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Posted Date: 8 April 2026

doi: 10.20944/preprints202604.0444.v1

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Article

The lncRNA011760/miR-novel-91/*NIPA2* ceRNA Network Regulates Salinity Stress Response in Sea Cucumber (*Apostichopus japonicus*)

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Abstract

Low-salinity stress poses a critical constraint on the commercial aquaculture and survival of the sea cucumber (*Apostichopus japonicus*). This study investigated the regulatory network involving lncRNA011760, miR-novel-91, and their target gene *NIPA2* in response to salinity fluctuations. Using integrated in vivo and in vitro functional assays, we demonstrate that lncRNA011760 acts as a competitive endogenous RNA (ceRNA) to sponge miR-novel-91, thereby alleviating the post-transcriptional repression of *NIPA2*. Based on these molecular dynamics, we propose a novel inhibition-adaptation-survival three-stage model. Initially (0–3h), acute *NIPA2* upregulation enhances Mg^{2+} transport efficiency to mitigate osmotic shock. During the mid-stage (3–24 h), miR-novel-91-mediated *NIPA2* suppression creates a transient biosynthetic window, facilitating a shift from passive tolerance to active metabolic adaptation. Ultimately (24–48 h), lncRNA-driven *NIPA2* restoration sustains Mg^{2+} homeostasis, allowing the organism to enter a low-metabolism survival mode. These stage-specific shifts reflect the inherent physiological strategies of sea cucumbers as osmoconformers. Our findings elucidate the complex epigenetic orchestration of osmotic stress tolerance and highlight the lncRNA011760/miR-novel-91/*NIPA2* axis as a promising molecular target for the marker-assisted breeding of salt-tolerant strains.

Keywords: *Apostichopus japonicus*; salinity stress; ceRNA network; *NIPA2*

1. Introduction

The sea cucumber (*Apostichopus japonicus)* is an important marine aquaculture species in East Asia. The growing market demand has made the sustainable farming of sea cucumbers particularly important. However, intensifying global climate change and human activities have led to significant fluctuations in coastal salinity, posing a serious threat to the sustainable production of sea cucumbers [1,2]. As stenohaline invertebrates, sea cucumbers lack specialized osmoregulatory organs and are strictly confined to a narrow salinity range and are highly sensitive to changes in the salinity of their surrounding environment[3–5]. Consequently, hypoosmotic shock leads to a rapid disruption of intracellular osmotic equilibrium, triggering physiological dysfunctions, suppressed metabolic activity, and high mortality rates in aquaculture systems [6–8].

As an osmo-conforming marine invertebrate, sea cucumbers are considered to be strictly marine animals confined to a narrow salinity range [9] because sea cucumbers lack specialized osmoregulatory organs, rendering them particularly susceptible to salinity changes [10,11]. To overcome these challenges, sea cucumbers and other echinoderms have evolved a range of strategies to enhance their adaptability. According to Binyon (1972) and Diehl (1986), sea cucumbers exhibit hypo-osmotic regulatory mechanisms at low salinity and hyperosmotic regulatory strategies at high salinity [10,12]. Gray sea cucumber (*Holothuria grisea*) showed obvious protective behaviors:

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temporally regulate the osmotic pressure of its coelomic fluids by possibly contracting ambulacral feet and oral tentacles to reduce body wall permeability and slow coelomic fluid flow [13,14]. In extreme cases, sea cucumbers may discharge their intestines to adapt to salinity changes in the environment [15]. Adult *Echinus esculentus* can acclimate to decreased salinity, thereby increasing their resistance to subsequent hypo-osmotic stress, yet their capacity for acute tolerance is restricted [16]. Physiologically, they rely on cellular-level adjustments, primarily the active regulation of osmotic pressure through ion transport mechanisms [14,17,18]. Hypo-salinity stress induced an increase in the activity of Na^+/K^+-ATPase activity and antioxidant enzymes to mitigate oxidative damage from osmotic stress [19]. Inorganic ions, amino acids, nucleotides, and their derivatives play crucial osmoregulatory roles in sea cucumbers under hypo-osmotic [20]. In a sea star and two sea urchin species (*Asterias rubens*, *Psammechinus milliaris*, and *Strongylocentrotus droebachiensis*) could utilize various combinations of FAAs to maintain osmotic equilibrium [21]. While these physiological and metabolic responses are documented, the upstream molecular networks—specifically the epigenetic mechanisms orchestrating these complex processes—remain largely elusive.

Recent research has highlighted non-coding RNAs (ncRNAs), such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), as master regulators of gene expression under environmental stress [22,23]. Specifically, lncRNAs can function as competitive endogenous RNAs (ceRNAs), acting as molecular sponges to sequester miRNAs and thereby alleviating the post-transcriptional repression of target mRNAs [24]. Transcriptomic studies have found that lncRNA001074 acts as a ceRNA and can regulate the expression of the Na^+/K^+-ATPaseαsubunit (NKAα) under low-salt stress through let-7[25]. The over-expression of miR-novel-3 suppressed *SLC17a9*, whereas lncRNA015168 acted as a competitive endogenous RNA (ceRNA) to sequester miR-novel-3 to deal with salinity stress [26]. The emphasized the critical role of ncRNA networks in maintaining ionic gradients. However, the specific ceRNA axes governing small-molecule metabolic pathways and cation transport during salinity adaptation have not been fully characterized.

Among the various cations, Mg^{2+} is the second most abundant cation in cells, present in high concentrations and tightly regulated within cells, playing an important role in biochemical functions, particularly those involving ATP hydrolysis [27,28]. In vertebrates, Mg^{2+} homeostasis is tightly controlled by a diverse array of transporters, including *MAGT1*, *MRS2*, and the *SLC41* family [29]. Magnesium transporter protein 1 (*MAGT1*) is upregulated by low Mg^{2+} concentrations and is an essential protein in Mg^{2+} uptake into cells [27,28]. The *SLC41A1* and *SLC41A2* encode magnesium transport proteins 1 and 2 respectively (*MagT1* and *MagT2*), which both mediate the uptake of Mg^{2+} into cells [27,30]. *MAGT1* and *SLC41* families mediate generalized cellular uptake. Magnesium transporter *MRS2* homolog, mitochondrial (*MRS2*) mediates the influx of Mg^{2+} into the mitochondrial matrix [31]. *TRPM6* along with its close relative *TRPM7* may play an important role in regulating Mg^{2+} homeostasis in vertebrates[32]. Membrane magnesium transporter 1 (*MMGT1*) mediates the uptake of Mg^{2+} across the Golgi membrane [33]. *NIPA2* (Non-imprinted in Prader-Willi syndrome region gene 2) has emerged as a highly selective Mg^{2+} transporter localized in the plasma membrane and early endosomes [34]. Given that seawater Mg^{2+} concentrations correlate linearly with salinity, dynamic changes in environmental osmotic pressure directly impact the intracellular demand for Mg^{2+} to sustain metabolic flux. However, whether *NIPA2* is integrated into a ceRNA regulatory network to facilitate the active salinity adaptation of echinoderms remains entirely unknown.

Based on previous high-throughput transcriptome screening under simulated low-salinity stress (18 psu) and the evolutionary conservation of the ceRNA mechanism, we hypothesized that the lncRNA011760/miR-novel-91/*NIPA2* axis acts as a critical molecular switch mediating salinity adaptation in *A. japonicus* by orchestrating Mg^{2+} homeostasis and associated metabolic pathways. To test this, we employed quantitative real-time PCR (qRT-PCR) and rigorous in vivo and in vitro functional validations (including agomirs, mimics, and siRNA interference). Our objectives were to verify the regulatory interaction within the lncRNA011760/miR-novel-91/*NIPA2* axis; to elucidate the temporal dynamics of this axis during salinity stress; and to propose a mechanistic model linking molecular oscillations to physiological survival. This work provides a theoretical foundation for the

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molecular mechanisms of stress tolerance in echinoderms and identifies potential molecular biomarkers for the marker-assisted breeding of salt-tolerant sea cucumber varieties.

2. Materials and Methods

2.1. Tissue Sample Collection and Low-Salt Stress

The experiment was conducted at the Key Laboratory of Marine Aquaculture and Stock Enhancement in the Northern Seas of the Ministry of Agriculture and Rural Affairs, Dalian Ocean University. The animal research was approved by the Institutional Animal Care and Use Committee (IACUC) of Dalian Ocean University (Approval No.: DOU-IACUC-2023-0901). All experiments were conducted in accordance with the Guidelines for the Management and Use of Laboratory Animals of the Chinese Academy of Fishery Sciences and local regulations. Healthy sea cucumbers (average weight 20.2 g ± 1.1 g) were collected from a commercial farm in Liaoning Province, China. Before the experiment, the sea cucumbers were acclimated for 14 days under controlled conditions in 1000-liter fiberglass tanks (30 individuals per tank)：the temperature was 16-18℃, the pH is 7.8-8.0, and the dissolved oxygen is 5.0-6.0 mg/L (measured using the YSI ProODO® multiparameter meter, Yellow Springs Instruments, USA). Sediment (sieved to remove debris) was replaced every two weeks, and a customized diet plan (60% *Sargassum thunbergii*, 40% sea mud) was provided daily at 3–5% of body weight.

After acclimation, sea cucumbers were randomly distributed to the control and experimental groups with three replicate groups (n=3 tanks/group, with 30 sea cucumbers per tank). For salinity challenge, the seawater salinity was gradually reduced from 30 PSU (control) to 18 PSU within 24 hours using deionized water to minimize osmotic shock. Control groups were maintained at 30 PSU throughout the experiment. Five experimental subgroups were established to verify the function under 18 PSU stress: negative control (NC) group (transfected with non-targeting oligonucleotides); miR-novel-91 agomir-transfected group; miR-novel-91 mimics-transfected group; si-NIPA2-transfected group; lncRNA011760 mimics-transfected group. Transfection was carried out 24h before salinity stress by intraperitoneal injection (50 μL per individual). Sea cucumber samples were randomly collected at six time points after salinity stress (1.5, 3, 6, 12, 24, and 48h), with 3 individuals per group at each time point. Coelomic fluid was extracted by puncturing the coelom with a syringe, centrifuged at 3000 × g for 5 minutes at 4 °C, and then the supernatant was immediately frozen in liquid nitrogen and stored at -80 °C for subsequent RNA extraction and sequencing. The entire experimental design used in this study was shown in Figure 1.

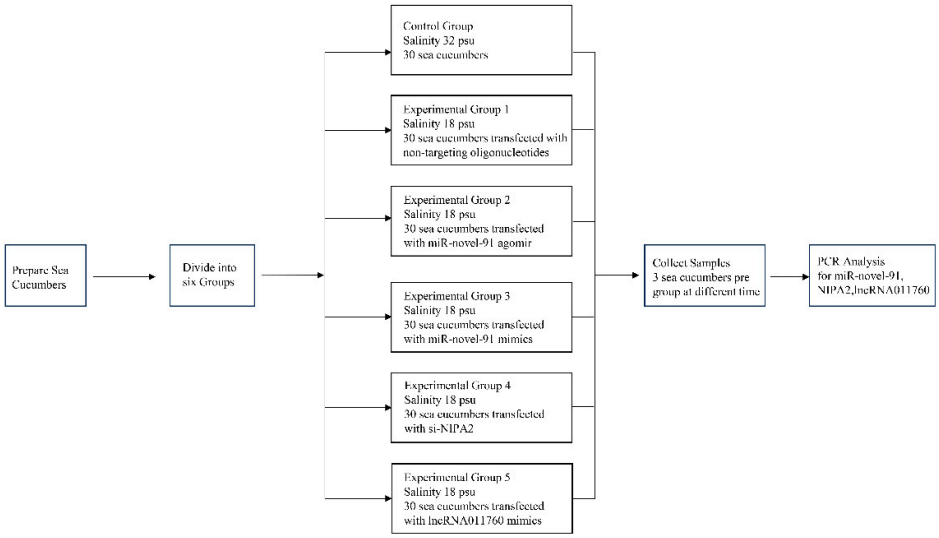

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Figure 1. The entire experimental procedure used in this study.

2.2. RNA Extraction, cDNA Synthesis, mRNA, miRNA and LncRNA Expression

Total RNA was extracted from body cavity fluid samples using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions [35]. The purity and integrity of the RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and 1.5% agarose gel electrophoresis, respectively. miRNA-specific cDNA was synthesized using the miRcute Plus miRNA First-Strand Kit (Sangon Biotech, Shanghai). The 20 μL reaction system contained 100 ng of total RNA, 10 μL of 2× miRNA P-RT Solution mix, 2 μL of miRNA P-RT Enzyme mix, and nuclease-free water to reach the final volume. The reaction was carried out at 37 °C for 60 minutes, followed by incubation at 85 °C for 5 minutes. The first-strand synthesis of lncRNA cDNA was performed using the lnRcute lncRNA cDNA First-Strand Synthesis Kit (Degenmol, Tiangen Company). The preparation and handling of the reaction system were as follows: 100 ng of RNA, 2 μL of 5× gDNA Buffer, and RNase-free water were added in a 10 μL volume. The mixture is combined and incubated at 42 °C for 3 minutes, then placed on ice. Into the mixture, 2 μL of 10× lnR RT Buffer, 1 μL of lnR RT Enzyme mix, 2 μL of lnR-RT Primer mix, and RNase-free water were added to reach a final volume of 20 μL. The reaction mixture is incubated at 42 °C for 15 minutes, then at 95 °C for 3 minutes, and stored at 80 °C in preparation for subsequent experiments.

The qRT-PCR was performed using the LightCycler® 96 (Roche Diagnostics, Basel, Switzerland) with 2×SG Fast qPCR Master Mix (Sangon Biotech, Shanghai, China). The 20 μL reaction system consisted of 10 μL of 2×SG Fast qPCR Master Mix, 1.0 μL of cDNA template, 0.4 μL of each primer (10 mmol/L), and nuclease-free water to a final volume. The reaction program was as follows: pre-denaturation at 95 °C for 30 seconds; 55 cycles of denaturation at 95 °C for 3 seconds and annealing at 60 °C for 30 seconds. All reactions were performed with three technical replicates and three biological replicates, with *Cytb* and *RNU6B* serving as reference genes for mRNA and miRNA/lncRNA, respectively[25,36]. All primers were designed using Primer 5.0 software and synthesized by Sangon Biotech (Shanghai, China) (Table 1). Each sample was analyzed in three technical replicates and three biological replicates. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. miR-novel-91, *NIPA2,* and lncRNA011760 homologous gene primers used for expression.

Categroy	Sequences name	Sequence (5′-3′)
qRT-PCR Primers	*NIPA2*-F	CTGACCTGCCTTCCGTGAGTAAG
	NIPA2-R	TGGCTGCTCTCCTCCTCTGAC
	miR-novel-91	CATGTGACCGTTACAATGGGCG
	lncRNA011760-F	GGAGAGCCTAGATTATGATACCGTTAC
	lncRNA011760-R	TTCACCACTAATCGTTCCACCAAG
	Cytb-F	TGAGCCGCAACAGTAATC
	Cytb-R	AAGGGAAAAGGAAGTGAAAG
	U6	ACGCAAATTCGTGAAGCGTT
Functional oligonucletides	Negative Control	UUGUACUACACAAAAGUACUG GUACUUUUGUGUAGUACAAUU
	miR-novel-91	AUGUGACCGUUACAAUGGGCG
	agomir/minics	CCCAUUGUAACGGUCACAUUU
	si-*NIPA2*	GCGAUAUCUACAAGAAACUTT AGUUUCUUGUAGAUAUCGCTT

2.3. Culture of Sea Cucumber Coelomocytes

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Collected 1 mL of coelomic fluid from healthy sea cucumbers and mix it 1:1 with ice-cold anticoagulant solution, centrifuged at 1000 × g for 5 minutes at 4 °C (Eppendorf 5810R centrifuge, Hamburg, Germany), then washed twice with isotonic buffer (Leibovitz's L-15 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin). Resuspend the coelomic cells in Leibovitz 's L-15 medium, adjusting the cell density to 6×10^5 cells/mL. Dispensed 500 μL of the cell suspension (6×10^5 cells per well) into 24-well culture plates. Cultured in the dark under humid conditions at 16 °C with 5% CO_2. After 24 hours of culture, washed the adherent coelomic cells twice with ice-cold PBS (pH 7.4) and collected them by centrifugation at 1000g for 5 minutes for transfection.

2.4. miRNAs, lncRNA and Targeted Gene Overexpression and Knockdown Experiments in Sea Cucumber Coelomocytes

Selected miR-novel-91, lncRNA011760, and their target gene *NIPA2* for functional validation through overexpression (agomir) and inhibition (antagomir) to elucidate their roles in salinity stress response. Used Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, transfected 30 pmol miR-novel-15 agomir/mimic, si-*NIPA2*, lncRNA011760 mimics, or the corresponding negative controls into adherent co-cultured cells. In short, diluted the oligonucleotides and Lipofectamine 2000 in 50 μL of serum-free L-15 medium, mixed and incubated at room temperature for 20 minutes. Then added the transfection mixture to each culture well and continued incubating the cells for 24 hours. After transfection, exposed the cells to low salinity stress (18 PSU), diluting seawater with deionized water, and collected samples at 6, 24, and 48 hours post-stress for RNA extraction and qRT-PCR analysis.

2.5. Statistical Analysis

All data are presented as mean ± standard deviation (SD). The statistical differences in the temperature and spatial expression of miR-novel-91, *NIPA2*, and lncRNA011760 were analyzed using one-way ANOVA (post-test using LSD) tested in SPSS software (version 22.0). A significance level of $P<0.05$ was set. Graphs were generated using GraphPad Prism 8.0 software (GraphPad Software, San Diego,CA, USA).

3. Results

3.1. Identification of miR-novel-91 and Target Gene NIPA2

The corresponding target genes of miR-novel-91 were predicted by using the software miRanda v3.3a and the local database, and the miR-novel-91 and *NIPA2* corresponding binding sites are shown in Figure 2D. The length of the mature region of miR-novel-91 was 21, the length of the target gene *NIPA2* was 1000, the sequence alignment score was 154, the minimum free energy was -24.0 kcal/mol, and the $P<0.05$ were correlated, and the 2-20 on the mature region of miR-novel-91 was combined with the 433-450 locus on the 3'-UTR region of *NIPA2*.

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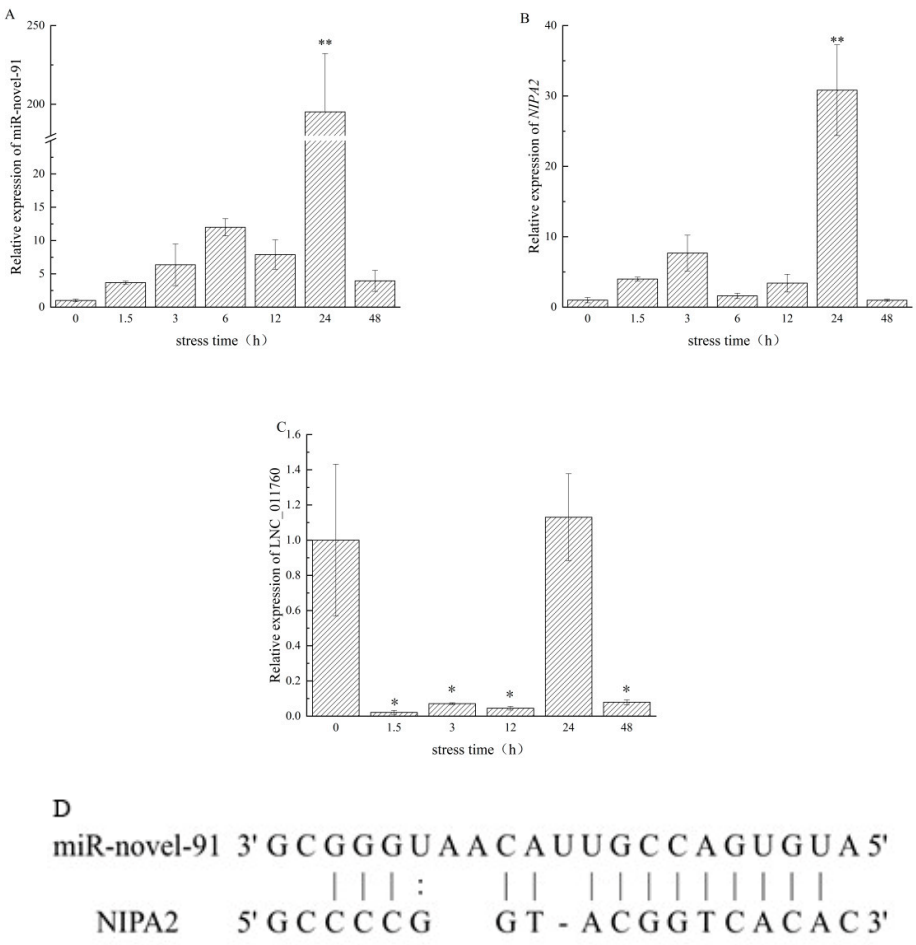

Figure 2. Expression profiles of miR-novel-91 and *NIPA2* during low-salinity adaptation in *A. japonicus*. (A) Relative expression of miR-novel-91. (B) Relative expression of *NIPA2*. (C) Relative expression of lncRNA011760. Data are presented as mean ± standard error of the mean (SEM) (n=3 biological replicates). Note: * indicates a significant difference compared to the control group ($P < 0.05$), ** indicates a highly significant difference compared to the control group ($P < 0.01$). (D) Binding site maps of miR-novel-91 and its corresponding target genes.

3.2. Expression Profiles of miR-novel-91, lncRNA011760 and NIPA2 Under Salinity Stress

The temporal expression patterns of miR-novel-91, lncRNA011760, and target gene *NIPA2* during salinity stress response were determined by real-time quantitative PCR (Figure 2). The expression level of miR-novel-91 gradually increased with the duration of stress, peaking at 24 hours with a significantly higher expression relative to the control group (Figure 2A). Exhibiting a similar to that of miR-novel-91, *NIPA2* showed a gradual upregulation from 0 to 3 hours and peaked significantly at 24 hours, before returning to the basal, unstressed level by 48 hours (Figure 2B). The relative expression of lncRNA011760 displayed significant fluctuations across the time course. Notably, its expression dropped below the detection limit at 6 hours—a result confirmed by repeated assays—yet rebounded at 24 hours to a level showing no significant difference from the unstressed control group (Figure 2C). These dynamic patterns indicate that the 24-hour mark represents a critical temporal window for the involvement of miR-novel-91 and its target *NIPA2* in the salinity adaptation process of the sea cucumber.

3.3. Validation of the miR-novel-91/NIPA2 Regulatory Axis In Vivo

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To verify the regulatory relationship between miR-novel-91 and *NIPA2*, in vivo functional assays were conducted under low-salinity stress using miR-novel-91 agomir and small interfering RNA targeting *NIPA2* (si-*NIPA2*) (Figure 3). The transcript levels of both genes exhibited significant dynamic alterations depending on the delivery conditions. In the miR-novel-91 agomir-treated group, the basal expression of miR-novel-91 was initially lower than that of the control group prior to stress induction; however, it increased progressively with the duration of the low-salinity stress. This upregulation reached a highly significant level at 24 hours ($P<0.01$) and remained significantly elevated at 48 hours ($P<0.05$) (Figure 3A). Concurrently, the expression of *NIPA2* displayed a highly significant variation at 6 hours and was notably repressed compared to the control group by 48 hours (Figure 3B), demonstrating an inverse expression pattern relative to the microRNA. Conversely, in the si-*NIPA2*-treated group, the expression of miR-novel-91 showed a highly significant difference prior to stress and was subsequently downregulated as the stress duration extended, dropping below the control group level after 24 hours (Figure 3C). Meanwhile, the expression of *NIPA2* was upregulated over the stress time course, peaking with a highly significant difference at 24 hours (Figure 3D). Collectively, these temporal expression dynamics confirm a negative regulatory relationship between miR-novel-91 and *NIPA2* in *A. japonicus* during low-salinity adaptation.

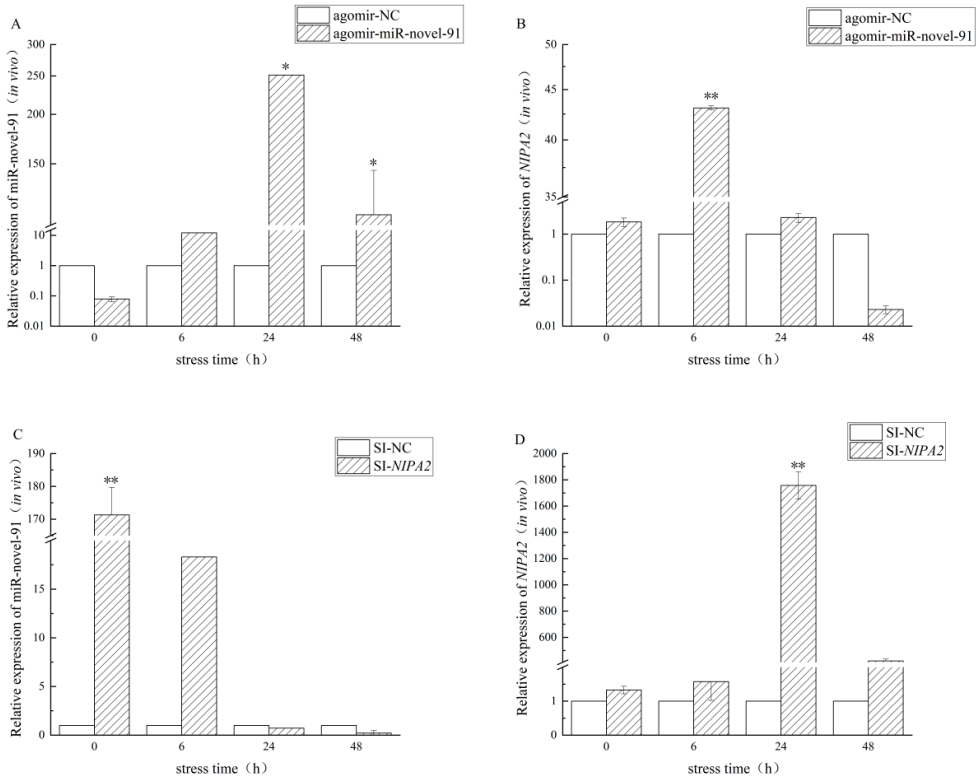

Figure 3. Expression profiles of miR-novel-91 and *NIPA2* in *A. japonicus* under low-salinity stress following in vivo delivery of miR-novel-91 agomir or si-*NIPA2*. (A) Expression of miR-novel-91 after injection of miR-novel-91 agomir; (B) Expression of *NIPA2* after injection of miR-novel-91 agomir; (C) Expression of miR-novel-91 after transfection with si-*NIPA2*; (D) Expression of *NIPA2* after transfection with si-*NIPA2*. Data are presented as mean ± SEM (n=3 biological replicates). Note: * indicates a significant difference compared with the control group ($P<0.05$), ** indicates a highly significant difference compared with the control group ($P<0.01$).

3.4. Validation of the miR-novel-91/NIPA2 Regulatory Axis In Vitro

The regulatory interaction was further validated in vitro using cultured *A. japonicus* coelomocytes transfected with miR-novel-91 mimics or si-*NIPA2* (Figure 4). In the miR-novel-91

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mimics-transfected group, the expression of miR-novel-91 peaked immediately post-transfection (0 hours) and maintained a highly significant elevation at 6 hours ($P < 0.01$). Subsequently, with prolonged stress exposure, its expression was downregulated, dropping below that of the control group at 24 hours (Figure 4A). The expression of the target gene *NIPA2* was significantly repressed at 24 hours compared to the control, but later exhibited a highly significant rebound at 48 hours (Figure 4B). In the experimental group transfected with si-*NIPA2*, the expression of miR-novel-91 was progressively upregulated throughout the stress period, reaching a highly significant peak at 48 hours (Figure 4C). Meanwhile, the expression of *NIPA2* displayed a highly significant peak at 0 hours, sharply declined below the control level at 6 hours, and subsequently rebounded, showing a significant upregulation again by 48 hours (Figure 4D). Collectively, these in vitro temporal dynamics substantiate that miR-novel-91 actively regulates *NIPA2* expression, further confirming the critical role of this microRNA-target interaction in the cellular response to salinity stress.

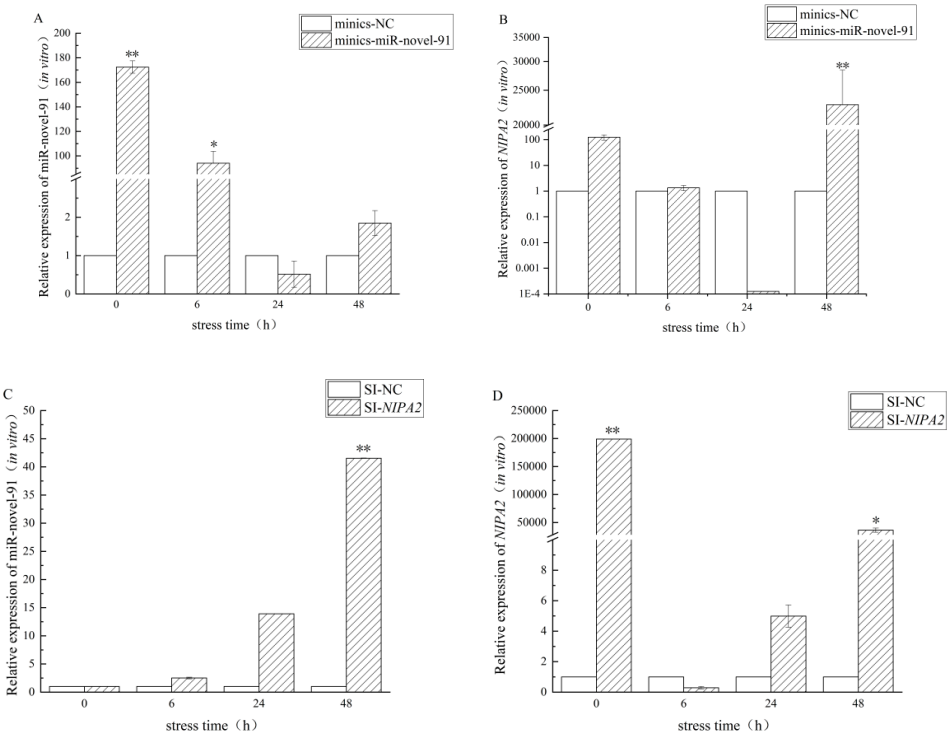

Figure 4. Expression profiles of miR-novel-91 and *NIPA2* in *A. japonicus* coelomocytes (in vitro) under low-salinity stress following the transfection with miR-novel-91 mimics or si-*NIPA2*. (A) Expression of miR-novel-91 after transfection with miR-novel-91 mimics; (B) Expression of *NIPA2* after transfection with miR-novel-91 mimics; (C) Expression of miR-novel-91 after transfection with si-*NIPA2*; (D) Expression of *NIPA2* after transfection with si-*NIPA2*. Note: * indicates a significant difference compared with the control group ($P < 0.05$), ** indicates a highly significant difference compared with the control group ($P < 0.01$).

3.5. Proposed Mechanism of Salinity Response via the lncRNA011760/miR-novel-91/NIPA2 Axis and Small Molecule Pathway

Based on the observed expression profiles and functional validation results, a three-stage adaptive model for sea cucumber salinity adaptation was proposed (Figure 5). This model operates through a ceRNA regulatory network, in which the lncRNA011760-miR-novel-91 axis tightly regulates *NIPA2* expression, thereby fine-tuning Mg^{2+} transport and associated small-molecule metabolic pathways. The chronological cascade of this osmotic stress response is categorized into the following three distinct physiological phases. The initial stage (0-3 hours) is acute osmotic shock and passive tolerance. Upon exposure to acute salinity fluctuations, the intracellular ion balance of the

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sea cucumber is rapidly disrupted, challenging the homeostasis of inorganic cations such as Mg^{2+}. At 0 hours, lncRNA011760 is significantly upregulated to act as a ceRNA sponge, sequestering miR-novel-91. This event effectively relieves the post-transcriptional repression of *NIPA2*, driving a marked increase in its expression (Figure 2). As an essential cation transporter, the enriched *NIPA2* facilitates the transmembrane flux of Mg^{2+} and other inorganic cations. This immediate molecular response stabilizes fundamental intracellular cation homeostasis and mitigates the structural and metabolic damage induced by the early osmotic shock.

Mid-stage (3-24 hours) was active physiological adaptation period. As the stress duration extends, the sea cucumbers gradually transition from passive tolerance to an active osmotic remodeling state. During this critical window, lncRNA011760 expression is markedly downregulated, which releases the sequestered miR-novel-91, leading to its significant upregulation and subsequent direct targeting of *NIPA2* (Figure 5). The targeted suppression of *NIPA2* strategically modulates the transport efficiency of inorganic cations. This regulatory bottleneck recalibrates the metabolic rhythm of small-molecule pathways, accommodating the dynamic energetic and ionic demands required for the cells to establish a newly adapted intracellular ionic equilibrium.

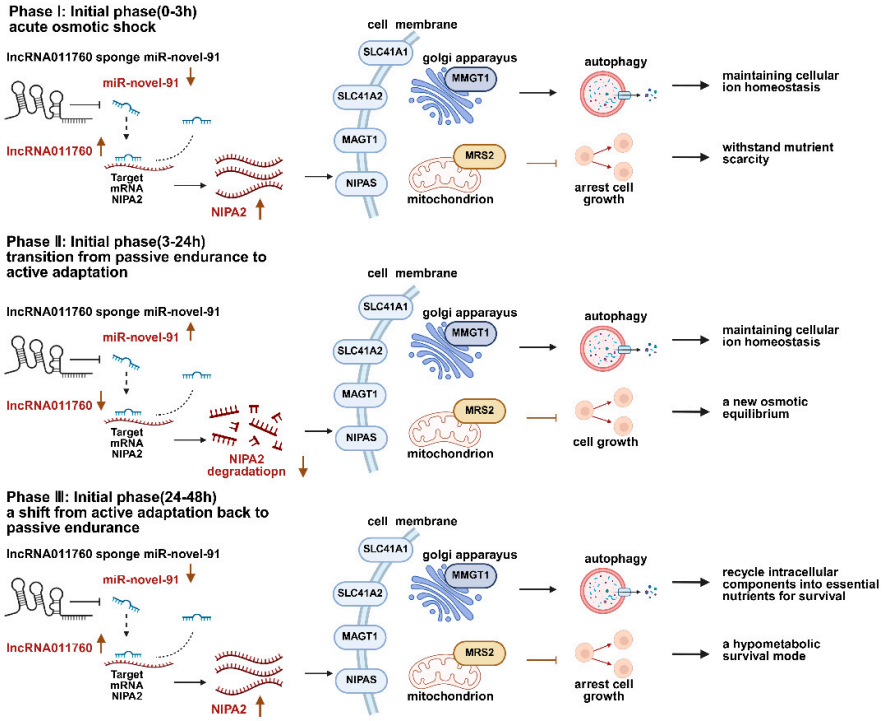

Figure 5. A three-stage adaptive model for salinity acclimation in sea cucumbers based on the results and small molecule pathway. (Created in https://BioRender.com).

Late stage (24–48 hours) is low-metabolism survival and potential autolysis. Under prolonged osmotic stress, the intracellular energy and nutrient reserves approach critical depletion. By 48 hours, the expression of lncRNA011760 significantly rebounds, re-engaging its sponge effect on miR-novel-91 and consequently restoring *NIPA2* expression levels. The recovered *NIPA2* sustains the basal transport of Mg^{2+} and maintains the essential functions of small-molecule pathways. This shift ensures the homeostasis of critical intracellular cations while minimizing global energy expenditure, effectively transitioning the organism into a low-metabolism survival mode to endure extreme stress. However, if long-term salt stress persists beyond physiological limits, the prolonged energetic deficit may trigger excessive autophagy and an irreversible arrest of anabolic metabolism, ultimately culminating in autolysis and visceral damage.

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4. Discussion

4.1. The miR-novel-91/NIPA2 Regulatory Axis in Salinity Stress

MicroRNAs (miRNAs) are key post-transcriptional regulatory factors that trigger mRNA degradation or translational repression by binding to the 3′ untranslated region (UTR) of target mRNAs [22,37]. In this study, we identified a negative regulatory relationship between miR-novel-91 and its target gene *NIPA2* in sea cucumbers under salinity stress. Experimental data revealed that under low-salinity conditions, miR-novel-91 and *NIPA2* exhibited coordinated temporal dynamics, both reaching peak significance at 24 hours. The involvement of non-coding RNAs in echinoderm osmoregulation has recently gained considerable attention. Recent evidence reveals that parallel ceRNA networks, such as the lncRNA001074/let-7/NKAα axis and the lncRNA015168/miR-novel-3/*SLC17A9* transmembrane transporter axis, also exhibit critical inflection points at 24 hours under salinity stress[19, 32]. These consistent temporal dynamics across different RNA networks strongly validate our findings and suggest that ceRNA-directed modulation of ion transporters is a universally conserved epigenetic strategy for osmoregulatory plasticity in sea cucumbers.

In vivo functional validation further demonstrated that overexpression of miR-novel-91 first upregulated and then downregulated *NIPA2*, whereas knockdown of *NIPA2* expression led to upregulation of miR-novel-91. It is speculated that sea cucumbers consume miR-novel-91 in response to low-salinity stress, temporarily relieving the inhibition of *NIPA2*. The miR-novel-91 overexpression elicited a biphasic response in *NIPA2*, while *NIPA2* knockdown triggered a compensatory upregulation of miR-novel-91. This feedback loop suggests that *A. japonicus* may modulate miR-novel-91 levels to transiently alleviate *NIPA2* inhibition, thereby facilitating a rapid response to osmotic fluctuations. Liu et al. found that *NIPA2* deficiency led to an increased frequency of spontaneous action potentials in neurons and a decrease in BK channel currents, whereas a specific BK channel activator NS11021 was able to reduce neuronal excitability in *NIPA2* knockout mice [38].

4.2. Physiological Implications of Metabolic Modulation in Salinity Adaptation

NIPA2 is a highly selective magnesium transporter localized primarily on the plasma membrane and early endosomes, functioning to transfer extracellular Mg^{2+} into the cytoplasm [39]. The study also found that downregulation of *NIPA2* expression can induce mitophagy, thereby affecting the osteogenic ability of osteoblast [40]. *NIPA2* is involved in 29 entries, including 13 biological processes, 5 cellular components, and 11 molecular functions. *NIPA2* is mainly located on the plasma membrane and, together with *NIPA1*, functions as a magnesium ion transport channel. *NIPA2* is mainly found in the plasma membrane and functions together with *NIPA1* as a magnesium ion transport channel. Studies have shown that *NIPA2* mediates selective magnesium ion transport in the plasma membrane of Xenopus laevis. Goytain et al. found that *NIPA2* expression was upregulated under low magnesium conditions, and its function as a selective magnesium transporter was confirmed in Xenopus laevis oocyte[34]. *NIPA2* protein is primarily localized to early endosomes and the cell membranes and redistributes to the cell membranes under low magnesium conditions. Mutant *NIPA2* proteins are unable to efficiently transport magnesium ions to the cell membrane, leading to reduced intracellular magnesium ion concentration [41]. Some studies have identified *NIPA2* as a potential target for treating osteoporosis in type 2 diabetes. AGEs can dose-dependently reduce *NIPA2* expression, thereby affecting intracellular magnesium levels, osteoblast apoptosis, and osteoclast activity [39]. Downregulation of *NIPA2* expression activates mitochondrial autophagy, which in turn affects the osteogenic capacity of osteoblasts. At the same time, *NIPA2* can regulate PINK1/Parkin-mediated mitophagy through the PGC-1α/FoxO3a/MMP pathway [40].

Mg^{2+} is an abundant cation that is important for many intracellular biochemical functions, especially as a cofactor for ATP. The process by which cells produce energy consists of many Mg^{2+}-dependent enzymatic reactions, and disruption of Mg^{2+} homeostasis impairs ATP production by affecting energy metabolism and morphological changes[42]. *NIPA2* acts as a selective Mg^{2+} transporter and helps maintain Mg^{2+} influx [41], is involved in the transport of inorganic cations such

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as Mg^{2+} in small molecule pathways. Small molecule signaling pathways can regulate the physiological state of cells in response to changes through intracellular and extracellular signal transduction [43]. As a key mediator linking Mg^{2+} transport with the regulation of small molecule pathways, the loss or downregulation of *NIPA2* can have a significant impact on small molecule pathways. Mg^{2+} is an indispensable cofactor for ATP-dependent enzymatic reactions, linking its homeostasis intrinsically to energy metabolism [44,45]. At the early stage of acute low-salinity stress (0–3h), the rapid decline in environmental and coelomic fluid Mg^{2+} concentration acts as an intracellular starvation signal, triggering the immediate upregulation of *NIPA2* (Figure 5). Previous physiological studies on intertidal holothurians demonstrate that while sodium and chloride levels fluctuate passively with external salinity, Mg^{2+} exhibits a distinct pattern of active homeostatic control [45]. The acute upregulation of *NIPA2* enhances the efficiency of Mg^{2+} transmembrane transport and uptake, thereby reshaping intracellular Mg^{2+} homeostasis [46], systemically regulating small molecule pathways and inorganic cation balance, and alleviating damage caused by initial osmotic stress.

As stress progresses to the mid-stage (3–24 h), *A. japonicus* undergoes a strategic shift from passive endurance to active physiological adaptation. This transition involves a complex synergy of ion transport—particularly the fluctuant activation of Na^+/K^+-ATPase and the mobilization of intracellular osmolytes [19,20,47]. *NIPA1* expression increases as Mg^{2+} concentration decreases, enhancing Mg^{2+} uptake to maintain homeostasis [48]. Behaviorally, adaptive strategies are manifested through defensive mechanisms, such as reducing body wall permeability. This was observed in the gray sea cucumber (*Holothuria grisea*), which lowers body wall permeability by contracting its tube feet and oral tentacles, thereby reducing water influx and alleviating osmotic stress[45]. The miR-novel-91 is significantly upregulated, inhibiting *NIPA2* and reducing the transport efficiency of inorganic cations such as Mg^{2+} (Figure 2A-B). The significant upregulation of miR-novel-91 during this phase suppresses *NIPA2*, effectively recalibrating the transport efficiency of Mg^{2+}. This regulatory bottleneck adjusts the metabolic process of small-molecule pathways, allowing cells to establish a newly adapted ionic equilibrium and providing support for adapting to the osmotic environment [39,40].

As an organism that lacks specialized organs for regulating osmotic pressure, sea cucumbers can only live within a certain salinity range and are very sensitive [17]. Prolonged salinity stress eventually leads to the depletion of cellular energy reserves, such as glucose and branched-chain amino acids [49]. As cellular energy and amino acid reserves are depleted, they shift from active adaptation to passive tolerance. At 48 hours, the rebound of lncRNA011760 acts as a ceRNA sponge to sequester miR-novel-91, effectively restoring *NIPA2* levels and maintaining basal Mg^{2+} influx (Figure 4). This shift ensures the persistence of essential metabolic functions while transitioning the organism into a low-metabolism survival mode [46].

Crucially, Mg^{2+} availability and *NIPA2* expression are tightly linked to mitochondrial physiology and autophagy. In vertebrate models, the downregulation or absence of *NIPA2* leads to intracellular Mg^{2+} deficiency, which directly activates PINK1/Parkin-mediated mitophagy via the AMPK/mTOR or PGC-1α signaling pathways to clear damaged mitochondria [50]. We postulate a similar mechanism in *A. japonicus*: to minimize energy expenditure under prolonged stress, the sea cucumber halts energy-intensive protein synthesis and fully activates systemic autophagy to recycle intracellular components. Unlike the early protective autophagy, the late-stage autophagy mainly recycles intracellular components into essential nutrients needed to maintain energy [49]. This shift is a direct result of the sea cucumber limited salinity tolerance—because it cannot sustain active osmoregulation, it prioritizes saving energy to prolong survival. However, this survival strategy has limitations: with prolonged salinity stress, the continued activation of autophagy and complete cessation of anabolic metabolism may exceed physiological thresholds, and the sea cucumber may adopt its ultimate survival strategy—evisceration [15].

This study demonstrates the pivotal role of the lncRNA011760/miR-novel-91/*NIPA2* axis in the salinity adaptation of *Apostichopus japonicus*, specifically by modulating metabolic pathways and

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cation homeostasis. Our findings offer a novel framework for understanding how stenohaline echinoderms multi-stage adaptive strategies at the epigenetic level. Nevertheless, several limitations warrant further consideration. The intricate interplay between *NIPA2*-mediated Mg^{2+} flux and downstream effectors within the broader metabolic network—including secondary ion transporters and specific osmolytes—requires more further research to fully delineate the regulatory hierarchy. Additionally, the macroscopic physiological consequences of manipulating this ceRNA axis, particularly its impact on long-term osmotic equilibrium, remain to be empirically validated. Future research should address these gaps through the integration of multi-omics (transcriptomics, proteomics, and metabolomics) and functional genomics to refine this regulatory network. Such efforts will be instrumental in exploring the potential of this axis as a molecular target for the genetic enhancement and marker-assisted breeding of salt-tolerant strains in this economically significant but salinity-sensitive species.

5. Conclusions

This study elucidates the pivotal role of the lncRNA011760/miR-novel-91/*NIPA2* axis in orchestrating the salinity adaptation of *Apostichopus japonicus* via metabolic pathways. Through integrated in vivo and in vitro functional assays, we confirmed a competitive endogenous RNA mechanism where lncRNA011760 acts as a molecular sponge for miR-novel-91, thereby alleviating the post-transcriptional repression of the magnesium transporter *NIPA2*. Based on these molecular dynamics, we propose a novel inhibition-adaptation-survival three-stage model. Initially, the acute upregulation of *NIPA2* enhances Mg^{2+} transport efficiency to mitigate immediate osmotic shock. Subsequently, miR-novel-91-mediated suppression of *NIPA2* creates a transient biosynthetic window, facilitating the transition from passive tolerance to active metabolic adaptation. Ultimately, the lncRNA-driven restoration of *NIPA2* maintains Mg^{2+} homeostasis, enabling the transition into a low-metabolism survival mode. These stage-specific adaptive shifts reflect the inherent physiological constraints of sea cucumbers as osmoconformers. Our findings provide deep insights into the epigenetic regulation of stress tolerance in echinoderms and identify the lncRNA011760/miR-novel-91/*NIPA2* axis as a promising molecular target for the marker-assisted breeding of salt-tolerant strains.

Author Contributions: Yi Tian, Junwei Chen, Yudi Zhao, Jiawei Zhong, Haotian Xue, Project administration, Visualization, Writing. Xin Wei, Qing Gao,: Data curation, Supervision, review & editing.

Funding: This work was supported by the National Natural Science Foundation of China (No. 32273117).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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