cells at 24 hr after transfection with the miR-2008 mimics. (G) Relative expression of miR-2008 in primary cultured cells at 24 hr after miR-2008 inhibitor transfection. (H) *AjBHMT* mRNA expression in primary cultured cells at 24 hr after miR-2008 inhibitor transfection.

further confirmed by sequencing prior to the luciferase reporter assay. For the transfection experiment, HEK293-T cells were seeded into a 96-well white TC plate with a total volume of 100 μ l. Two solutions were prepared in each well as follows: the first solution contained 0.2 μ g of pMIR-REPORT containing either the wild-type or mutated *AjBHMT* 3'-UTR and 0.01 μ g of pRL-CMV constructs with 0.25 μ l of transfection reagent. The second solution comprised 100 nM miR-137 or miR-2008 mimics and 0.25 μ l of transfection reagent. Twenty-five microliters of each solution was mixed together and incubated at room temperature for 20 min. Subsequently the solutions were replaced by 50 μ l of medium in each well. At 48-hr post-transfection, the cells were collected for activity determination using the Dual-Luciferase Reporter Assay System (E1910, Promega). The efficiency of each miRNA transfection was confirmed by fluorescence microscope BX53 (Olympus). Luciferase activity was calculated based on the luciferase signal ratio between the two constructs, pMIR-REPORT and pRL-CMV, which could be used to normalize the protein content among different samples. All of the experiments were performed in six replicates.

To explore the effect of combined miR-137 and miR-2008 on *AjBHMT*, HEK-293T cells were cotransfected with the pMIR-REPORT-BHMT-WT vector, pRL-CMV-Renilla-luciferase and control or serial concentration of miR-137, miR-2008 mimics (50 nM, 100 nM, and 150 nM) or their combinations (50 nM miR-137 + 50 nM miR-2008, 50 nM miR-137 + 100 nM miR-

2008, and 100 nM miR-137 + 50 nM miR-2008). The experiments were conducted as mentioned above.

Pathogen microorganism challenge in sea cucumber

An overnight culture of *V. splendidus* was centrifuged at $5,000 \times g$ for 5 min to harvest the bacteria, and live *V. splendidus* was subsequently resuspended in filtered seawater (FSW). For the challenge experiments, three tanks were used as controls, and the other three tanks were immersed with a high density of *V. splendidus* at a final concentration of 10^7 cfu·ml⁻¹. Coelomic fluids were collected from the individuals in each tank and centrifuged at $800 \times g$ for 5 min to harvest the hemocytes at 0, 48, and 96 hr, and the cells were subsequently stored at -80° before RNA extraction.

Expression analysis of mir-137, mir-2008, and AjBHMT in vivo

Total RNA was extracted using RNAiso plus reagent (Takara) according to the manufacturer's instructions. The SYBR green qRT-PCR assay was used for miRNA and mRNA quantification in the same samples. Briefly, 500 ng of RNA-containing miRNAs was polyadenylated using poly(A) polymerase and converted to cDNA through reverse transcription using the miScript Reverse Transcription Kit (Qiagen). For miRNA analysis, qRT-PCR was performed using the miScript SYBR Green PCR Kit (Qiagen) and the provided miScript Universal primer and miRNA-specific forward primers (for mRNA, the

Figure 5 Western blot analysis of AjBHMT expression in *in vitro* assays. NCM, negative control of miRNA mimics; 137M, miR-137 mimics; 2008M, miR-2008 mimics; NCI, negative control for the miRNA inhibitor; 137I, miR-137 inhibitor; 2008I, miR-2008 inhibitor; siNC, negative control for the AjBHMT siRNA; and siAjBHMT, AjBHMT siRNA.

primers were gene-specific forward and reverse primers) on a Rotor-Gene Q 6000 Real-Time PCR cycycler (Qiagen).

The miRNA-specific primers were designed based on previously published miRNA sequences (Li *et al.* 2012), and the gene-specific primers were designed based on previously published mRNA sequences (Zhang *et al.* 2013) (Table S1). Each reaction was performed in a final volume of 20 μ l containing 2 μ l of the cDNA, 1 μ l of each 10- μ M primer, 6 μ l of RNase-free water, and 10 μ l of SYBR Green PCR Master mix (Qiagen). The amplification profile included initial denaturation at 94° for 15 min, followed by 40 cycles of 94° for 15 sec, 60° for 30 sec, and 70° for 30 sec, in which fluorescence was acquired. Immediately following amplification, a melting curve analysis was performed. Each sample was run in triplicate with three biological replications. The miRNA expression levels were normalized to RNU6B, and the expression levels of mRNAs were normalized to 18S ribosomal RNA.

The $2^{-\Delta\Delta C_t}$ method was used to analyze the expression levels of both miRNA and mRNA, and the value obtained represented the *n*-fold difference relative to the control (untreated samples). The data were presented as the relative expression levels (means $\pm$ SD, *n* = 9), and all experimental data were subjected to one-way analysis of variance (ANOVA), followed by Duncan's multiple range test to determine differences in the mean values among the controls. Significant differences between the treated and corresponding control groups at each time point were indicated as **P* < 0.05 and ***P* < 0.01. The error bars in the graphs represent standard deviations.

Primary coelomocyte culture

Sea cucumbers were sterilized in 7% benzalkonium bromide and 75% ethanol for ~2 min, respectively. Subsequently, the sea cucumbers were dissected using an aseptic surgery technique as previously described (Xing *et al.* 1998). The coelomic fluids were collected and mixed with an equal volume of anticoagulant solution (0.02 M EGTA, 0.48 M NaCl, 0.019 M KCl, and 0.068 M Tris-HCl, pH 7.6). The cell suspension was filtered through a 100- μ m nylon mesh to remove large tissue debris and centrifuged at 800 $\times$ *g* for 10 min at 18°. The cells were washed twice with isotonic buffer (0.001 M EGTA, 0.53 M NaCl, and 0.01 M Tris-HCl, pH 7.6) and resuspended in the Leibovitz's L-15 cell culture medium (Invitrogen) supplemented with penicillin (100 units·ml⁻¹) and streptomycin sulfate (100 μ g·ml⁻¹), and NaCl (0.39 M) was added to adjust the osmotic pressure. The cell suspension was adjusted

to 6 $\times$ 10⁵ cells·ml⁻¹. Aliquots of 500 μ l of cell suspension were dispensed into the wells of 24-well culture microplates. The coelomocytes were incubated for 24 hr at 18° in the darkness prior to use.

Expression analysis of miR-137, miR-2008, and AjBHMT in LPS-exposed primary coelomocytes

For LPS (Sigma) challenge, the primary cultured coelomocytes were stimulated with different concentrations of LPS (0, 0.1, and 1 μ g·ml⁻¹) for the indicated times. After 3 and 6 hr, the cells were washed once with cold PBS, centrifuged at 800 $\times$ *g* for 5 min at 4°, and then stored at -80° for RNA extraction. The expression levels of miR-137, miR-2008, and AjBHMT were analyzed as described above. Each sample was run in triplicate with three biological replications.

Functional analysis of miRNA *in vitro* and *in vivo*

The miRNAs mimics and inhibitors were synthesized at GenePharma and are shown in Table S1. For miR-137 mimics and inhibitor transfections, HiPerFect transfection reagents (Qiagen) were used for the transfection experiment. The miR-137 mimics or inhibitor (2.5 μ l of 50 nM), as well as each negative control, were mixed with an equal volume of HiPerFect transfection reagents. The mixture was then transfected into 1000 μ l of primary cultured cells. For miR-2008, 100 nM of the mimics or inhibitor were utilized for transfection analysis. At 24 hr post-transfection, the cells were harvested and washed with cold PBS, and centrifuged at 800 $\times$ *g* and 4° for 5 min in preparation for the subsequent expression analysis. The miR-137 and miR-2008 modified mimics specific for *in vivo* as well as the negative control were designed for the *in vivo* assay and synthesized by GenePharma (Shanghai). The sequence information is shown in Table S1. These modified mimics were then dissolved into RNase-free water to obtain a working solution of 20 μ M. We mixed 10 μ l of each modified mimics with 10 μ l of transfection reagent and 80 μ l of PBS to serve as the transfection solution. Twenty sea cucumbers (60 $\pm$ 10 g) were infected with 100 μ l of each modified mimic or the negative control. After 24 hr, the treated and control coelomocytes were collected for expression analysis. The assays described above were biologically repeated three times and run in triplicate.

Silencing of AjBHMT using small interfering RNA *in vitro* and *in vivo*

Small interfering RNA (siRNA) targeting AjBHMT and a negative control were designed and synthesized by GenePharma. The detailed sequence information is shown in Table S1. For *in vitro* assay, specific siRNA oligonucleotides (1.0 μ l of 20 nM) or the negative control were mixed with 1.0 μ l of siRNA-specific transfection reagent (GenePharma). The mixture was added to 1000 μ l of primary cultured cells for 24 hr. The treated and control coelomocytes were collected for expression analysis. For *in vivo* AjBHMT knockdown, 5 μ l of AjBHMT-specific siRNA (20 nM) and negative control were mixed with 5 μ l of transfection reagent and 90 μ l of PBS to

Figure 6 Functional analysis of miR-137 and miR-2008 and *AjBHMT* *in vivo*. (A) Relative miR-137 and miR-2008 expression levels after transfection with miRNA modified mimics. (B) Relative *AjBHMT* expression after transfection with miRNA modified mimics. (C) Relative *AjBHMT* expression after *AjBHMT* siRNA transfection. (D) Western blot analysis after transfection with miRNA modified mimics. (E) Western blot analysis after *AjBHMT* silencing *in vivo*.

serve as the transfection solution. Twenty sea cucumbers (60 ± 10 g) were injected with $100 \mu\text{l}$ of each *AjBHMT* siRNA or the negative control. At 24 hr post-transfection, the coelomocytes were collected and stored at -80° until further RNA and protein extraction. *AjBHMT* expression was analyzed as described above.

***AjBHMT* recombinant protein expression and polyclonal antibody preparation**

PCR fragments encoding *AjBHMT* and *Ajβ-actin* mature peptides were amplified using gene-specific primers (Table S1) with 5'-end *Bam*HI and *Not*I, respectively. The PCR products were cloned into pMD18-T simple vector (Takara), completely digested using the restriction enzymes *Bam*HI and *Not*I (NEB), and subsequently cloned into the pET-28a (+) expression vector (Novagen) between the *Bam*HI and *Not*I restriction sites. The recombinant plasmids (pET-28a-*Ajβ-actin* and pET-28a-*AjBHMT*) were transformed into *Escherichia coli* BL21 (DE3)-plysS (Novagen) and subjected to DNA sequencing. The positive clones were subsequently incubated in LB medium containing $50 \mu\text{g}\cdot\text{ml}^{-1}$ of kanamycin at 37° and 220 rpm. IPTG, at a final concentration of 1 mM, was added when the culture reached an OD_{600} of 0.6 and then incubated for an additional 3 hr under the same conditions. The bacteria were harvested through centrifugation at $10,000 \times g$ for 2 min and resuspended in buffer containing 50 mM Tris, 5 mM EDTA, 50 mM NaCl, and 5% glycerol, pH 7.9. After sonication, the recombinant *Ajβ-actin* and *AjBHMT* proteins were purified using HiTrap chelating columns (Amersham Biosciences) according to the manufacturer's instructions. The concentration of the purified proteins was

measured using the BCA Protein Assay Kit (Sangon), followed by 15% SDS-PAGE. For polyclonal antibody preparation, 4-week-old rats were intradermally injected with a mixture containing $100 \mu\text{g}$ of purified His-tagged *AjBHMT* or *Ajβ-actin* recombinant protein and an equal volume of complete Freund's adjuvant (Promega). After 2 weeks, the rats were intramuscularly boosted twice with $75 \mu\text{g}$ of His-tagged *AjBHMT* or *Ajβ-actin* recombinant protein mixed with an equal volume of incomplete Freund's adjuvant at a 1-week interval. Two weeks after the last immunization, the antiserum was harvested from rat eyes and stored at -80° until further use.

Western blot analysis

For Western blot assay, 48-hr post-transfected cells were washed twice in ice-cold PBS, and the protein was extracted using a Total Protein Extraction Kit (Sangon) according to the manufacturer's instructions. Subsequently, the protein concentration was measured using the BCA Protein Assay Kit (Sangon). For SDS-PAGE, $25 \mu\text{g}$ of protein from each sample was used, followed by electrophoretic transfer to a 0.45-mm pore nitrocellulose membrane using an ECL Semi-Dry Blotter (Amersham Biosciences). After blocking with 5% skim milk (in 20 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20) at 37° for 1 hr, the membranes were incubated with *AjBHMT* or *Ajβ-actin* polyclonal antibodies diluted to 1:500 in 5% skim milk at room temperature for 2 hr. The membranes were washed three times with TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20) and then incubated with goat-anti-rat IgG (Beyotime) diluted to 1:1000 in 5% skim milk at room temperature for 1.5 hr. After washing three times with TBST for 10 min each, the membrane was incubated in

Figure 7 Correlation analysis of Hcy contents with miR-137, miR-2008, and *AjBHMT* *in vitro* and *in vivo*. (A) miR-137 or miR-2008 inhibitor transfection *in vitro*. (B) miR-137 or miR-2008 mimics transfection *in vitro*. (C) *AjBHMT* silencing *in vitro*. (D) miR-137 or miR-2008 modified mimics transfection *in vivo*. (E) *AjBHMT* silencing *in vivo*.

Western Lightning-ECL substrate (Perkin Elmer) prior to exposure onto X-OMAT AR X-ray film (Eastman Kodak, Rochester, NY). The protein bands were quantified using the BioRad Quantity One software package, and the results were derived from the statistical analysis of three independent experiments.

Detection of Hcy concentration

For the *in vitro* detection of the Hcy concentration at 48 hr post-transfection, the levels of Hcy in the culture medium of cells transfected with miRNA mimics, inhibitor, or siRNA were measured using a cycling enzymatic method. Briefly, the culture medium from each treatment was collected and analyzed using Liquid Stable (LS) 2-Part Homocysteine Reagent (Axis-Shield) on an Olympus AU400 Chemistry Analyzer. Subsequently, the cells from each sample were collected for protein extraction.

For the *in vivo* detection of the Hcy concentration, 10 μ l of miR-137 and miR-2008 modified mimics, 5 μ l of *AjBHMT* siRNA, and the respective negative controls were mixed with transfection reagent and PBS buffer and then injected into sea cucumbers as described above. At 24 hr post-transfection, the coelomocytes were collected and stored at -80° until further use for Hcy detection.

Reactive oxygen species production in vitro

Intracellular reactive oxygen species (ROS) production after Hcy exposure or *AjBHMT* knockdown was measured as pre-

viously described (Wang *et al.* 2009), with slight modifications. Primary cultured cells were treated with 0, 0.5 or 1 mM Hcy (Sigma). Silencing of *AjBHMT* expression was conducted as described above. After 24 hr, the cells were incubated with 0.1% nitroblue tetrazolium (NBT) and 0.01 μ M phorbol 12-myristate 13-acetate (PMA) for 1 hr. Subsequently, the supernatant was removed through centrifugation at $540 \times g$ for 10 min, and the cells were fixed with 35% methanol. After washing twice with 70% methanol, the cells were resuspended in 0.92 M potassium hydroxide containing 54% dimethyl sulfoxide in a final volume of 1.3 ml to dissolve the reduced NBT (in the form of formazan). The optical density of the sample was measured at 625 nm.

Colony-forming unit assay

Primary cultured cells were treated with 0, 0.5, or 1 mM Hcy and 10 M *AjBHMT* siRNA and the respective negative controls for 24 hr prior to infection. Subsequently, *V. splendidus* was added to the cultured cells at a ratio of ~ 100 bacteria/cell. After incubation at 18° for 3 and 6 hr, the infected cells were extensively washed with PBS to remove extracellular bacteria, followed by lysing in 1 ml of distilled water. Quantitative culturing was performed using 10-fold serial dilutions. Aliquots of each dilution were inoculated in triplicate onto 2216E agar plates. The plates were incubated overnight at 28° , and subsequently, the colonies were then counted.

Figure 8 Determination of ROS production and bacterial survival rate after *AjBHMT* knockdown *in vivo*. (A) ROS production assay. (B) Bacterial survival rate assay.

ROS production *in vivo*

In vivo ROS production analysis was performed by flow cytometry (FCM). The FCM analysis was performed using a FACScan (Becton-Dickinson, San Jose, CA). For each sample, a total of 10,000 events were acquired, and the data in forward-scatter (FSC) and side-scatter (SSC) parameters were collected and analyzed using the Cell Quest software.

Before FCM analysis, 0, 10, and 50 mM Hcy were mixed with PBS buffer in a final volume of 100 μ l and then injected into 30 sea cucumbers. About 5 μ l of *AjBHMT* siRNA or negative control was mixed with 5 μ l of transfection reagent and 90 μ l of PBS and injected into 20 sea cucumbers. After treatment for 24 hr, the coelomocytes were collected and adjusted to 6×10^5 cells·ml⁻¹ with L15 culture medium. For ROS analysis, ROS was detected using the Reactive Oxygen Species Assay Kit (Beyotime Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Briefly, 1000 μ l of cells was centrifuged at $800 \times g$ and 18° for 5 min and then resuspended in 10 μ M DCFH-DA (diluted in L15 culture medium). After incubation at 18° for 20 min in HulaMixer Sample Mixer (Invitrogen), the cells were washed three times by L15 culture medium and then used for FCM test.

Data availability

All list plasmids and strains may be requested from Li Lab. Current versions of protocols will be maintained in our laboratory.

Results

Characterization of miR-137 and miR-2008 expression in response to pathogenic infection *in vivo* and *in vitro*

MiR-2008 has been identified as a regulatory factor in sea cucumber SUS outbreaks using deep sequencing (Li *et al.* 2012). MiR-137 is widely accepted as a tumor suppressor in many human cancer developments (Wu *et al.* 2007; Liu *et al.* 2011). To fully understand the roles of miR-137 and miR-2008 in the host-pathogen interaction process, we used qPCR to examine the expression profiles of these two miRNAs in response to pathogenic infection or LPS exposure (Figure 1). As shown in Figure 1, miR-137 expression decreased in

V. splendidus-challenged sea cucumbers (Figure 1A), with 0.37- and 0.28-fold reductions observed at 48 and 96 hr, respectively. By contrast, miR-2008 expression increased by 1.96-fold and 6.82-fold at 48 hr and 96 hr, respectively (Figure 1C). Further exploration in LPS-exposed primary cultured cells revealed similar patterns of miR-137 (Figure 1B) and miR-2008 (Figure 1D) expression, with a 0.22-fold decrease or 2.74-fold increase at 6 hr, respectively, after exposure to 1 μ g·ml⁻¹ LPS. However, in our previous study, the expression level of miR-137 displayed no significant change between diseased and healthy juvenile individuals (3–5 g) collected from the indoor ponds of Dalian. The discrepant expression level of miR-137 might result from the different samples or the different states of samples. However, the expression level of miR-2008 exhibited similar expression tendencies between the diseased and pathogen-infected samples. Thus, we suppose that the discrepant results between infected and diseased experiment might indicate a progressively pathogenic process from infection to pathogenesis.

Identification of miR-137 and miR-2008 targets

To understand the functional roles of these two miRNAs, candidate targets were predicted using the miRanda software and the available transcriptome RNA-seq data (Zhang *et al.* 2013) (see Table S2). A total of 22 and 29 genes were predicted as putative targets of miR-137 and miR-2008, respectively. Among these putative targets, *AjBHMT* was identified as a common target of both miR-137 and miR-2008. More importantly, *AjBHMT* modulated the Hcy content, which may directly or indirectly lead to cell apoptosis, oxidative stress, or endoplasmic stress. Therefore, *AjBHMT* was selected as a promising target for functional validation.

Cloning the full-length cDNA of *AjBHMT* and its response to immune induction *in vitro* and *in vivo*

The full-length 1415-bp *AjBHMT* cDNA was obtained using overlapping ESTs and RACE fragments and deposited in GenBank under the accession no. KJ855191. The full-length cDNA of *AjBHMT* contained a 5'-UTR of 54 bp, a 3'-UTR of 281 bp, and a putative ORF of 1080 bp encoding a polypeptide of 359 amino acid residues with a predicted molecular mass of 39.72 kDa and theoretical isoelectric point (pI) of

Figure 9 ROS production assay after *AjBHMT* knockdown *in vivo* by flow cytometry. (A) Hcy control; (B) 10 mM Hcy injection; (C) 50 mM Hcy injection; (D) negative control for siBHMT; (E) *AjBHMT* silencing; and (F) bar chart representing ROS production as described in A–E.

5.49. SMART analysis indicated that *AjBHMT* had a conserved S-methyl_trans domain (24–323 aa) (see Figure S1).

AjBHMT expression was increased not only in the bacteria-challenged individuals, but also in the LPS-treated cells. As shown in Figure 2, a 5.53- and 7.64-fold increase was detected at 48 hr and 96 hr in bacteria-infected samples, respectively, compared with the control group (Figure 2A), and a 3.06-fold increase was detected at 6 hr after exposure to $1 \mu\text{g}\cdot\text{ml}^{-1}$ LPS (Figure 2B). However, the *AjBHMT* protein level initially increased by 4.01-fold at 24 hr postinfection, but gradually decreased to control levels at 48 hr and continuously declined at 96 hr in *V. splendidus*-challenged sea cucumbers from our iTRAQ qualification (Zhang *et al.* 2014) (Figure 2C). These findings indicated that *AjBHMT* should be tightly regulated at different stages of infection.

Validation of the interaction between 3'-UTR of *AjBHMT* and miR-137 or miR-2008 through dual-luciferase reporter assays

To further validate the predicted regulation of *AjBHMT* through miR-137 and miR-2008 at the 3'-UTR of this gene, two reporter plasmids containing the putative binding and mutation sites of each miRNA were constructed and used in classical reporter assays in HEK-293T cells. Information concerning the binding and mutant sites of each miRNA in the 3'-UTR of *AjBHMT* is shown in Figure 3A. These constructs were named pMIR-REPORT-*AjBHMT*-WT and pMIR-REPORT-*AjBHMT*-MT. Following the transient cotransfection of HEK-293T cells with pMIR-REPORT-*AjBHMT*-WT/MT, pRL-CMV-Renilla-luciferase and 100 nM miR-137 or miR-2008 mimics, we observed a 51.39% ($P = 0.0002$) and 25.07% ($P = 0.0359$) reduction in luciferase activity in HEK293 cells transfected with pMIR-REPORT-*AjBHMT*-WT compared with controls for miR-137 and miR-2008, respectively (Figure 3B). No significant variation in luciferase activity in HEK293 cells transfected with pMIR-REPORT-*AjBHMT*-MT was observed (Figure 3C).

For the cooperative functional identification of miR-137 and miR-2008 on the *AjBHMT*, we used serial concentrations of miR-137 or miR-2008 mimics as well as their combination to cotransfect HEK-293T cells with pMIR-REPORT-*AjBHMT*-WT, pRL-CMV-Renilla-luciferase. Compared with the mimics control group, cells transfected with 50 nM miR-137 mimics or miR-2008 mimics exhibited no significant change in luciferase activity. However, in the miR-137 and miR-2008 cotransfected cells (50 nM miR-137 + 50 nM miR-2008), we found a 35.17% ($P = 0.0032$) reduction in luciferase activity (Figure 3D). For higher concentration transfection groups (150 nM), miR-137 mimics and miR-2008 mimics displayed 71.92% ($P = 0.0005$) and 31.66% ($P = 0.0049$) reduction in luciferase activity, respectively (Figure 3D). Interestingly, two miR-137 and miR-2008 combined groups (50 nM miR-137 + 100 nM miR-2008, 100 nM miR-137 + 50 nM miR-2008) both exhibited higher inhibitory effect by 74.24% ($P = 0.0005$) and 82.24% ($P = 0.0005$) reduction compared with 150 nM miR-137 and miR-2008 group, respectively (Figure 3D). Thus we think that these two miRNAs may target to the *AjBHMT* collaboratively.

miR-137 modulates *AjBHMT* at the post-transcriptional level, whereas miR-2008 modulates *AjBHMT* at the translational level *in vitro* and *in vivo*

To fully understand the potential roles of miR-137 and miR-2008 in differentially regulating *AjBHMT*, loss-of-function or gain-of-function assay of miR-137 and miR-2008 was performed *in vitro* and *in vivo*. As shown in Figure 4, the qRT-PCR results indicated that the overexpression of miR-137 (Figure 4A) decreased the cellular levels of *AjBHMT* mRNA (Figure 4B). In contrast, the knockdown of miR-137 (Figure 4C) increased *AjBHMT* gene expression (Figure 4D). Western blot analysis of miR-137 overexpression and knockdown at 48 hr post-transfection revealed that the protein abundance of *AjBHMT* was decreased upon miR-137 overexpression and increased upon miR-137 inhibition (Figure 5), consistent

Figure 10 Effect of Hcy on ROS production and the bacterial survival rate in primary cultured cells. (A) ROS production after Hcy stimulation. (B) Bacterial survival rate after Hcy stimulation.

with the *AjBHMT* mRNA expression levels. *In vivo* analysis further revealed that the overexpression of miR-137 (Figure 6A) decreased the expression of *AjBHMT* at both the mRNA (Figure 6B) and protein levels (Figure 6D). These results suggested that miR-137 directly targeted *AjBHMT* by inducing the degradation of *AjBHMT* mRNA.

The expression of *AjBHMT* in primary cultured cells transfected with miR-2008 mimics or the miR-2008 inhibitor is shown in Figure 4. Neither the overexpression nor the inhibition of miR-2008 (Figure 4, E and G) significantly changed *AjBHMT* mRNA expression (Figure 4, F and H). In contrast, Western blot analysis revealed that the overexpression of miR-2008 decreased *AjBHMT* protein abundance, and miR-2008 knockdown increased *AjBHMT* protein abundance (Figure 5). Consistent with the result of *in vitro* analysis, the results of *in vivo* assay also showed that the overexpression of miR-2008 (Figure 6A) decreased the expression of *AjBHMT* protein (Figure 6B), without affecting the levels of *AjBHMT* mRNA (Figure 6D). Collectively, these observations suggested that miR-2008 decreases *AjBHMT* protein expression without affecting *AjBHMT* mRNA expression.

The Hcy concentration is directly modulated through changes in *AjBHMT* in vitro and in vivo

As a substrate of BHMT metabolism, the Hcy content was considered as an important index for changes in BHMT expression. Therefore, we explored alterations in the Hcy concentration in the culture medium of cells transfected with miRNA mimics or inhibitors at 48 hr post-transfection. The inhibition of miR-137 and miR-2008 decreased the levels of Hcy in the culture medium compared with the negative control (Figure 7A), whereas the overexpression of miR-137 and miR-2008 increased the levels of Hcy in the culture medium (Figure 7B). The abundance of Hcy was also increased upon siRNA-mediated *AjBHMT* silencing (Figure 7C), further suggesting that miR-137 and miR-2008 modulated the Hcy concentration by targeting *AjBHMT* *in vitro*. *In vivo*, the overexpression of miR-137 and miR-2008 (Figure 7D), as well as the siRNA-mediated *AjBHMT* silencing (Figure 7E) also causes the accumulation of the Hcy concentration, consistent with the result of *in vitro* analysis.

siRNA-mediated *AjBHMT* silencing decreases the bacterial survival rate through the promotion of Hcy expression and ROS production in vitro and in vivo

The injection of *AjBHMT* siRNA specifically inhibited *AjBHMT* mRNA expression (Figure 6C) and protein synthesis *in vivo* (Figure 6E). A dramatic increase in Hcy expression was observed after *AjBHMT* deletion (Figure 7E). Previous studies have indicated that Hcy elevates respiratory burst in immune-competent cells (Vladychenskaya and Boldyrev 2009). Therefore, we investigated the ROS production and bacterial survival rate in cells transfected with *AjBHMT* siRNA. As shown in Figure 8, *AjBHMT* silencing significantly accelerated intracellular ROS production (Figure 8A) and decreased the bacterial survival rate by 35.3% and 25.7% at 3 and 6 hr postinfection, respectively (Figure 8B). We used the 2',7'-dichlorofluorescein diacetate (DCFH-DA) method to directly evaluate the effects of *AjBHMT*-mediated respiratory burst on sea cucumbers and assayed the results using ECM. As shown in Figure 9, *AjBHMT* silencing increased intracellular ROS production BY 65.4% (Figure 9, E and F) compared with the control group (Figure 9, D and F).

Hcy exposure also minimizes the bacterial survival rate through an increase in ROS production in vitro and in vivo

To evaluate the effect of Hcy on ROS production and the bacterial survival rate, we stimulated primary cultured cells with different concentrations of Hcy. As shown in Figure 10, exposure to 0.5 and 1 mM Hcy significantly increased intracellular ROS production (Figure 10A) and decreased the bacterial survival rate (Figure 10B). In response to stimulation with 0.5 mM Hcy, 55.1% and 41.0% bacteria survived at 3 and 6 hr postinfection, respectively, and 1 mM Hcy exposure decreased the bacterial survival rate to 37.0% and 21.1% at 3 and 6 hr postinfection (Figure 10B). To further confirm Hcy-mediated ROS production *in vivo*, we injected sea cucumbers with different concentrations of Hcy and assayed ROS production in the coelomocytes using ECM. The results shown in Figure 10 demonstrated that 10 mM Hcy exposure increased ROS production to 50.6% (Figure 9, B and F) and 50 mM Hcy stimulation increased ROS production to 92.7% (Figure 9, C and F) compared with the respective control groups showing 40% ROS production (Figure 9, A and F).

Figure 11 Schematic representation of the involvement of miR-137 and miR-2008 in the host-pathogen interaction by cotargeting *AjBHMT*.

Discussion

MicroRNA-mediated gene silencing is a widespread regulatory mechanism implicated in host-pathogen interactions and improvements in cell-specific targeting for gene and cell therapy (Brown and Naldini 2009). Accumulative evidence indicated that miRNAs modulate target gene expression at the mRNA or protein level, and a given mRNA can be targeted by several miRNAs. However, scarce information is available concerning the modulation of a specific target through different miRNAs at different levels, particularly in nonmodel invertebrates. In the present study, we explored the expression patterns of miR-137, miR-2008, and *AjBHMT* in pathogen-challenged individuals and LPS-exposed cells using qRT-PCR. The results suggested an inverse relationship between miR-137 and *AjBHMT* expression, as well as a parallel relationship between miR-2008 and *AjBHMT* expression. In a previous study, we performed a iTRAQ-based proteomics analysis of pathogen-challenged sea cucumbers, showing that the abundance of *AjBHMT* protein increased at 24 hr postinfection but decreased at 48 and 96 hr postinfection. Thus, *AjBHMT* could be regulated through miR-137 at the post-transcriptional level via direct mRNA degradation and through miR-2008 at the translational level via translation repression. To confirm this hypothesis, we applied the overexpression and inhibition of miR-137 and miR-2008 to explore the gene or protein levels of *AjBHMT* using qRT-PCR and Western blotting *in vitro* and *in vivo*. The results indicated that miR-137 and miR-2008 differentially regulated *AjBHMT* expression. This differential regulation might represent a highly efficient mechanism for the tight regulation of target proteins. Given that a single transcript can produce hundreds of proteins, the different mechanisms of miR-137 and miR-2008 act in cooperation to post-transcriptionally control *AjBHMT* through a “fine-tuning” model (Sevignani

et al. 2006). Meanwhile the miR-137-mediated degradation of *AjBHMT* mRNA may lead to a sharp decline in the abundance of *AjBHMT* protein abundance, the translation repression of *AjBHMT* induced by miR-2008 only reduced the *AjBHMT* protein levels, which could be quickly recovered as this kind of repression often is relatively mild (Baek *et al.* 2008; Selbach *et al.* 2008). The discrepancy between the mRNA expression level and the protein abundance of *AjBHMT* may also result from some other regulatory factors. However, this topic is beyond the scope of the present study.

BHMT was first identified as a facilitator of methyl group donation for the remethylation of Hcy into methionine (Pajares and Pérez-Sala 2006), and reduced BHMT functions can elevate Hcy levels (Ji *et al.* 2007). Consistently, in the present study, the overexpression of miR-137 and miR-2008 and silencing of *AjBHMT* elevated the Hcy content, whereas the inhibition of miR-137 and miR-2008 decreased the Hcy levels. In the past decade, Hcy has been identified as a risk factor for numerous diseases. Many studies have demonstrated that a high Hcy concentration increases intracellular ROS generation, and the imbalance of Hcy metabolism results in cellular immune dysfunction in metabolic syndromes. ROS are important intracellular second messengers that mediate the production of inflammatory cytokines (Gorowara *et al.* 1998). Abundant evidence indicated that excessive Hcy in endothelial cells induces endothelial dysfunction through the inhibition of arginine transport (Jin *et al.* 2007), elevates ROS levels through autooxidation, and diminishes the activities of antioxidative enzymes, such as glutathione peroxidase-1 (Handy *et al.* 2005) and superoxide dismutase (Yamamoto *et al.* 2000). Furthermore, recent studies indicate that Hcy induces the production of superoxide anion (Alvarez-Maqueda *et al.* 2004; Au-Yeung *et al.* 2006), exerts important effects on cellular NO production (Tsen *et al.* 2003; Zhang *et al.* 2000), and interferes with *de novo* GSH synthesis

(Toroser and Sohal 2007) to improve intracellular ROS levels. In a previous study (Li *et al.* 2012), we demonstrated that the extremely high cellular abundance of miR-2008 can reduce the activity of AjBHMT significantly in SUS-diseased samples, whereas the normal abundance of miR-137 maintains low abundance of AjBHMT mRNA. Both of these events resulted in difficult recovered AjBHMT activity and continued high Hcy concentration. As described above, elevated Hcy concentrations induce inflammatory responses and increase ROS production, which play a critical role in cellular pathophysiology and induce damage to organisms (Lee *et al.* 2013). This kind of excess accumulation of Hcy and ROS production may contribute to the SUS outbreak of *A. japonicus*. Numerous studies have implicated ROS in oxidative defense mechanisms responsible for bacterial death (Slauch 2011). Thus, we evaluated the effect of Hcy on intracellular ROS generation and bacterial clearance, and we confirmed that Hcy induced ROS generation and observably decreased the bacterial survival rate. A schematic representation of the involvement of miR-137 and miR-2008 in the host–pathogen interaction by regulating intracellular ROS generation through cotargeting of *AjBHMT* to modulate cellular Hcy concentration is shown in Figure 11.

Intriguingly, in the early phase of *V. splendidus* infection, the down-regulation of miR-137 expression could increase the level of *AjBHMT* expression, resulting from an increasing demand for methionine in protein synthesis or methyl donation. Previous studies have confirmed that the aberrant methylation of the miR-137 promoter contributes to miR-137 silencing (Langevin *et al.* 2010, 2011; Dang *et al.* 2013). In the present study, we speculated that the continuous down-regulation of miR-137 might reflect an increasing level of methylation, forming a feedback loop that increases AjBHMT levels and decreases in miR-137 levels to further increase the concentration of AjBHMT. Elevated AjBHMT activity might promote the expression of miR-2008 to maintain appropriate cellular AjBHMT levels. Thus, our future studies should focus on the upstream regulation of these two miRNAs.

In the present study, we confirmed that miR-137 and miR-2008 played a pivotal role in host–pathogen interactions through cotargeting of the *AjBHMT* gene to regulate Hcy metabolism, thereby affecting cellular oxidative-redox stress and improving bacterial elimination. Immunometabolism is an emerging frontier that demonstrates the interplay between immunological and metabolic processes. The multilevel interactions between metabolic and immune systems suggest the importance of understanding the pathogenic mechanisms (Mathis and Shoelson 2011). The results of the present study suggested a new horizon for dissecting the pathogenesis of infectious diseases from the perspective of miRNA-mediated cross-talk between immunology and metabolism.

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GENETICS

Supporting Information

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The Roles of Two miRNAs in Regulating the Immune Response of Sea Cucumber

**Pengjuan Zhang, Chenghua Li, Ran Zhang, Weiwei Zhang, Chunhua Jin, Lingling Wang,
and Linsheng Song**

<i>Apostichopus japonicus</i>	- - M K S K M P S T K G L L E R L D N G E K V V V A E G Y L F N F E K R C Y L Q A G H F V P T V V L	48
<i>Strongylocentrotus purpuratus</i>	- - - - - M S K A K G L L Q R I Q D G E T V I V A E G Y I F A F E R L G Y L K A G P Y T P E V V M	44
<i>Saccoglossus kowalevskii</i>	M E R N N D S K M V K G L M E R L N D G E H V I I A E G F V F Y F E R M G Y L Q A G S W V P E V V L	50
<i>Aplysia californica</i>	- - - - M S A G K V K G L R E R L R D G E S I I C A E G Y M W E L E R R G F V K C G C F T P E V V L	46
<i>Crassostrea gigas</i>	- - - - M A T - - - K G L V E R L K N G E D I L I A E G Y M W E L E R R G Y L T A G G F I P E I V L	43
<i>Mus musculus</i>	M A P V A G K K A K K G I L E R L N A G E V V I G D G G F V F A L E K R G Y V K A G P W T P E A A V	50
<i>Rattus norvegicus</i>	M A P I A G K K A K R G I L E R L N A G E V V I G D G G F V F A L E K R G Y V K A G P W T P E A A V	50
<i>Homo sapiens</i>	M P P V G G K K A K K G I L E R L N A G E I V I G D G G F V F A L E K R G Y V K A G P W T P E A A V	50

<i>Apostichopus japonicus</i>	D H P E L V T Q L H K E F V R A G S D V V Q A F T Y Y A H R E K L Q L I D - - - - K T A D L E T I N	94
<i>Strongylocentrotus purpuratus</i>	D H P E L V R Q M Y K D F V R A G S D V V Q A F T Y Y G N R Y K M G I V G - - - - R G D E L E K Q N	90
<i>Saccoglossus kowalevskii</i>	N H P D K V K S L Y R E F V D A G S D V V L A F T Y Y A T R E K T R L I G - - - - K E D T L E Q L N	96
<i>Aplysia californica</i>	D Y P E R V T S L H E E Y V L S G S D V V E A Y T Y Y G H R E K M K V I G - - - - R E D E L E K L N	92
<i>Crassostrea gigas</i>	D N P E V V R A L H M E F I H A G T D V I E A F T Y Y G H R E K L R T I G - - - - R E D D L E K L N	89
<i>Mus musculus</i>	E H P E A V R Q L H R E F L R A G S N V M Q T F T F Y A S E D K L E N R G N Y V A E K I S G Q K V N	100
<i>Rattus norvegicus</i>	E H P E A V R Q L H R E F L R A G S N V M Q T F T F Y A S E D K L E N R G N Y V A E K I S G Q K V N	100
<i>Homo sapiens</i>	E H P E A V R Q L H R E F L R A G S N V M Q T F T F Y A S E D K L E N R G N Y V L E K I S G Q E V N	100

<i>Apostichopus japonicus</i>	R N A L R I A R D V A R E T G T L F A G N I C N T N I Y N L S E A D N K D K V K Q I F K E Q I E W A	144
<i>Strongylocentrotus purpuratus</i>	R L A L K M A R E I A D S T G T L M C G D L S N T F V Y Q P D N D E A I E K T K E M F K E Q V E W T	140
<i>Saccoglossus kowalevskii</i>	K N A L K M A R E V A D E K G V L M A G N I C N T N I Y Q P G K K D R E D E I K G M F K E M I E W S	146
<i>Aplysia californica</i>	L N S L S M A R E V A D K Y G K L M A G N L S N S T T F V P N D K E S A R K T A E I F K E Q V E W A	142
<i>Crassostrea gigas</i>	R V A L Q M A K E V A R E N G K L L A G G I C N S G I Y D P Q D E S T F A A V T A M F K E Q I E W A	139
<i>Mus musculus</i>	E A A C D I A R Q V A D E G D A L V A G G V S Q T P S Y L S C K S E V - - E V K K I F R Q Q L E V F	148
<i>Rattus norvegicus</i>	E A A C D I A R Q V A D E G D A L V A G G V S Q T P S Y L S C K S E T - - E V K K I F H Q Q L E V F	148
<i>Homo sapiens</i>	E A A C D I A R Q V A D E G D A L V A G G V S Q T P S Y L S C K S E T - - E V K K V F L Q Q L E V F	148

<i>Apostichopus japonicus</i>	V D E G V D F I I A E T F V K F E E A A I A L E S I K E Y G K G V P A V V T M A A T G L A T V E G H	194
<i>Strongylocentrotus purpuratus</i>	V D E G A D Y I V G E T F G Y V G E A I L A A Q A I K E Y G K G V P A V V T L S S H V K Y D D E G R	190
<i>Saccoglossus kowalevskii</i>	V E A G A D F I L A E T Y A H L G E A L L A V E A I K E H G K G L P A V V T F A P F V T A T K N G K	196
<i>Aplysia californica</i>	V K G N A D Y M I A E T F S D F A E A L I A L E V I K K Y G N G I P V V V T I A L Y S P D - - - - -	187
<i>Crassostrea gigas</i>	V E Y E V D Y I I A E T F N D L G E A M L A L K A I Q Q Y G K G V P A V I T M T A Y I P D - - - - -	184
<i>Mus musculus</i>	M K K N V D F L I A E Y F E H V E E A V W A V E A L K A S G K P V A A T M C I G P - - - - - - - -	189
<i>Rattus norvegicus</i>	M K K N V D F L I A E Y F E H V E E A V W A V E A L K T S G K P I A A T M C I G P - - - - - - - -	189
<i>Homo sapiens</i>	M K K N V D F L I A E Y F E H V E E A V W A V E T L I A S G K P V A A T M C I G P - - - - - - - -	189

<i>Apostichopus japonicus</i>	P	T	T	V	D	K	V	E	V	A	E	A	M	K	R	L	E	A	A	G	A	D	V	V	G	L	N	C	F	N	G	P	E	T	A	L	G	V	M	S	H	I	K	K	S	G	V	K	A	P	244						
<i>Strongylocentrotus purpuratus</i>	P	C	T	I	D	G	V	E	M	N	E	A	L	R	R	L	V	E	A	G	A	D	V	V	G	F	N	C	T	R	G	P	E	T	I	M	K	L	V	E	S	A	T	N	A	G	I	K	V	P	240						
<i>Saccoglossus kowalevskii</i>	A	I	T	A	D	K	V	L	L	T	E	A	C	K	Q	V	K	E	A	G	A	D	V	V	G	L	N	C	G	R	G	P	N	T	M	L	P	L	M	K	D	I	M	K	V	-	C	P	G	P	245						
<i>Aplysia californica</i>	-	L	T	T	D	D	V	P	A	V	E	A	L	R	R	L	E	E	A	G	A	D	V	V	G	L	N	C	A	R	G	P	A	T	M	L	P	V	L	R	E	A	R	K	V	-	C	K	G	P	235						
<i>Crassostrea gigas</i>	-	M	T	T	D	D	V	P	F	P	E	A	C	R	R	L	E	E	A	G	A	A	V	V	G	V	N	C	G	R	G	P	R	T	M	L	P	L	V	K	E	I	K	K	V	-	C	K	G	P	232						
<i>Mus musculus</i>	E	G	D	L	H	G	V	P	P	G	E	C	A	V	R	L	V	K	A	G	A	S	I	V	G	V	N	C	H	F	D	P	S	V	S	L	Q	T	V	K	L	M	K	E	G	-	-	-	-	-	234						
<i>Rattus norvegicus</i>	E	G	D	L	H	G	V	S	P	G	E	C	A	V	R	L	V	K	A	G	A	A	I	V	G	V	N	C	H	F	D	P	S	T	S	L	Q	T	I	K	L	M	K	E	G	-	-	-	-	-	234						
<i>Homo sapiens</i>	E	G	D	L	H	G	V	P	P	G	E	C	A	V	R	L	V	K	A	G	A	S	I	I	G	V	N	C	H	F	D	P	T	I	S	L	K	T	V	K	L	M	K	E	G	-	-	-	-	-	234						
<i>Apostichopus japonicus</i>	L	A	C	L	P	V	P	Y	R	T	T	T	K	E	P	T	F	L	Q	L	T	D	P	I	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	E	K	V	F	S	A	N	L	D	C	V	M	C	283				
<i>Strongylocentrotus purpuratus</i>	L	A	V	L	P	V	L	Y	R	T	T	D	E	Y	P	S	F	H	S	L	P	D	P	K	T	G	-	-	-	-	-	-	-	-	-	-	-	-	E	R	A	F	P	-	D	M	D	C	L	L	C	278					
<i>Saccoglossus kowalevskii</i>	F	A	A	I	P	V	P	Y	R	T	C	D	K	E	P	T	F	F	M	L	T	D	P	V	T	G	-	-	-	-	-	-	-	-	-	-	-	-	K	K	V	F	P	T	E	T	E	V	C	S	C	284					
<i>Aplysia californica</i>	L	A	A	L	P	V	P	F	R	T	T	E	K	E	R	T	F	Q	A	L	T	D	P	K	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	K	S	V	Y	P	L	D	L	A	C	L	Q	C	274				
<i>Crassostrea gigas</i>	V	A	M	L	P	V	T	F	R	C	R	D	N	C	R	T	F	Q	S	L	R	D	P	E	T	G	K	Y	L	S	P	A	R	L	S	S	R	K	P	L	Y	P	T	D	L	E	A	A	R	C	282						
<i>Mus musculus</i>	L	E	A	A	R	L	K	A	Y	L	M	S	Q	P	L	A	Y	H	T	P	D	C	G	K	Q	G	-	-	-	-	-	-	-	-	-	-	-	-	F	I	D	L	P	E	F	P	F	G	L	E	P	R	V	A	276		
<i>Rattus norvegicus</i>	L	E	A	A	R	L	K	A	Y	L	M	S	H	A	L	A	Y	H	T	P	D	C	G	K	Q	G	-	-	-	-	-	-	-	-	-	-	-	-	-	F	I	D	L	P	E	F	P	F	G	L	E	P	R	V	A	276	
<i>Homo sapiens</i>	L	E	A	A	R	L	K	A	H	L	M	S	Q	P	L	A	Y	H	T	P	D	C	N	K	Q	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	I	D	L	P	E	F	P	F	G	L	E	P	R	V	A	276
<i>Apostichopus japonicus</i>	S	R	D	D	I	F	K	F	G	R	R	C	D	E	I	G	I	D	Y	L	G	L	C	C	G	N	S	P	H	Y	T	R	T	L	C	E	S	I	G	-	-	-	-	-	-	-	-	-	-	-	322						
<i>Strongylocentrotus purpuratus</i>	S	R	R	D	V	I	E	F	G	Q	R	C	A	Q	L	K	I	P	Y	V	G	L	C	C	G	N	A	P	H	Y	T	R	A	L	A	E	S	Y	G	-	-	-	-	-	-	-	-	-	-	-	-	317					
<i>Saccoglossus kowalevskii</i>	S	R	E	D	F	S	D	F	G	K	Q	C	K	D	L	G	I	Q	F	V	G	V	C	C	G	N	R	H	Y	Y	T	R	S	L	A	E	A	L	G	-	-	-	-	-	-	-	-	-	-	-	-	323					
<i>Aplysia californica</i>	N	R	S	D	I	R	N	F	A	K	E	A	K	E	I	G	I	Q	Y	I	G	L	C	C	G	S	S	S	N	F	L	R	E	L	A	E	V	Y	G	-	-	-	-	-	-	-	-	-	-	-	-	313					
<i>Crassostrea gigas</i>	S	R	S	D	I	R	S	W	A	E	E	A	K	A	A	G	I	N	Y	I	G	L	C	C	G	N	A	S	F	Y	F	R	E	L	A	E	A	Y	G	-	-	-	-	-	-	-	-	-	-	-	-	321					
<i>Mus musculus</i>	T	R	W	D	I	Q	K	Y	A	R	E	A	Y	N	L	G	V	R	Y	I	G	G	C	C	G	F	E	P	Y	H	I	R	A	I	A	E	E	L	A	P	E	R	G	F	L	P	P	A	S	E	326						
<i>Rattus norvegicus</i>	T	R	W	D	I	Q	K	Y	A	R	E	A	Y	N	L	G	V	R	Y	I	G	G	C	C	G	F	E	P	Y	H	I	R	A	I	A	E	E	L	A	P	E	R	G	F	L	P	P	A	S	E	326						
<i>Homo sapiens</i>	T	R	W	D	I	Q	K	Y	A	R	E	A	Y	N	L	G	V	R	Y	I	G	G	C	C	G	F	E	P	Y	H	I	R	A	I	A	E	E	L	A	P	E	R	G	F	L	P	P	A	S	E	326						
<i>Apostichopus japonicus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	R	P	P	A	S	E	Y	S	P	D	M	D	L	H	G	F	F	G	T	D	343					
<i>Strongylocentrotus purpuratus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	R	P	E	A	S	K	Y	S	P	D	M	A	M	H	G	W	L	G	T	K	338				
<i>Saccoglossus kowalevskii</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	K	P	A	S	T	T	F	S	P	D	M	T	K	H	Y	V	Y	G	S	D	344				
<i>Aplysia californica</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	K	P	A	S	S	V	Y	S	P	D	L	K	K	S	Y	V	F	G	S	D	334				
<i>Crassostrea gigas</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	K	P	P	T	S	K	Y	T	P	N	V	G	L	S	H	V	F	G	K	T	342				
<i>Mus musculus</i>	K	H	G	S	W	G	S	G	L	D	M	H	T	K	P	W	I	R	A	R	A	R	K	E	Y	W	Q	N	L	R	I	A	S	G	R	P	Y	N	P	S	M	S	R	P	D	A	W	G	V	T	376						
<i>Rattus norvegicus</i>	K	H	G	S	W	G	S	G	L	D	M	H	T	K	P	W	I	R	A	R	A	R	K	E	Y	W	Q	N	L	R	I	A	S	G	R	P	Y	N	P	S	M	S	K	P	D	A	W	G	V	T	376						

<i>Homo sapiens</i>	K H G S W G S G L D M H T K P W V R A R A R K E Y W E N L R I A S G R P Y N P S M S K P D G W G V T	376
<i>Apostichopus japonicus</i>	E R V T K N - - - V T K D V Q D A L K - - - - - - - - -	359
<i>Strongylocentrotus purpuratus</i>	K D V V D E H Y S V T I K S F Q N N F - - - - - - - - -	357
<i>Saccoglossus kowalevskii</i>	A K V R K D V S E - E S M D L Y H T L R D I - - - - - - - -	365
<i>Aplysia californica</i>	V T G H A K K V A E H M R G T T L M E F S - - - - - - - -	355
<i>Crassostrea gigas</i>	S E A D K Y K R S T K I K E F M I G K D N A - - - - - - - -	364
<i>Mus musculus</i>	K G A A E L M Q Q K E A T T E Q Q L R E L F E K Q K F K S A Q	407
<i>Rattus norvegicus</i>	K G A A E L M Q Q K E A T T E Q Q L R A L F E K Q K F K S A Q	407
<i>Homo sapiens</i>	K G T A E L M Q Q K E A T T E Q Q L K E L F E K Q K F K S Q -	406

Figure S1 Multiple alignment of BHMTs from different organisms

Primers	Sequence(5'-3')	Application
AjBHMT5-1	GCCCAACCACATCTGCTCC	5'RACE
AjBHMT5-2	CCTTCATCAACAGCCCATTC	5'RACE
AjBHMT3-1	CTTCAATGGACCAGAAACAGCG	3'RACE
AjBHMT3-2	TTCAAGTTTGGTCGTCGCTGTG	3'RACE
Adapter dT	GGCCACGCGTCGACTAGTACT(17)	RACE adapter
Adapter dG	GGCCACGCGTCGACTAGTACG(10)	RACE adapter
AjBHMTF	AGAGTTTGTTCAGAGCAGGTAGCG	Real-Time PCR
AjBHMTTR	CTCCTTCATCAACAGCCCATTC	Real-Time PCR
3'UTR-W-MluI	ATAGGCCGGCATAGACGCGTAGCTGGCGGGAAGTAAATG	Wildtype plasmid construction
3'UTR-W-HindIII	AAAGATCCTTTATTAAGCTTCGACTAGTACTTTTTTTTTTC	Wildtype plasmid construction
3'UTR-M-MluI	ATAGGCCGGCATAGACGCGTAGCTGGCGGGAAGTAAATG	Mutant plasmid construction
miR-137-M-1	ATACTGACTCCGTATACATTTACTTCCCGCCAGCT	Mutant plasmid construction-137 binding
miR-137-M-2	GTAAATGTTCCGTATGTCAGTATTTTGGTTGACTTGATC	Mutant plasmid construction-137 binding
miR-2008-M-1	AGTCCCCACCCAAGAAACATTACATCTAGAAGAGATA	Mutant plasmid construction-2008 binding
miR-2008-M-2	TGTAATGTTCCCAAGATCAGGGGTGTCAATAAATGAA	Mutant plasmid construction-2008 binding
3'UTR-M-HindIII	AAAGATCCTTTATTAAGCTTCGACTAGTACTTTTTTTTTTC	Mutant plasmid construction
AjBHMT-BamHI	CGCGGATCCGCGGTCAGAGCAGGTAGCGATGTAG	Plasmid construction
AjBHMT-NotI	AAATATGCGGCCGCTATAAATGTTCCGAAGAACCCGTGTA	Plasmid construction
Ajβ-actin-BamHI	CGCGGATCCGCGCTGCGTGTAGCCCCAGAGGAG	Plasmid construction
Ajβ-actin-NotI	AAATATGCGGCCGCTATAAAGTATTTCTCTCTGGTGGAGC	Plasmid construction
miR-137	TATTGCTTGAGAATACACGTAG	Real-Time PCR
	Qiagen miScript universal primer	
miR-2008	AGGCAAGATGTTGGCATAGCT	Real-Time PCR
	Qiagen miScript universal primer	
RNU6B	ACGCAAATTCGTGAAGCGTT	Real-Time PCR
	Qiagen miScript universal primer	
NCM	UUCUCCGAACGUGUCACGUTT	Negative control of miRNA mimics
	ACGUGACACGUUCGGAGAATT	

137M	UAUUGCUUGAGAAUACACGUAG	miR-137 mimics
	ACGUGUAUUCUCAAGCAAUAU	
2008M	AUCAGCCUCGCUGUCAAUACG	miR-2008 mimics
	UAUUGACAGCGAGGCUGAUUU	
NCI	CAGUACUUUUGUGUAGUACAA	Negative control of miRNA mimics
137I	CUACGUGUAUUCUCAAGCAAUA	miR-137 inhibitor
2008I	CGUAUUGACAGCGAGGCUGAU	miR-2008 inhibitor
siNC	UUCUCCGAACGUGUCACGUTT	Negative control of siRNA
	ACGUGACACGUUCGGAGAATT	
AjBHMT-siRNA	GGAUCAUCCUGAACUUGUUTT	AjBHMT specific siRNA
	AACAAGUUCAGGAUGAUCCTT	

Table S1 Primers and sequences used in the study

miRNA	putative targets
miR-137	DNA polymerase
	Tudor domain-containing protein 1
	Multiple coagulation factor deficiency 2
	Betaine homocysteine S-methyltransferase
	Trace amine associated receptor 20v
	Histone H2B
	Probable heat shock protein ssa2
	Probable heat shock protein ssa1
	ZZ-type zinc finger-containing protein
	FAT atypical cadherin 1
	von Willebrand factor type A, EGF and pentraxin domain containing 1
	Chromatin licensing and DNA replication factor 1
	Multiple EGF-like-domains 10
	Solute carrier family 5, member 9
	Fibrillin 2
	Histone methyltransferase Pr-set7/Set8
	Potential rRNA biogenesis helicase Dhr
	Aldehyde dehydrogenase
	SAM domain and HD domain-containing protein 1
miR-2008	Isocitrate dehydrogenase [NADP]
	Leucine-rich repeat-containing protein 15
	Allatostatin receptor
	Replication factor C subunit 2
	Phospholipase A2, group IB
	Matrix metalloproteinase 1
	Transcription termination factor, RNA polymerase II
	Group IID secretory phospholipase A2
	Superoxide dismutase [Cu-Zn] 5
	Leucine-rich repeat-containing protein 59
	Deleted in malignant brain tumors 1 protein
	Major heat shock 70 kDa protein Ab
	DNA-directed RNA polymerase
	ZZ-type zinc finger-containing protein
	Organic solute transporter subunit alpha
	Zinc finger protein Noc
	Inter-alpha trypsin inhibitor, heavy chain 3
	Cytochrome P450, family 26, subfamily b, polypeptide 1
	Nephronectin
	Zinc transporter ZIP4
	Betaine homocysteine S-methyltransferase
	Chaperone protein GP96
	Alkaline serine protease
	Heat shock protein 83
	Peptidase inhibitor 16
	G protein-coupled receptor 98
	Topoisomerase I beta
	Sodium/glucose cotransporter 4
	DNA replication licensing factor mcm5
	endothelial-specific receptor tyrosine kinase

Table S2 Putative targets of miR-137 and miR-2008