A norepinephrine-responsive miRNA directly promotes CgHSP90AA1
expression in oyster haemocytes during desiccation

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Abstract

Oyster *Crassostrea gigas* is one model mollusc inhabiting in the intertidal zone and is frequently stressed by desiccation. The adaptation mechanism of oyster to environmental stress involves multiple levels, and miRNA is one of the most important regulators in post-transcriptional level. In the present study, an oyster norepinephrine-responsive miRNA cgi-miR-365 was proved to contribute to the host adaptation against desiccation by directly promoting the expression of CgHSP90AA1.

Briefly, a significant increase of cgi-miR-365 was observed from the first day after aerial exposure and the up-regulation was vigorously repressed when oysters were injected with adrenoceptors antagonists. A total of 15 genes involved in biological regulation, metabolic process and response to stimulus were predicted to be modulated by cgi-miR-365. Among these genes, CgHSP90AA1 was up-regulated significantly during desiccation and could be down-regulated after simultaneous injection of adrenoceptors antagonists. The interaction between cgi-miR-365 and CgHSP90AA1 was subsequently verified *in vitro*, and a significant promotion of CgHSP90AA1 transcripts was observed after overexpressing cgi-miR-365 in either *in vitro* luciferase reporter assay or primarily cultured haemocytes. Meanwhile, CgHSP90AA1 transcripts decreased *in vivo* when cgi-miR-365 was repressed by its inhibitor during desiccation. Collectively, it was suggested that cgi-miR-365 could be induced by norepinephrine during desiccation and promote CgHSP90AA1 expression directly after binding to its 3'-UTR, which would provide new evidence in miRNA-mediated adaptation mechanism in oysters against intertidal stress.

Key words: Desiccation; Norepinephrine; HSP90; miRNA-mediated gene promotion

1. Introduction

The intertidal zone is a unique area connecting the ocean and land, which has been regarded as one of the harshest environment on the earth as its physical environment (tidal levels, temperatures, salinity, pH and *etc*) could alter harshly and dynamically in a short period [1, 2]. Notwithstanding, the intertidal region is also known as flourishing habitat for multiple organisms, such as Mollusca, Arthropoda, Echinodermata, and Annelida [1]. Meanwhile, the communities of inhabiting organisms as well as their morphology and physiology have been vigorously shaped

by the high variety of intertidal environment for a better adaptation [3-5]. For instance, previous studies have suggested that geographical distribution of intertidal organisms could be shaped by their thermal tolerance [6] and some intertidal barnacles could survive in a wide range of temperatures [7]. There has been growing efforts to investigate how those organisms respond to the dynamic environments. For example, some reports indicated that organisms could rapidly adjust the free amino acid concentration inside cell to thrive under osmotic stress [8]. However, more investigations are still needed to clarify the adaptation mechanisms of intertidal organisms.

The oyster *Crassostrea gigas* is a dominant sessile mollusc in intertidal zone throughout the world and weight greatly in aquaculture. The life span of adult oysters, unlike other intertidal organisms, is geologically limited to rock surfaces after their settlement at spat stage. Therefore, the oysters have evolved a complex mechanism to respond against the vigorously changing environments and were regarded as an ideal model in stress adaptation, especially the adaptation against desiccation [9]. It was observed that oysters could survive for several days during aerial exposure [10] and extensive biological activities might be modulated at the meantime [10-12]. For example, some ion channels and transporters would be altered significantly during desiccation in accordance with osmotic changes, and some protein chaperons including heat shock protein (HSP) 70 could be up-regulated to maintain homeostasis in protein synthesis [13]. Recently, it is found that stress hormones, such as norepinephrine (NE), also increased rapidly after desiccation in oyster haemolymph, indicating its unique role in maintaining homeostasis (unpublished data).

NE is a representative neurotransmitter in catecholaminergic nervous system and plays important roles in diverse physiological processes [14-16]. Accumulating

evidences have suggested that NE could modulate the emotion [17, 18], promote glucose production and uptake [19], and regulate vessels constriction [20] as well as the immune responses during pathogen infection [21, 22]. NE was regarded as a crucial stress hormone responsive to biotic or abiotic stresses [23, 24]. In invertebrates, the neurotransmitter NE was also found to be rapidly released after either acute or chronic stress [25-27] such as osmotic stress [28], cold stimulation [29] and pathogen infection [27]. Dozens of immune related genes could be modulated by NE, shaping the host immune response [30]. Recently, some reports also suggested that some miRNAs could be modulated by NE, demonstrating the complexity of NE-mediated modulation [31].

As an important class of gene regulators at post-transcriptional level, miRNAs have been identified in numbers of organisms and proved indispensable in almost all biological processes, including cell proliferation, differentiation, maturation and apoptosis [32]. It was found that stress responses of mammal cells could also be mediated by miRNAs. As an example, miR-21 was found induced in endothelial cells by shear stress and it modulated apoptosis and eNOS activity subsequently [33]. In addition, miR-29b, miR-144 and miR-200c were also up-regulated in oxidative stress, resulting in the alternation in oxidative stress tolerance [34-36]. Recently, sixteen miRNAs were found differentially expressed in oyster haemocytes after NE stimulation [31]. Given that NE concentration increased in oyster haemolymph after desiccation, it was speculated that above miRNAs might participate in cellular response against desiccation. Among those 16 miRNAs, cgi-miR-365 was of interest as its homologues was identified as a mechanosensitive miRNA and could target multiple genes to modulate cell proliferation, differentiation, adhesion and apoptosis [31]. The purpose of the present study was to (1) investigate the tissue distribution

and phylogenetic relationships of cgi-miR-365, (2) survey its expression pattern during aerial exposure and interaction with NE, (3) predict and verify the potential target genes of cgi-miR-365 during desiccation and hopefully provide new insights into the miRNA-mediated adaptation mechanism of oysters living in intertidal zone.

2. Methods and Materials

2.1. Oysters, desiccation stress and sample collection

Oysters *C. gigas* (averaging 150 mm in shell length, 70 mm in width) employed in the present study were collected from a local oyster farm in Qingdao, China and sawed at the shell close to adductor muscle for injection. All oysters were cultured with continuous aeration for two weeks before any experiment.

Fifteen healthy oysters were employed to survey the distribution of cgi-miR-365 in different tissues including haemocytes, hepatopancreas, gills, adductor muscle, mantle and gonad. The tissues from five random oysters were mixed together as one sample, and there were three biological replicates for each tissue. Expression level of cgi-miR-365 was examined by quantitative real-time PCR (qRT-PCR) after the miRNA extraction and cDNA synthesis.

A total of 180 oysters were subjected for aerial exposure assay and sampled every 24 h for transcriptional detection of cgi-miR-365 and CgHSP90AA1 (alternative name as CgHSP90- α). Briefly, oysters were placed in an empty tank with ambient temperature at 16°C and relative humidity at 40%. Haemocytes from five oysters were sampled randomly every 24 h from the posterior adductor muscle sinus for qRT-PCR analysis of cgi-miR-365. Haemocytes from another five oysters were collected simultaneously for subsequent qRT-PCR of CgHSP90AA1. Each trial contains three biological replicates.

2.2. Phylogenetic analysis cgi-miR-365

Homologues of cgi-miR-365 were first searched globally by blastn program at miRBase website (<http://www.mirbase.org/>) and subjected to ClusterX for sequence alignment. A phylogenetic tree was then constructed by MEGA 6.0 with neighbour-joining algorithm (bootstrap resampling for 1000 times).

2.3. Concomitant stimulation of adrenoceptor antagonists with desiccation

A total of 90 oysters were divided randomly into three groups, designated as seawater, prazosin and propranolol group, and they were subjected to concomitant stimulation of 100 μL sterilized seawater, 100 μL prazosin (α -adrenoceptor antagonist, 10 $\mu\text{mol L}^{-1}$, Sigma Aldrich) and 100 μL propranolol (β -adrenoceptor antagonist, 10 $\mu\text{mol L}^{-1}$, Sigma Aldrich) at the beginning of desiccation stress, respectively [37]. The haemocytes of five oysters from each group were collected randomly at 48 h post stimulation for further detection of cgi-miR-365 transcripts. Haemocytes from another five oysters were obtained at the same time for detection of CgHSP90AA1 transcripts.

2.4. Primary oyster haemocytes culture and NE stimulation

Primary oyster haemocytes culture was conducted following the methods described previously [37, 38]. In brief, a total of 5×10^6 haemocytes were first obtained with acid citrate-dextrose anticoagulant agent (22 g L^{-1} sodium citrate, 8 g L^{-1} citric acid, 24.5 g L^{-1} glucose, pH 7.4) at ratio of 8:1 and seeded into a 6-well plate in modified Leibovitz's L-15 medium (Gibco). After cultured overnight (at 16°C and relative humidity at 40%), cells were stimulated with NE (Sigma Aldrich) at a final

concentration of 10^{-7} mol L⁻¹ for 4 and 8 h and collected for detection of CgHSP90AA1 transcripts by qRT-PCR. Three replicates were employed in the trial.

2.5. Target prediction of cgi-miR-365 and GO analysis

Target genes of cgi-miR-365 were searched globally by miRanda software (parameter: score higher than 160, energy less than -25 kcal/mol) using oyster 3'-UTR sequences obtained according to genome information [39]. GO annotation was carried out by Blast2GO software and subjected to WEGO for illustration. GO terms were classified based on cellular component, molecular function and biological process.

2.6. miRNA mimics and inhibitors, and CgHSP90AA1 3'-UTR luciferase reporter assay

miRNA mimics of cgi-miR-365 were designed and synthesized by GenePharm and diluted into 20 μ mol L⁻¹ before employed in gain-of-function assay. miRNA control which could not mimic any miRNA were also synthesized by GenePharma as negative control. Cgi-miR-365 inhibitors in precise complementation with cgi-miR-365 were prepared for loss-function assay *in vivo*. Control inhibitor with no complementation against any oyster miRNAs was synthesized as well.

CgHSP90AA1 3'-UTR luciferase reporter assay was conducted with methods as previous reports [40]. Briefly, CgHSP90AA1 3'-UTR containing putative binding site of cgi-miR-365 (404 nt to 421 nt) was cloned using a pair of gene-specific primers (designed as wild type) and inserted into psiCHECK-2 vector (Promega) for subsequent co-transfection. A total of 100 μ g recombined vectors were then transfected into 1×10^5 HEK293T cells in blank group using Lipofectamine 2000

reagent (Invitrogen) and cells in cgi-miR-365 group or miRNA control group were also co-transfected with 5 pmol cgi-miR-365 mimics or miRNA control. At 24 h post transfection, cells were harvested for luminescence detection and qRT-PCR of CgHSP90AA1 3'-UTR. Point mutations at putative binding site were also prepared (mutated type 1: GGTAGGATTGGGGCATTT → CCATCCTAAGGGGCATTT; mutated type 2: GGTAGGATTGGGGCATTT → GGTAGGATTCTGGGGGCATT) and subjected for luciferase reporter assay. All trials were conducted with three replicates.

2.7. Assays of Gain-of-function of cgi-miR-365 *in vitro* and loss-of-function *in vivo*

Gain-of-function assay of cgi-miR-365 was conducted *in vitro* in primary haemocytes using Neon Transfection System (Invitrogen). In short, a total of 1×10^6 oyster haemocytes harvested in anticoagulant agent were first washed in phosphate buffered saline without Ca^{2+} and Mg^{2+} and diluted at a final density of $1 \times 10^7 \text{ mL}^{-1}$ by Resuspension Buffer. Haemocytes were then mixed with either cgi-miR-365 mimics at a final concentration of 10 nM (designated as cgi-miR-365 group) or miRNA control at a final concentration of 10 nM (designated as miRNA control group) and subjected to the Neon device for electroporation (parameter: voltage, 1400 V; width, 20 ms; pulses, 1 pulse). After cultured at modified L-15 medium for 24 h, the cells were harvested for transcriptional detection of cgi-miR-365 and CgHSP90AA1 by qRT-PCR.

Loss-of-function assay of cgi-miR-365 was conducted *in vivo* by injecting cgi-miR-365 inhibitors. Briefly, a total of 90 oysters were averagely divided into three groups including seawater, cgi-miR-365 inhibitor and control inhibitor group. They were firstly exposed in air (ambient temperature at 16°C and relative humidity at 40%)

and injected with 100 μ L sterilized seawater, 2.5 nmol cgi-miR-365 inhibitors (in 100 μ L sterile seawater) and 2.5 nmol control inhibitors (in 100 μ L sterile seawater), respectively at 24 h post air exposure. At the third day after desiccation, haemocytes from five oysters were collected for qRT-PCR of cgi-miR-365. The expression level of CgHSP90AA1 was also surveyed by qRT-PCR using haemocytes obtained from another five oysters. All trials were conducted with three replicates.

2.8. RNA isolation, cDNA synthesis and SYBR Green fluorescent qRT-PCR

The extraction of total RNA was conducted with Trizol reagent (Life technology) according the instructions. Extraction of total miRNA was conducted with PureLink miRNA Isolation Kit (Invitrogen). Subsequent cDNA synthesis qRT-PCR assay were conducted using methods described before [41]. All primers employed in the present study were listed in Table 3. The relative mRNA or miRNA expression was calculated using $2^{-\Delta\Delta C_t}$ method [42] after normalization with human endogenous glyceraldehyde phosphate dehydrogenase gene (HsGAPDH), oyster elongation factor (CgEF) or oyster 5S rRNA.

9. Statistical analysis

All data obtained were subjected to one-way analysis of variance (one-way ANOVA) followed by a multiple comparison (LSD algorithm) and given as mean $\pm$ S.D. Asterisks (**) or letters (a, b, c etc.) were marked on the top of error bars of the figures to indicate statistical differences (letters if $p < 0.05$; “**” if $p < 0.01$).

3. Results

3.1. Phylogenetic analysis of cgi-miR-365 and its tissue distribution pattern

Homologues of cgi-miR-365 were first searched across phylum by blastn program at miRBase. A total of 12 miRNAs (Table 1) were obtained and subjected to sequence alignment and phylogenetic analysis. Cgi-miR-365 was found highly conserved among all members of miR-365 family, sharing same seed region at 2-8 nt (AAUGCCC, Fig. 1, A). Meanwhile, most members of miR-365 family, except for cgi-miR-365 and sko-miR-365 (from *Saccoglossus kowalevskii*), are consistent in sequences, while substitutions were observed at the 1st, 9th, 10th and 11th nucleotide of cgi-miR-365. As a consequent, cgi-miR-365 was found branched separately with its homologues in vertebrates or in *S. kowalevskii* (Fig. 1, B).

The tissue distribution of cgi-miR-365 was determined by qRT-PCR. Cgi-miR-365 could be detected in different tissues including haemocytes, hepatopancreas, gill, adductor muscle, mantle and gonad. The expression level of cgi-miR-365 was found most abundant in gill, which was 12.33-fold of that in haemocytes (Fig. 1, C).

3.2. cgi-miR-365 was induced by NE during aerial exposure

The expression alternations of cgi-miR-365 during aerial exposure were then surveyed by qRT-PCR. The transcripts of cgi-miR-365 increased robustly since the first day of desiccation (12.54-fold of basal level) and peaked at the fourth day, which was 105.10-fold of that before challenge ($p < 0.01$). At the fifth day of desiccation, the cgi-miR-365 transcripts level decreased drastically in comparison with that at fourth day, which was 15.72-fold of basal level ($p < 0.01$) (Fig. 1, D).

Prazosin and propranolol were widely used antagonists for α -adrenoceptors and β -adrenoceptors respectively, and they were injected into oysters to block NE-mediated signalling pathway during desiccation. A significant decrease of cgi-miR-365 transcripts was observed in oyster haemocytes when adrenoceptors antagonists

were injected during desiccation stress. In detail, the transcripts level of cgi-miR-365 decreased to 0.33-fold of that in control group after prazosin injection ($p < 0.05$), and it declined to 0.75-fold of that in control group after propranolol injection ($p < 0.05$) (Fig. 1, E).

3.3. Gene Ontology (GO) analysis of target genes of cgi-miR-365

The target genes of cgi-miR-365 were searched globally by miRanda software using 3'-UTR sequences from all oyster genes. A number of 15 genes were annotated finally, including CgHSP90AA1, RNA 3'-terminal phosphate cyclase, sulfhydryl oxidase 1, acetyl-CoA carboxylase 1 and Ras-related protein Rab-20 (Table 2). The GO distribution was then annotated by Blast2GO and WEGO and multiple biological processes, including biological regulation, biosynthetic process, cellular metabolic process and responses to stimulus were predicted to be modulated by cgi-miR-365 (Fig. 2).

3.4. CgHSP90AA1 could also be induced by NE during aerial exposure

Among the target genes, HSP90 was known to be responsive against multiple stresses maintaining the homeostasis of cell. The mRNA expression level of CgHSP90AA1 was investigated in primary haemocytes after NE stimulation. The transcripts of CgHSP90AA1 increased rapidly after the NE incubation and reached 9.01-fold of basal level at 8 h ($p < 0.05$) (Fig. 3, A). The mRNA level of CgHSP90AA1 also increased during desiccation, which peaked at the third day after the stress (4.02-fold of basal level, $p < 0.05$) and returned to the basal level at the fifth day (Fig. 3, B). Meanwhile, a significant decrease of CgHSP90AA1 transcripts was observed when oysters were injected with either prazosin (0.23-fold of that in the first

day after desiccation, $p < 0.05$) or propranolol during the desiccation (0.64-fold of that in the first day after desiccation, $p < 0.05$) (Fig. 3, C).

3.5. The interaction between *cgi-miR-365* and *CgHSP90AA1* 3'-UTR *in vitro*

The interaction between *cgi-miR-365* and *CgHSP90AA1* was first verified *in vitro* by *CgHSP90AA1* 3'-UTR luciferase reporter assay. Two mutated types of *CgHSP90AA1* 3'-UTR along with the wild one were prepared, and all of them remained precise base-pair complementation with the seed region of *cgi-miR-365* (Fig. 4). The relative luminescence ratio of cells transfected with wild type *CgHSP90AA1* 3'-UTR increased remarkably after overexpression of *cgi-miR-365* (1.14-fold of that in miRNA control group, $p < 0.01$) (Fig. 5A). Similarly, the relative luminescence ratio in mutated types also increased when it was transfected with *cgi-miR-365* in comparison with miRNA control group (1.29-fold with mutated type1, 1.69-fold with mutated type 2, $p < 0.01$) (Fig. 5, B, C). The relative expression level of *CgHSP90AA1* 3'-UTR against *HsGAPDH* gene in transfected cells was also examined by qRT-PCR. Consequently, the *CgHSP90AA1* 3'-UTR transcripts in *cgi-miR-365* group increased to 1.46-fold of that in miRNA control group when the cells were transfected with wild type 3'-UTR ($p < 0.01$) (Fig. 5, D). When the cells were transfected with mutated type 3'-UTR, the relative expression level increased to 1.28-fold (mutated type 1, $p < 0.01$) and 2.35-fold (mutated type 2, $p < 0.01$), respectively (Fig. 5, E, F).

3.6. *cgi-miR-365* promoted *CgHSP90AA1* expression *in vivo*

Gain-of-function assay of *cgi-miR-365* was subsequently conducted in primary haemocytes. Compared with those in miRNA control group, *cgi-miR-365*

transcripts in cgi-miR-365 group ascended to 66.28-fold at 24 h post transfection. Meanwhile, the CgHSP90AA1 mRNA level increased to 8.92-fold in cgi-miR-365 group in comparison with miRNA control group ($p < 0.01$) (Fig. 6, A).

Loss-of-function assay of cgi-miR-365 was then conducted *in vivo* by injecting cgi-miR-365 inhibitors at the first day post desiccation. The cgi-miR-365 transcripts in cgi-miR-365 inhibitor group declined to 0.11-fold of that in control inhibitor group at the third day post desiccation ($p < 0.05$) (Fig. 6, B). The mRNA level of CgHSP90AA1 also declined significantly after cgi-miR-365 was repressed by cgi-miR-365 inhibitors (0.88-fold of that in control inhibitor group, $p < 0.05$) (Fig. 6, C). No significant changes of either cgi-miR-365 or CgHSP90AA1 transcripts were observed between seawater injection group and control inhibitor group.

4. Discussion

Catecholaminergic neuroendocrine system is one of the most important neuroendocrine systems in chordate and it can innervate multiple biological processes robustly, including metabolism, emotion and immune response [43, 44]. Recently, the catecholaminergic system was characterized in bivalves with crucial role in the response against bacteria [27]. The intertidal bivalves are frequently stressed by aerial exposure with daily fluctuations in tidal levels, and a robust mechanism is vital for bivalves to maintain the homeostasis during desiccation [9, 13]. In our previous studies, NE was found to be significantly increased in haemolymph of scallops [28] and oysters (unpublished data) during desiccation. Meanwhile, some miRNAs identified from oyster were also found to be modulated by NE [31]. It was audaciously deduced that the responses of oyster against desiccation might be vigorously modulated by NE via miRNA-mediated networks. In the present study, the

expression of cgi-miR-365, one of those NE-responsive miRNAs [31], was surveyed during aerial exposure. The transcripts of cgi-miR-365 increased remarkably at the first day after aerial exposure when NE was significantly up-regulated, reaching at 12.54-fold of basal level (Fig. 1, D). Moreover, when NE-mediated signalling was blocked with adrenoceptors antagonists, a significant repression on cgi-miR-365 transcripts were also observed (0.33-fold in prazosin group, and 0.75-fold in propranolol group, Fig. 1, E), demonstrating its transcriptional activation by NE, which was crucial during desiccation. To date, there is no report about miRNA transcriptionally activated by NE, while Sp1, NF- κ B and p53 are reported to activate mammalian miR-365 expression [45, 46]. Meanwhile, the translocation of CgRel to nucleus, one subunit of oyster NF- κ B, was found significantly promoted by NE in oyster haemocytes [30]. It was therefore deduced that the expression promotion of cgi-miR-365 by NE might be NF- κ B-dependent. Nevertheless, more investigations should be conducted to reveal the detailed mechanism of NE-mediated transcriptional activation on cgi-miR-365 during desiccation.

In mammals, miR-365 could modulate multiple biological processes by targeting histone deacetylase 4, interleukin 6, Bcl-2 and thyroid transcription factor 1 [45, 47-49]. In the present study, the target genes of cgi-miR-365 were searched globally by using oyster 3'-UTR information, and a total of 15 target genes including CgHSP70, RNA 3'-terminal phosphate cyclase, sulfhydryl oxidase 1 and acetyl-CoA carboxylase 1 (Table 2) were found, which were involved in biological regulation, metabolic process and response to stimulus (Fig. 2). However, none of the above genes was targeted by miR-365 homologues in other organisms, indicating the unique function of cgi-miR-365 in oysters. Besides, most of the target genes could play

fundamental roles in regulating biological events or metabolic processes, possibly suggesting a housekeeping role of cgi-miR-365.

Among the 15 potential target genes, only CgHSP90AA1 and acetyl-CoA carboxylase 1 were found significantly fluctuated in mRNA expression level in oysters after aerial exposal (unpublished transcriptome data, CgHSP90AA1 was up-regulated and acetyl-CoA carboxylase 1 was down-regulated). Acetyl-CoA carboxylase 1 was a rate-limiting enzyme in long-chain fatty acids biogenesis while HSP90 has been known as a highly conserved molecular chaperone in eukaryotic cells [50]. There are normally two HSP90 genes (HSP90AA1 and HSP90AB1) in mammalian genome [51], and both of them could stabilize and refold denatured proteins during stress, and help proteins to transport across the organelle membranes by cooperating with other protein chaperones [50]. HSP90 genes are therefore vital for almost all biological events as well as the homeostasis of host [52]. In the present study, CgHSP90AA1 expression was found significantly induced in primary oyster haemocytes after NE stimulation (Fig. 3, A), while it was also up-regulated during desiccation and down-regulated when NE-mediated signalling was blocked by adrenoceptors antagonists (Fig. 3, B, C). It indicated that CgHSP90AA1 could be promoted by cgi-miR-365 during desiccation.

Most miRNAs are reported to repress gene expression by binding mRNAs to form a RNA-induced silencing complex and leading to the degradation or translation inhibition [53]. Notwithstanding, some miRNAs from vertebrates and invertebrates could also promote gene expression directly after binding[54]. For example, miR-346 in p19 cells could promote the expression of a specialized splice variant of receptor-interacting protein 140 mRNA by binding to its 5'-UTR [55], and miR34a/b-5 could advance the translation of mouse cytoplasmic β -actin without increasing mRNA level

[56]. In the present study, the interaction between CgHSP90AA1 and cgi-miR-365 was first investigated by luciferase reporter assay *in vivo*. Consequently, the significant increases of both the relative luminescence ratio and the CgHSP90AA1 3'-UTR transcripts were observed in cells co-transfected with wild type CgHSP90AA1 3'-UTR and cgi-miR-365 (Fig. 5, A, D), indicating a promoting role of cgi-miR-365 in CgHSP90AA1 3'-UTR expression. In most circumstance, the base-pair complementation between seed region of miRNA and target mRNA is decisive in the miRNA-mRNA interaction while complementation at other parts of miRNA could further advance the modulatory effect [53]. To further confirm the promotion on CgHSP90AA1 3'-UTR by cgi-miR-365 in the assay, mutations were constructed at the putative binding site in the 3'-UTR (Fig. 4). As a result, the relative luminescence ratio and CgHSP90AA1 3'-UTR transcripts both increased in either mutation type 1 or type 2 and peaked when mutated CgHSP90AA1 3'-UTR was in complete complementation with cgi-miR-365 (mutated type 2) (Fig. 5, B, C, E and F), reconfirmed their interaction *in vitro*. Moreover, CgHSP90AA1 transcripts were also up-regulated significantly in oyster primary haemocytes transfected with cgi-miR-365 in comparison with that in the miRNA control group (Fig. 6, A), while decreased during desiccation when cgi-miR-365 was repressed by its inhibitor (Fig. 6, B, C), demonstrating the promotion of CgHSP90AA1 by cgi-miR-365 *in vivo*. Though there are accumulating reports about miRNAs to promote gene expression, the underlying mechanism is still remained largely unclear [54]. It has been reported that the miRNA-mediated gene promotion is condition-limited and cell-limited [57, 58], while the mRNAs without cap and typical poly(A) tail are reported to be enhanced more easily by miRNAs [59]. Additionally, some other special proteins are needed while the structure of mRNA is decisive during the modulation [60]. Unfortunately, the

present results failed to demonstrate the exact mechanism of how CgHSP90AA1 was promoted by cgi-miR-365. It has been reported that miRNAs could augment expression of target genes by either activating gene translation without increasing mRNA level or stabilizing mRNA from degradation [54]. Considering that CgHSP90AA1 transcripts decreased more significantly in NE receptor blocking assay (Fig. 3, C) than that in cgi-miR-365 loss-of-function assay (Fig. 6, C), it was speculated that the cgi-miR-365 could protect CgHSP90AA1 mRNA from degradation, which might be crucial in oysters during aerial exposure.

Inhabiting in the intertidal region, the oyster has accustomed to the environmental fluctuations including desiccation by evolving a robust modulation mechanism in maintaining its homeostasis [61]. The great expansion of genes coding for heat shock protein and inhibitors of apoptosis has been observed in oyster genome, which could contribute decisively to its sessile life in intertidal zone [61]. Meanwhile, only one copy of CgHSP90AA1 was identified in oyster genome, indicating its indispensable role in stress adaptation. On the other hand, miR-365 is an evolutionary conserved miRNA identified from multiple organisms across vertebrates and invertebrates. In the phylogenetic tree of miR-365 homologues (Fig. 1, A, Table 1), cgi-miR-365 was branched separately from others (Fig. 2, B), suggesting that it might evolved differently in oyster. Moreover, cgi-miR-365 was found to be constitutively expressed in all the tested tissues, especially in gill and mantle which could be easily exposed in air. It was speculated that the cgi-miR-365 mediated protection of CgHSP90AA1 transcripts could be ubiquitous in oyster tissues during desiccation or other stresses after NE was up-regulated, and contribute crucially to the maintaining of cellular homeostasis (Fig. 6, D). Though more works are still needed to fully elucidate the interaction between CgHSP90AA1 and cgi-miR-365, the present result

provided evidence for miRNA-mediated gene promotion, which would dedicate indispensably in oyster adaptation against desiccation.

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Figure Legends

Fig. 1. Phylogenetic analysis of cgi-miR-365 and its expression during desiccation.

Homologues of cgi-miR-365 were obtained from miRBase and subjected to ClusterX software for sequence alignment (A). Phylogenetic tree of cgi-miR-365 homologues were constructed by MEGA with neighbour-joining algorithm (B). Expression level of cgi-miR-365 in different tissues of oyster was surveyed by qRT-PCR (C). Changes of cgi-miR-365 transcripts were also investigated in oyster haemocytes during desiccation (D). Antagonists of α - and β -adrenoceptors were injected during desiccation to survey cgi-miR-365 expression changes (E).

Fig. 2. GO distribution of target genes of cgi-miR-365.

Target genes of cgi-miR-365 were predicted by miRanda software using oyster 3'-UTR sequences and subjected to WEGO for GO analysis.

Fig. 3. Expression pattern of CgHSP90AA1 after NE or desiccation stimulation.

Expression level of CgHSP90AA1 was investigated in oyster primary haemocytes at 4 and 8 h post NE stimulation (A). A significant increase of CgHSP90AA1 transcripts was observed since the third day post desiccation (C). The expression level of CgHSP90AA1 decreased remarkably when adrenoceptors were inhibited by antagonists during aerial exposure (C).

Fig. 4. Binding site of cgi-miR-365 on CgHSP90AA1 3'-UTR.

Putative binding site of cgi-miR-365 was found at wild type CgHSP90AA1 3'-UTR from 404 nt to 421 nt.

Fig. 5. Interaction between cgi-miR-365 and CgHSP90AA1 *in vitro*.

Interaction between cgi-miR-365 and CgHSP90AA1 was verified *in vitro* by CgHSP90AA1 3'-UTR luciferase reporter assay. Significant increases in the relative luminescence ratio were observed at 24 h post co-transfection of cgi-miR-365 and wild type (A) or mutated type 1 (B) or mutated type 2 (C) CgHSP90AA1 3'-UTR. The expression level of CgHSP90AA1 3'-UTR were determined simultaneously by qRT-PCR in above cells (D for wild type, E for mutated type 1, F for mutated type 2). Data were shown in boxplot with lines representing measured value.

Fig. 6. Interaction between cgi-miR-365 and CgHSP90AA1 *in vivo*.

Expression alternations of cgi-miR-365 and CgHSP90AA1 were surveyed at 24 h post gain-of-function assay of cgi-miR-365 in oyster primary haemocytes (A). Loss-of-function assay was conducted *in vivo* by injecting cgi-miR-365 inhibitors during desiccation and expression level of cgi-miR-365 (B) and CgHSP90AA1 (C) were determined subsequently. Cgi-miR-365 was suggested to promote the expression of CgHSP90AA1 directly during aerial exposure (D).

Table 1. Homologues of cgi-miR-365

ID	Sequence	Organism
cgi-miR-365	AAAUGCCCCCAGAAAUCCUAAA	<i>Crassostrea gigas</i>
sko-miR-365-3p	UAAUGCCCUUUGAAAUCCUAAA	<i>Saccoglossus kowalevskii</i>
hsa-miR-365a-3p	UAAUGCCCCUAAAAAUCCUUAU	<i>Homo sapiens</i>
hsa-miR-365b-3p	UAAUGCCCCUAAAAAUCCUUAU	<i>Homo sapiens</i>
mmu-miR-365-3p	UAAUGCCCCUAAAAAUCCUUAU	<i>Mus musculus</i>
rno-miR-365-3p	UAAUGCCCCUAAAAAUCCUUAU	<i>Rattus norvegicus</i>
dre-miR-365	UAAUGCCCCUAAAAAUCCUUAU	<i>Danio rerio</i>
fru-miR-365	UAAUGCCCCUAAAAAUCCUUAU	<i>Fugu rubripes</i>
gga-miR-365-3p	UAAUGCCCCUAAAAAUCCUUAU	<i>Gallus gallus</i>
xtr-miR-365	UAAUGCCCCUAAAAAUCCUUAU	<i>Xenopus tropicalis</i>
aca-miR-365-3p	UAAUGCCCCUAAAAAUCCUUAU	<i>Anolis carolinensis</i>
oan-miR-365-3p	UAAUGCCCCUAAAAAUCCUUAU	<i>Ornithorhynchus anatinus</i>
ame-miR-2788	CAAUGCCCUUCGAAAUCCCAA	<i>Apis mellifera</i>

Table 2. Target genes of cgi-miR-365

Gene ID	Gene Annotation	Accession Number
CGI_10024562	RNA 3'-terminal phosphate cyclase	EKC39161.1
CGI_10007524	Membrane progesterin receptor beta	EKC41258.1
CGI_10017200	Aryl hydrocarbon receptor nuclear translocator homolog	EKC32806.1
CGI_10024995	TBC1 domain family member 16	EKC30996.1
CGI_10024357	Protein pellino	EKC24574.1
CGI_10001195	Uncharacterized protein	EKC22910.1
CGI_10020580	Ras-related protein Rab-20	EKC40702.1
CGI_10016862	Sulfhydryl oxidase 1	EKC26420.1
CGI_10008463	Uncharacterized protein	EKC19491.1
CGI_10005358	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	EKC26599.1
CGI_10017621	Heat shock protein HSP 90 AA1	EKC25687.1
CGI_10015096	Acetyl-CoA carboxylase 1	EKC42778.1
CGI_10016631	FL cytokine receptor	EKC39899.1
CGI_10006244	Uncharacterized protein	EKC24196.1
CGI_10015190	ATP-binding cassette sub-family B member 8, mitochondrial	EKC24099.1

Table 3. Primers and RNAs used in this study

Category	Primer and RNA name	Sequence (5'-3')
RNAs	Cgi-miR-365(miRNA)	AAAUGCCCCCAGAAAUCCUAAA UAGGAUUUCUGGGGGCAUUUUU
	miRNA control	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT
	cgi-miR-365 Inhibitor	UUUAGGAUUUCUGGGGGCAUUU
	Control inhibitor	CAGUACUUUUGUGUAGUACAA
UTR clone primers qRT- PCR primers	CgHSP90AA1_wild_F_UTRclone	GTGTCATTTGAAAACGGGTGT
	CgHSP90AA1_wild_R_UTRclone	CGGCAAGTTATTTTGTAGCAGT
	CgHSP90AA1_mutated_1_F_in	GGCATCTGAAACTCCATCCTAAGGGG
	CgHSP90AA1_mutated_1_R_in	CATACAAAAATGCCCCCTTAGGATGGA
	CgHSP90AA1_mutated_2_F_in	GGTAGGATTTCTGGGGGCATTTTTG
	CgHSP90AA1_mutated_2_R_in	CAAAAATGCCCCCAGAAATCCTACC
	CgHSP90AA1_psi_XhoI	CTCGAGGTGTCATTTGAAAACGGGTGT
	CgHSP90AA1_psi_NotI	GCGGCCGCGCGCAAGTTATTTTGTAG
	CgHSP90AA1_RT_F	GACATGATTATCCCCATTCTGGA
	CgHSP90AA1_RT_R	ATACACTTGGCACTATTTGTGGATA
	HsGAPDH_F	AGGTCGGTGTGAACGGATTTG
	HsGAPDH_R	TGTAGACCATGTAGTTGAGGTCA
	CgEF-F	AGTCACCAAGGCTGCACAGAAAG
	CgEF_R	TCCGACGTATTTCTTTGCGATGT
	Cgi-miR-365	AAATGCCCCCAGAAATCCUAAA
	rRNA_5s	CAAGGATGACACGCAAAT