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Delivery of plant-derived sbi-miR5564a via exosome-like nanovesicles protects against ionizing radiation-induced oxidative damage

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ABSTRACT: Coix seed seedlings-derived exosome-like nanovesicles (CSSELNs) have demonstrated significant potential in alleviating ionizing radiation (IR)-induced damage. However, the role of bioactive substances, particularly whether microRNAs (miRNAs) significantly contribute to their radioprotective effects, remains unclear. In this study, we identified miRNAs enriched in CSSELNs by small RNA sequencing, followed by validation of the key miRNAs. It was found that sbi-miR5564a confers protection against IR-induced oxidative stress in THLE-2 cells. Bioinformatics analysis together with cell-based and animal experiments were conducted to explore their potential mechanism. The results indicated that sbi-miR5564a, when delivered through CSSELNs, targeted and downregulated EP300, thereby modulating the JNK/FOXO3a signaling pathway, leading to the restoration of reactive oxygen species to normal levels and protection against IR-induced damage. Notably, a single oral dose of sbi-miR5564a@CSSELNs, which were highly enriched with sbi-miR5564a, exerted highly effective protective effects *in vivo*, comparable to that achieved by 30-day oral gavage of CSSELNs alone. This study not only clarified the radioprotective effect of sbi-miR5564a but also established CSSELNs as a natural miRNA delivery platform. This advancement promotes the application of plant-derived nanovesicles in radioprotective effects and the development of functional foods.

Keywords: Exosome-like nanovesicles; Radioprotective factor; Sbi-miR5564a; Radiation damage protection; Regulation of oxidative stress

1. Introduction

Plant-derived exosome-like nanovesicles (PELNs), characterized by a phospholipid bilayer structure, encapsulate abundant bioactive substances including nucleic acids, proteins, and lipids [1-3]. Studies have demonstrated that PELNs stably exert their effects on the tissues or organs of mammals, playing a crucial role in human health [3-5]. For example, the *Panax notoginseng* - ELNs could be taken up by astrocytes and microvascular endothelial cells and target rat brain tissue to alleviate cerebral ischemia/reperfusion injury [6]. Additionally, the ginger-ELNs exhibited preferential uptake by a specific family of human intestinal bacteria, exerting their antitumor effects [7]. Similarly, the Biyang floral mushroom-ELNs exhibited

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efficient cellular uptake, and the ability to exert their radioprotective effects in mice [8]. These findings highlight PELNs as promising bioactive agents with significant health-promoting potential. However, the specific bioactive substances within these nanoparticles responsible for their health benefits remain to be fully characterized.

MicroRNAs (miRNAs), a class of small non-coding RNAs that can regulate gene expression in physiological and pathological processes, are recognized as key regulatory elements among nucleic acid [9]. The evidence indicates that plant-derived miRNAs can serve as the primary bioactive substances within PELNs [10, 11]. Emphatically, the evolutionarily conserved 2'-O-methylation at the nucleotide termini of plant-derived miRNAs can enhance their resistance to digestive nucleases [2, 12]. Moreover, the PELNs' lipid bilayer protects miRNAs from enzymatic degradation and facilitates their targeted delivery, thereby mediating their biological effects in recipients [13, 14]. These natural PELNs are increasingly attractive for miRNA delivery due to their biocompatibility, low immunogenicity, and inherent potential for biological functions [15-17]. For example, the *Brucea javanica*-ELNs could deliver functional miRNAs to 4T1 tumor cells, demonstrating their promising potential for cancer therapy [18]. In addition, miRNAs enriched in the garlic-ELNs alleviated colitis in mice by regulating intestinal flora [19]. Whereas, miRNAs in the *Phellinus linteus*-ELNs could effectively inhibit ultraviolet light-induced skin aging [20]. The ginger-ELNs loaded with osa-miR164d could effectively reprogram macrophages to alleviate colitis-associated symptoms [21]. The study has shown that the ginseng-ELNs loaded with miR-182-5p outperform the ginseng-ELNs in alleviating sepsis-induced acute lung injury [22]. These findings reveal that PELN delivers miRNAs to host cells and functions as a critical mediator of intercellular communication, thereby facilitating molecular exchange and cellular signaling [23].

Ionizing radiation (IR) has been extensively utilized in nuclear energy, military technology, medical diagnostics, and sterilization in the food industry [24, 25]. However, while IR offers convenience to human life, it also induces some unignorable adverse effects [26].

IR could induce damage to biological systems through the generation of reactive oxygen species (ROS) radicals, which interact with biomolecules like nucleic acids and proteins, consequently causing varying degrees of dysfunction in the hematopoietic and immune systems [27]. Prolonged IR exposure can severely damage to normal tissues and organs, potentially resulting in disease development [28]. The exploration and development of radioprotective agents derived from natural resources offer distinct advantages in terms of efficacy, cost-effectiveness, and safety, representing a key current research direction in radioprotection [29-31].

Previous studies have identified multiple bioactive substances in coix seed seedlings (*Coix lacryma-jobi* L.) [32, 33]. The preliminary research has demonstrated that coix seed seedlings-derived exosome-like nanovesicles (CSSELNs) have demonstrated promising radioprotective effects *in vivo* following 30-day oral gavage administration. However, it remains unclear whether CSSELNs can exert their radioprotective effects via the miRNA regulation. In this study, the small RNA sequencing and functional validation were applied to

screen radioprotective miRNAs within CSSELNs. Further analysis of the potential radioprotective mechanisms of the key protective factor sbi-miR5564a in CSSELNs was conducted through target gene prediction combined with quantitative real-time polymerase chain reaction (RT-qPCR), western blotting (WB), and dual-luciferase reporter assays. Additionally, CSSELNs highly enriched with *Sorghum bicolor* (sbi)-miR5564a (sbi-miR5564a@CSSELNs) were prepared by electroporation to investigate their radioprotective effects *in vitro* and *in vivo*, establishing a highly efficient radioprotective application system enriched with sbi-miR5564a. This provides a reference for developing novel radioprotective agents and functional foods based on PELNs.

2. Materials and methods

2.1 Reagents

Cell Counting Kit-8 (CCK-8, Cat No. C0005) was purchased from Topscience Co., Ltd (Shanghai, China). Superoxide dismutase (SOD, Cat No. A001-3), glutathione (GSH, Cat No. A006-2-1), aspartate aminotransferase (AST, Cat No. C010-2-1), alanine aminotransferase (ALT, Cat No. C009-2-1) and malondialdehyde kits (MDA, Cat No. A003-1) were all obtained from Nanjing Jiancheng Institute of Biochemical Co., Ltd (Nanjing, China). MiRNA 1st Strand cDNA Synthesis Kit (Cat No. MR201) and HiScript IV All-in-One Ultra RT SuperMix for qPCR (Cat No. R433) were obtained from Nanjing Novizan Biotechnology Co., Ltd. (Nanjing, China). All other reagents and chemicals were of analytical grade.

2.2 Preparation of CSSELNs

Coix seed seedlings were cultivated and harvested according to our previously method [34]. Then, CSSELNs were obtained through differential centrifugation combined with ultracentrifugation methodology [35].

2.3 Mice and cell lines

Male Kunming mice (6–8 weeks) were acquired from the Laboratory Animal Center of Zhengzhou University (Zhengzhou, China). All animal-related experimental protocols were approved by the Institutional Animal Care and Use Committee of Zhengzhou University (Approval No. ZZUIRB2023-327) in compliance with ethical guidelines. The transformed human liver epithelial-2 (THLE-2) lines was maintained in a specialized culture medium supplemented with specific growth factors under standard incubation conditions (37°C, 5% CO₂).

2.4 Small RNA sequencing analysis of CSSELNs

The small RNA sequencing was performed by Shanghai Personal Biotechnology Co., Ltd. Briefly, the total RNA was extracted from CSSELNs using TRIzol® Reagent (Thermo Fisher Scientific), followed by quantification of RNA concentration and assessment of purity (A260/A280 and A260/A230 ratios) using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Then, the RNA integrity was verified by agarose gel electrophoresis. Small RNA sequencing libraries were constructed using the NEBNext® Multiplex Small RNA Library Prep Kit for Illumina® (New England Biolabs), followed by paired-end

sequencing on an Illumina NovaSeq 6000 platform [36]. The filtered reads were aligned against mature miRNA sequences of all plants in miRBase22 (<http://www.mirbase.org/>; Griffiths-Jones et al., 2004) to screen conserved miRNAs.

2.5 Quantification and efficacy delivery of CSSELNs miRNA

The total RNA was extracted from CSSELNs using the freezol reagent (R711, Nanjing Novizan). The miRNA 1st Strand cDNA Synthesis Kit was used to add Poly(A) to the 3'-end of total RNA under the action of Poly A polymerase. Then, the reverse transcription was performed using a universal reverse transcription primer. Subsequently, the RT-qPCR analysis was carried out to detect the expression levels of the top 10 miRNAs identified by sequencing. Next, the cell-miR-39 (1 pmol, CR100-01, Tiangen) was added as an exogenous reference. The $2^{-\Delta\Delta C_t}$ method was used for RT-qPCR data analysis [37]. The primer sequences were shown in Table S1.

After treatment with 80 $\mu\text{g/mL}$ CSSELNs for 48 h, the miRNA expression in THLE-2 cells was detected using the same RT-qPCR method.

2.6 Transfection of miRNA mimic

The transfection was performed using the Lipofectamine™ 2000 transfection reagent (11668030, Thermo Fisher Scientific) according to the manufacturer's protocol [21]. THLE-2 cells were first cultured to the log phase and then evenly seeded into cell culture plates. Next, perform miRNA mimic transfection in Opti-MEM™. After transfection for 6 h, the culture medium was replaced with complete medium, and cells were further incubated until 24 h. Further incubated for 24 h after IR- treatment, cells were harvested for subsequent analysis [38]. The miRNA mimics mentioned above were provided by Sangon Biotech (Shanghai, China).

2.7 Cell viability

Cultivate THLE-2 cells to the logarithmic phase, and collect and count the cells. Then, evenly inoculate the cells into a 96-well cell plate. After 24 h of sample pretreatment, the IR-induced cell damage model was created using ^{60}Co γ -ray (2 Gy/min, 5 min, total 10 Gy) [8]. After another 24 h incubation, aspirate the medium, add CCK - 8 solution, and continue incubating in the incubator for 1 h. Cell viability was then quantified by measuring OD₄₅₀ using a Bio-Rad Model 1680 microplate reader.

2.8 Cell function assay

After 24 h of transfection with mimics or treatment with sbi-miR5564a@CSSELNs, the obtained THLE-2 cell were treated by ^{60}Co γ -ray (2 Gy/min, 5 min).

2.8.1 Detection of ROS level

Intracellular ROS levels were quantified with a ROS assay kit (S0033, Beyotime) following the manufacturer's protocol [39]. THLE-2 cells were collected and incubated with DCFH-DA probe diluted 1:5000 in serum-free medium. After resuspending cells in the probe solution, samples were incubated at

37°C for 15 min with gentle inversion every 3-5 min to ensure probe-cell contact. ROS levels were subsequently analyzed by flow cytometry (LSRFortessa, USA).

2.8.2 Detection of SOD and MDA levels

Levels of MDA and SOD were respectively detected according to the manufacturer's protocol of related assay Kits, including lipid peroxidation MDA assay kit and total superoxide dismutase (T-SOD) assay kit.

2.9 Target prediction and functional annotation of *sbi-miR5564a* for radioprotective effects

To identify high-confidence target genes of *sbi-miR5564a* (target score ≥ 80) [40], an *in silico* analysis was performed using the miRDB database (<http://mirdb.org/mirdb/custom.html>) based on its 5' - 3' sequence: UGGGGAAGCAAUUCGUCGAACA. Additionally, we conducted a parallel search for the target genes in the GeneCards database (<https://www.genecards.org/>) by looking up the entry "IR".

Subsequently, the online Venn diagram (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>) was applied to take the intersection of the target genes of *sbi-miR5564a* and those related to IR.

Gene ontology (GO) functional annotation, encompassing biological processes, molecular functions, and cellular components, was performed using the Metascape database (<https://metascape.org/gp/index.html#/main/step1>). In parallel, kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis was conducted to systematically characterize functional pathways.

2.10 Luciferase reporter assay

The potential binding sites of *sbi-miR5564a* within the 3' untranslated region (3' UTR) of EP300 were predicted via the RNAhybrid online tool, and mutant variants of these predicted binding sites were subsequently generated through site-directed mutagenesis. The luciferase reporter assay was utilized to detect the regulatory effect of *sbi-miR5564a* on the 3'UTR of the EP300 in 293T cells [20]. Specifically, miRNAs constructs containing wild-type (WT) or mutant-type (MUT) binding sites, along with their corresponding gene EP300 plasmid sequences, were custom synthesized by Hanhen biotechnology Co., LTD. *Sbi-miR5564a* mimics or mimic-NC, EP300-WT, and EP300-URT were co-transfected into 293T cells. 24 h after transfection, the luciferase activity was measured using a dual-luciferase assay kit (DL101, Nanjing Novizan). Using firefly luciferase as an internal control to correct for variations in transfection efficiency, relative luciferase activity was calculated by normalizing the ratio of the sea cucumber luciferase signal (reporting signal) to the firefly luciferase signal (internal control).

2.11 Assay for mRNA expression by RT-qPCR

Total RNA was isolated from cells or tissues with Freezol reagent per manufacturer's protocol. cDNA synthesis was then performed using reverse transcriptase with random or oligo(dT) primers. Following PCR amplification, target gene expression levels were normalized to GAPDH by comparative Ct ($2^{-\Delta\Delta C_t}$) analysis.

2.12 WB analysis

The total protein from THLE-2 cell lysates was separated by SDS-PAGE and transferred to PVDF membranes. Following a 10 min blocking step, the membranes were incubated overnight at 4°C with the following primary antibodies: p300 Rabbit mAb (Cat# A55035, Nature Biosciences), JNK1/2/3 Rabbit mAb (Cat# 9252, CST), Phospho-JNK1/2/3 Rabbit mAb (Cat# AP0631, Abclonal), FoxO3a Rabbit mAb (Cat# A9270, Abclonal) and Phospho-FoxO3a Rabbit mAb (Cat# 9465, CST). Subsequently, the membranes were washed three times with TBST, and then incubated with an HRP-conjugated secondary antibody. Membranes were visualized using hypersensitive ECL detection reagent (36208ES60, Yeasen) on a gel imaging system and quantified with grayscale imaging software. Protein quality in each lane was identified and standardized using the GAPDH antibody.

2.13 Preparation and characterization of *sbi-miR5564a*@CSSELNs

Preparation of engineered exosome-like nanovesicles enriched with *sbi-miR5564a* by loading *sbi-miR5564a* into CSSELNs using an electroporation technology [41]. In brief, 400 µL of CSSELNs (0.8 mg/mL) were co-incubated with 10 µL of mimics-*sbi-miR5564a* (20 pmol) on ice for 1 h, and then the electroporation was carried out. The setting parameters of the electroporator were 100 V and 2 ms. Following each electroporation pulse, the sample was incubated on ice for 5 min, and the electroporation was performed continuously for 5 times. Then, the sample was incubated on ice for 1 h, followed by incubation at 37°C for 1 h, resulting in CSSELNs highly enriched with *sbi-miR5564a* (*sbi-miR5564a*@CSSELNs).

The morphology of *sbi-miR5564a*@CSSELNs was characterized by transmission electron microscopy (TEM) (JEOL JEM 1400, Japan), and the hydrodynamic size and zeta potential were quantified using NanoZS Zeta sizer (Zetasizer Nano S90, UK) [42, 43].

2.14 Radioprotection effects of *sbi-miR5564a*@CSSELNs *in vivo*

2.14.1 Animals and treatments

Male Kunming mice (6–8 weeks) were housed in a specific pathogen-free animal care facility for one week of acclimatization. Then, mice were randomly divided into 6 groups (15 mice/group): (1) Normal control group (NC group); (2) Ionizing radiation model group (IR group); (3) Positive control group (PC group): Leucogen tablets (12 mg/kg/bw/day); (4) CSSELNs (8 mg/kg/bw/day); (5) *sbi-miR5564a*@CSSELNs (8 mg/kg/bw/day); (6) Free *sbi-miR5564a* group (200 pmoL). Each group of mice was treated by oral gavage once. Except for the NC group, the other mice were all subjected to a single-time whole-body uniform IR treatment with ⁶⁰Co γ-ray. The total IR dose was 6 Gy with a dose rate of 2 Gy/min. After IR treatment, the mice were deprived of food but had access to water for 24 h, and various indicators were measured [33].

2.14.2 Tissue histology

The liver tissues of mice were fixed in 10% neutral buffered formalin (24 - 48 h), paraffin-embedded, and sectioned at 4 µm. The sections were then subjected to a standard hematoxylin and eosin (H&E) staining

protocol[44]. All histological examinations were conducted using an upright fluorescence microscope (Nikon Eclipse C1, Japan).

2.14.3 Immunofluorescence

The sections of liver tissues were incubated with dihydroethidium at 37°C for 30 min to detect the levels of ROS, followed by nuclear counterstaining with DAPI. Fluorescence images were captured using an upright fluorescence microscope (Nikon Eclipse C1, Japan) and ROS levels were quantified as mean intensity (ImageJ) normalized to DAPI.

2.14.4 Detection of antioxidant indexes

Blood samples were collected from anesthetized mice and centrifuged (3,000 rpm, 10 min, 4°C) to isolate serum. Liver tissues were homogenized in ice-cold phosphate-buffered saline (PBS), centrifuged (12,000 × g, 15 min, 4°C), and supernatants stored at -80°C for analysis. The levels of MDA, GSH, and SOD in serum and hepatic were quantified, alongside hepatic injury biomarkers (AST/ALT) via enzymatic colorimetric assays.

2.15 Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). T-tests and one-way analysis of variance (ANOVA) were used to assess differences between two groups and differences between multiple groups, respectively. $P < 0.05$ was considered a level of statistically significant difference.

3. Results and discussion

3.1 Small RNA sequencing results

The small RNA sequencing was utilized to investigate the miRNA expression profiles in CSSELN. A total of 39,351,279 raw sequence reads were generated. After applying stringent filtering criteria, 12,998,721 clean reads (33.03% of raw reads) from all libraries were identified as reliable miRNA candidates (**Table S1**). Small RNA annotation profiles revealed distinct distribution patterns of various small RNA subtypes in CSSELN (**Fig. 1A**). Due to the absence of *Coix lacryma-jobi* L. genome sequence information, filtered reads were aligned against mature miRNA sequences of all plants in miRBase22 (<http://www.mirbase.org/>; Griffiths-Jones et al., 2004) to screen conserved miRNAs. Notably, 1.53% of small RNAs in CSSELN were annotated as known miRNAs. This proportion substantially exceeds the 0.25-0.31% miRNA content previously reported in the blueberry-ELNs small RNAs [45]. Observingly, the size distribution of miRNA-associated reads exhibited a distinct peak at 21-24 nt (**Fig. 1B**), consistent with the previous study across various plant species where most small RNAs range between 20-24 nt in length [36]. A total of 374 conserved miRNAs were identified in CSSELN samples (**Table S2**). To validate sequencing results, the expression levels of the top 10 miRNAs (with counts per million (CPM) exceeding 100 in the miRNA profile of CSSELN) were detected (**Fig. 1C-D**). The RT-qPCR results preliminarily validated the reliability of the sequencing data.

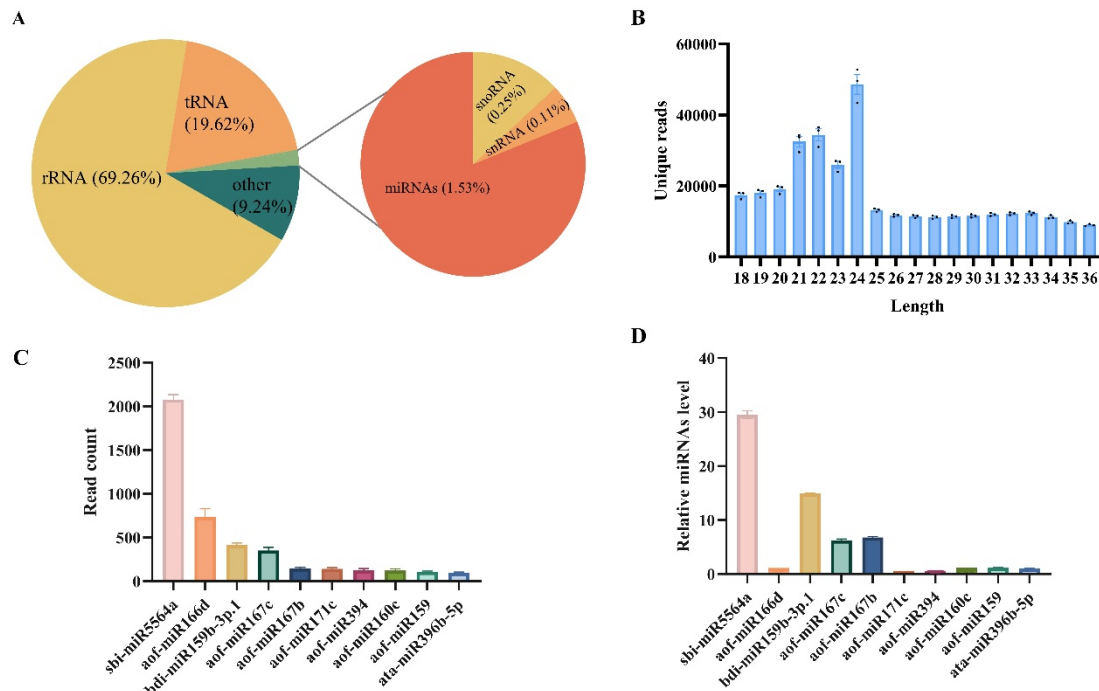


Fig.1 Expression profiles and identification of miRNAs derived from CSSELNs. (A) Classification of small nucleic acids contained in CSSELNs (n = 3). (B) Length distribution of clean reads of CSSELNs small RNA libraries (n = 3). (C-D) The relative expression levels of the top 10 miRNAs in CSSELNs were identified and validated by small RNA sequencing and RT-qPCR technology (n = 3).

3.2 Radioprotective effects of *sbi-miR5564a* in vitro

Previous studies have also suggested that miRNAs highly expressed in PELNs may possess biological regulatory potential. For instance, miR-2916-p3, the most abundantly expressed miRNA in the garlic-ELNs, holds potential for ameliorating colitis in mice [19]. To identify miRNAs with radioprotective functions in CSSELNs, first, the top 10 most highly expressed miRNAs in CSSELNs (*sbi-miR5564a*, *aof-miR166d*, *bdi-miR159b-3p.1*, *aof-miR167c*, *aof-miR167b*, *aof-miR171c*, *aof-miR394*, *aof-miR160c*, *ata-miR396b-5p*, and *aof-miR159*) were selected as candidate miRNAs. Subsequently, these candidate miRNA mimics were transfected into THLE-2 cells prior to IR treatment, and miRNAs with radioprotective functions were preliminarily screened by assessing cell viability. Results revealed that transfection with *sbi-miR5564a* significantly attenuated IR-induced cell viability reduction compared to the mimic-NC group (**Fig. 2A**). To verify the uptake of *sbi-miR5564a* by THLE-2 cells via CSSELNs, the THLE-2 cells were incubated with CSSELNs for 48 h. Remarkably, the expression level of *sbi-miR5564a* was detected in THLE-2 cells post-incubation (**Fig. 2B**), indicating that CSSELNs could mediate the stable uptake of their predominant miRNA component (*sbi-miR5564a*) to exert their radioprotective effects. Therefore, the radioprotective effects of *sbi-miR5564a* were further investigated.

IR can induce excessive ROS production, leading to oxidative stress and lipid oxidation, thereby causing organism damage [46]. MDA, a product of lipid peroxidation, serves as an indirect indicator of oxidative stress levels [47]. Conversely, SOD, an antioxidant metalloenzyme critical for cellular defense mechanisms, functions by neutralizing oxygen-derived free radicals [48]. Therefore, the effects of the identified *sbi-miR5564a* on the levels ROS, MDA and SOD were further detected (**Fig. 2 C-F**). Results

demonstrated that the sbi-miR556a could significantly reduce the levels of ROS and MDA while enhancing SOD activity in THLE-2 cells. These findings indicated that the identified sbi-miR556a within CSSELNs could serve as a key radioprotective factor.

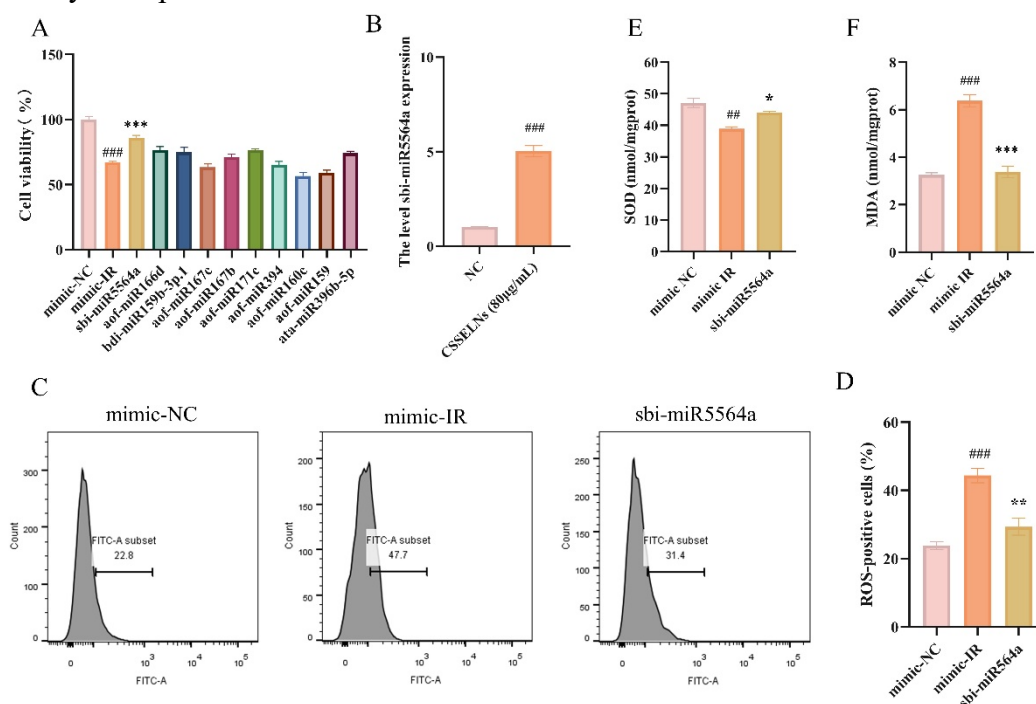


Fig. 2 Radioprotective effects of sbi-miR556a derived from CSSELNs. (A) Effects of 10 miRNAs on cell viability in IR-treated THLE-2 cells (n=5, one-way ANOVA). (B) Levels of sbi-miR556a expression in THLE-2 cells after treatment with CSSELNs at 80 μg/mL for 48 h (n=3, t-tests). (C-D) Effects of ROS expression in IR-treated THLE-2 cells with transfected sbi-miR556a (n=3, one-way ANOVA). (E-F) Effects of SOD enzyme activity and MDA content in IR-treated THLE-2 cells with transfected sbi-miR556a (n=3, one-way ANOVA). ### $P < 0.01$, ### $P < 0.001$ compared with NC group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with IR group. Data were presented as mean $\pm$ SEM.

3.3 Radioprotective mechanism of sbi-miR556a

Bioinformatic analysis via the miRDB database predicted 1,157 putative target genes of sbi-miR556a using its 5'-3' sequence (UGGGGGAAGCAAUUCGUCGAACA). Genes with a target score ≥ 80 (n=289) were selected as high-confidence candidates. Concurrently, 6,857 IR-associated genes were retrieved from the GeneCards database (<https://www.genecards.org/>) through keyword searching. Intersection analysis between these two gene sets revealed 107 overlapping genes (**Fig. 3A**), suggesting potential mechanistic links between sbi-miR556a and IR response pathways.

To elucidate the functional characteristics of the screened target genes, GO annotation and KEGG pathway enrichment analyses were conducted. GO annotation results (**Fig. 3B**) showed that these target genes were significantly enriched in the biological processes, including positive regulation of microtubule nucleation, synaptic vesicle maturation, regulation of glycolytic process, synaptic vesicle recycling, synaptic vesicle exocytosis, regulation of synaptic assembly and protein acetylation. Cellular component analysis revealed predominant localization of target gene products in clathrin-mediated transport vesicle membranes, presynaptic active zone membranes, and synaptic vesicle membranes. In the molecular function category, these targets were predominantly associated with binding and catalytic activities, including MAP kinase activity, transcription corepressor binding, transcription cofactor activity, transcription regulatory region

sequence-specific DNA binding, mRNA binding, macromolecular complex binding, ubiquitin protein ligase activity, transcription factor activity, and sequence-specific DNA binding. This observation aligns with the target gene annotation results from the blueberry-ELNs and the yam-ELNs [45, 49], indicating that miRNAs derived from PELNs may target diverse functional human genes.

KEGG pathway enrichment analysis (**Fig. 3C**) identified significant associations between the target genes and several signaling pathways, including ErbB signaling, Fc epsilon RI signaling and FoxO signaling. Notably, the FoxO signaling pathway is closely linked to IR-induced physiological damage [50]. The implicated target genes in the FoxO signaling pathway include MAPK10, NRAS, HDAC1, EP300, MAPK1, BRAF, ELK1, GSTM5, and BCL2L1. Among these, the EP300 (p300), a histone acetyltransferase family protein, functions as a transcriptional coactivator by binding to sequence-specific DNA-binding proteins, thereby playing a crucial role in regulating genomic stability, cellular proliferation, and programmed cell death [51]. A previous study found that EP300 could act as a vital and central regulator in transcriptional mechanisms influenced by IR [52]. Further research also demonstrated that IR treatment could lead to the upregulation of EP300 expression at the cellular level [53]. Additionally, the emerging evidence also indicated that miRNA-mediated regulation of EP300 occurs, particularly exemplified by the miR-106b~25 cluster, which could modulate γ -radiation-induced cellular senescence by suppressing EP300 expression [54]. Furthermore, EP300 was identified as a predicted target of sbi-miR5564a by sequence analysis in this study. These findings indicated that the sbi-miR5564a potentially exerted its radioprotective effects via EP300-mediated modulation, which merits in-depth exploration.

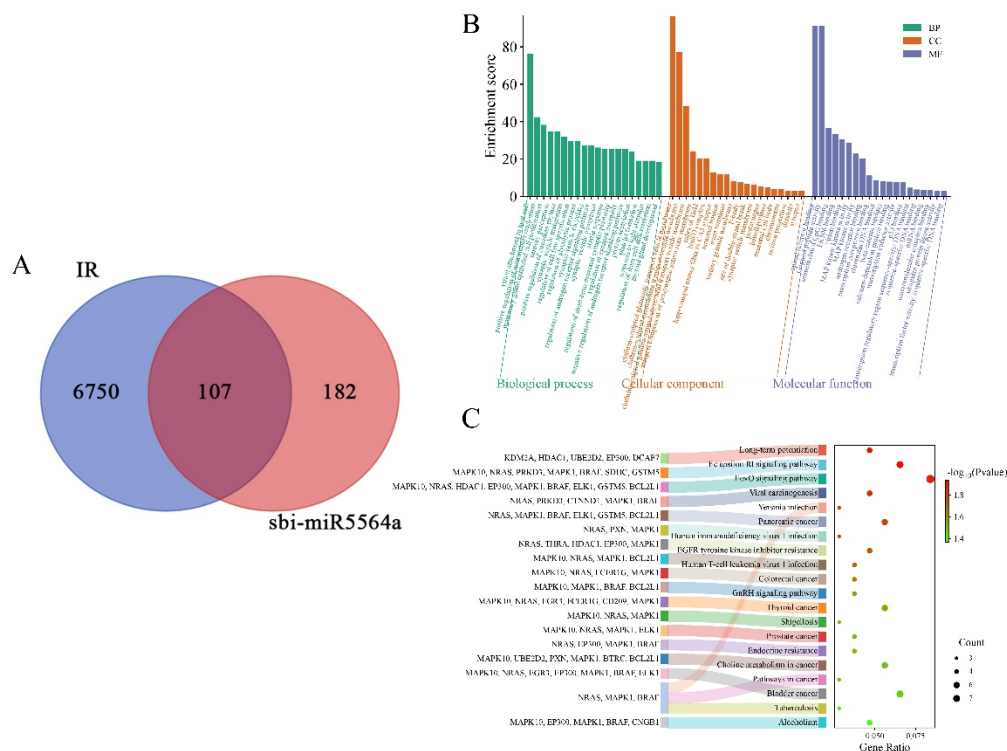


Fig. 3 Bioinformatics analysis of the predicted human target genes of sbi-miR5564a. (A) Venn diagram. (B) GO enrichment analysis. (C) KEGG enrichment analysis.

To confirm the direct targeting of EP300 by sbi-miR5564a, a potential binding site between the sbi-miR5564a seed sequence and the 3'-UTR of EP300 was predicted using TargetScan software (**Fig. 4A**). WT or MUT EP300 3'-UTR sequence fragments were cloned into dual-luciferase reporter plasmids and co-transfected with sbi-miR5564a mimics or negative control mimics (NC) into 293T cells. Luciferase activity was measured 48 h post-transfection. The luciferase reporter assay (**Fig. 4B**) demonstrated that sbi-miR5564a significantly suppressed relative luciferase activity in cells transfected with h-EP300-3'-UTR-WT ($P < 0.01$ vs. mimic NC + WT). However, no significant effect on relative luciferase activity was observed for the h-EP300-3'-UTR-MUT construct ($P > 0.05$ vs. mimic NC + MUT), confirming the direct targeting of EP300 by sbi-miR5564a. To investigate the involvement of EP300 in IR-induced damage in THLE-2 cells, siRNA targeting EP300 (siRNA-EP300) was designed (**Fig. 4C**). Flow cytometry analysis demonstrated that siRNA-mediated EP300 knockdown significantly attenuated IR-induced elevation of intracellular ROS levels ($P < 0.001$; **Fig. 4D**), suggesting EP300 plays a critical role in IR injury. In addition, the overexpression of EP300 increased the mRNA level of EP300, whereas co-transformation of EP300 and sbi-miR5564a restored the expression of EP300 (**Fig. 4F**). Notably, the overexpression of EP300 resulted in elevated cellular ROS levels, whereas co-transfection of EP300 and sbi-miR5564a restored ROS homeostasis (**Fig. 4G-H**).

C-Jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase family, plays an essential role in stress regulation, cell differentiation, and apoptosis [55]. JNK is activated by ROS-induced oxidative stress, leading to transcriptional regulation of downstream genes [56]. The Forkhead transcription factor (FOXO) is a key effector regulated by JNK [57]. Based on these established mechanisms, we hypothesized that the JNK/FOXO3a signaling pathway might mediate IR-induced oxidative stress. To verify this hypothesis, the expression levels of JNK and FOXO3a were evaluated. As shown in **Fig. 4I-Q**, compared to the NC group, the mRNA levels of JNK and FOXO3a were significantly elevated in the IR group, consistent with the previous study reporting oxidative stress-induced activation of the JNK/FOXO3a pathway [58]. Notably, the treatment of sbi-miR5564a effectively attenuated the IR-induced abnormal upregulation of JNK and FOXO3a mRNA levels. Concurrently, the protein-level analyses also demonstrated that the sbi-miR5564a could suppress IR-induced phosphorylation of JNK and FOXO3a. These findings suggested that IR-induced cellular damage generates ROS, which subsequently activates the EP300 and JNK/FOXO3a pathways. By targeting and inhibiting EP300 expression, sbi-miR5564a modulates the JNK/FOXO3a pathway to exert potential radioprotective effects.

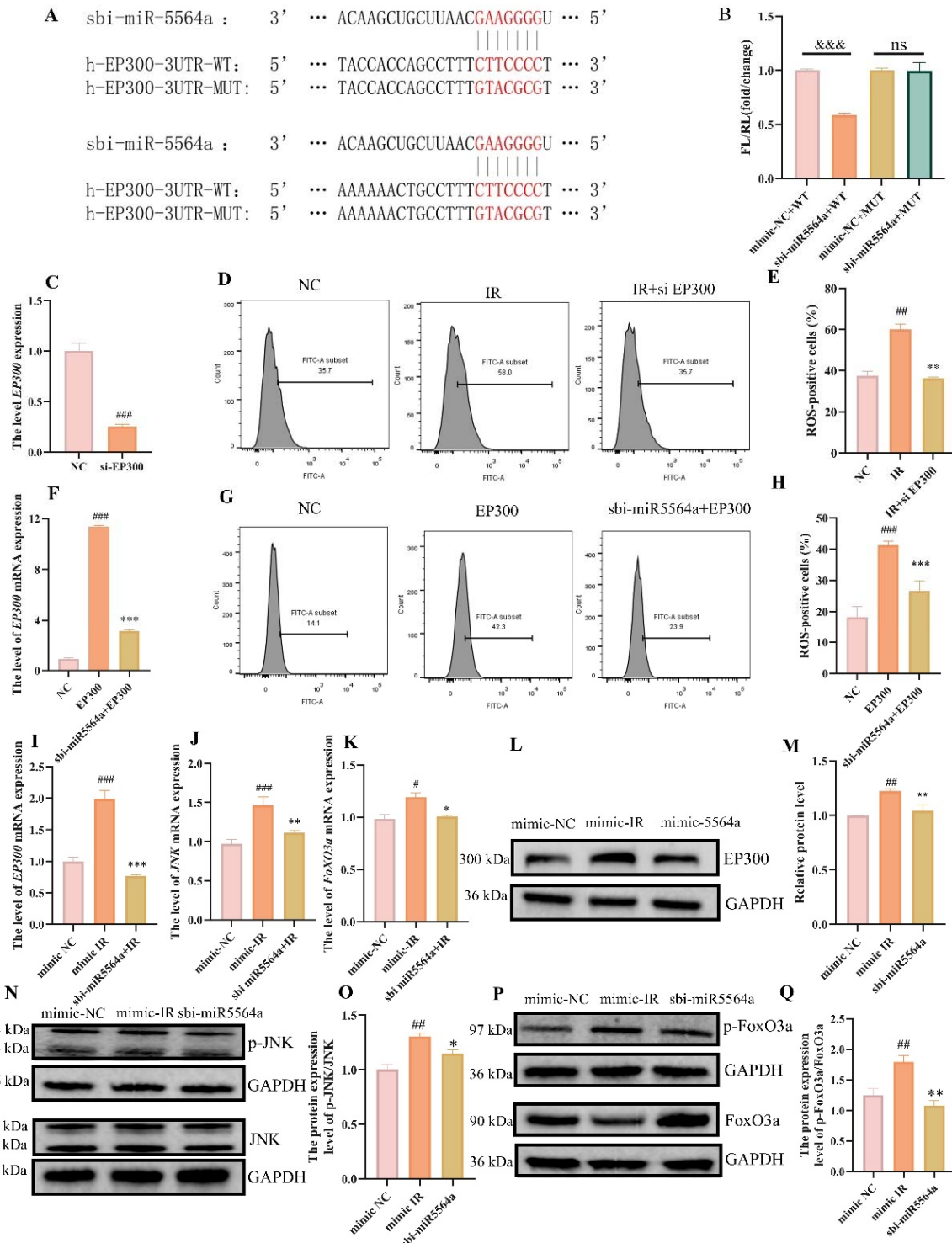


Fig.4 Sbi-miR5564a targets EP300 to regulate the JNK/FOXO3a pathway in IR-induced THLE-2 cells. (A) The matching results between the seed region of sbi-miR5564a and the 3'UTR region of EP300. (B) Relative luciferase activity of EP300 with sbi-miR5564a transfection by dual luciferase reporter gene assay ($n = 3$, one-way ANOVA). (C) The mRNA expression levels of EP300 in THLE-2 cells with EP300 transfection or co-transfection of EP300 and sbi-miR5564a tested ($n=3$, t-tests). (D-E) Levels of ROS in THLE-2 cells with EP300 transfection or co-transfection of both EP300 and sbi-miR5564a ($n = 3$, one-way ANOVA). (F) The mRNA expression levels of EP300 in THLE-2 cells with siRNA-EP300 transfection tested ($n = 3$, one-way ANOVA). (G-H) Levels of ROS in THLE-2 cells with IR treatment and siRNA-EP300 transfection ($n = 3$, one-way ANOVA). (I-K) The mRNA expression levels of *EP300*, *JNK*, *FOXO3a* after transfection with sbi-miR5564a from THLE-2 cells ($n = 3$, one-way ANOVA). (L-Q) The protein expression levels of EP300, p-JNK, JNK, p-FoxO3a, FoxO3a after transfection with sbi-miR5564a from THLE-2 cells ($n = 3$, one-way ANOVA). # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with NC group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with IR group. Significant differences between two groups were marked with &&& $P < 0.001$. Data were presented as mean $\pm$ SEM.

3.4 Radioprotective effects of sbi-miR5564a@CSSELNs *in vitro*

To explore the radioprotective potential of sbi-miR5564a enriched in CSSELNs at the animal level, we first prepared sbi-miR5564a@CSSELNs (**Fig. 5A**). The sbi-miR5564a expression in sbi-miR5564a@CSSELNs showed a 1,210-fold increase compared to native CSSELNs, confirming successful miRNA encapsulation via electroporation (**Fig. 5B**). The electroporation-based preparation of miRNA-loaded EVs is a well-documented approach. Examples include the ginseng-ELNs loaded with miR182-5p and small extracellular vesicle loaded with miR126, both engineered via electroporation [41, 59]. This method relies on low-voltage pulses to induce transient membrane permeabilization in vesicles, thereby facilitating passive diffusion of miRNA into PELNs [60]. The sbi-miR5564a@CSSELNs displayed a cup-shaped morphology with diameters ranging from 90 nm to 140 nm (**Fig. 5C**). The morphology of sbi-miR5564a@CSSELNs is similar to that observed after loading miRNA into the ginseng-ELNs [59]. Zeta potential measurements demonstrated a negative surface charge of -11.19 mV for sbi-miR5564a@CSSELNs (**Fig. 5D**), indicating the stability of the prepared samples. Dynamic light scattering (DLS) analysis further confirmed a mean hydrodynamic diameter of 113.67 nm (**Fig. 5E**), consistent with the size and morphology of CSSELNs. Similar to lipid-based nanoparticles reported previously, plant-derived nanovesicles could spontaneously self-assemble in aqueous solutions, facilitating efficient miRNA encapsulation [41, 61, 62]. To further validate the delivery efficiency of sbi-miR5564a@CSSELNs, the RT-qPCR analysis demonstrated a significant elevation of sbi-miR5564a levels in THLE-2 cells treated with sbi-miR5564a@CSSELNs compared to CSSELNs alone at 48 h post-treatment (**Fig. 5F**), confirming the superior miRNA delivery capability of sbi-miR5564a@CSSELNs. An IR-induced cellular damage model was employed to evaluate the radioprotective effects of sbi-miR5564a@CSSELNs. The results demonstrated that sbi-miR5564a@CSSELNs significantly alleviated the reduction in cell viability induced by IR (**Fig. 5G**). Furthermore, the treatment of sbi-miR5564a@CSSELNs could alleviate markedly IR-induced imbalances the levels of ROS, SOD and MDA (**Fig. 5H-K**). Notably, CSSELNs loaded with sbi-miR5564a exhibited superior efficacy in scavenging IR-induced ROS, likely attributed to their enhanced stability and efficient cellular uptake, which facilitate more stable and complete functional expression of the loaded miRNAs. These advantages highlight the potential of PELNs as miRNA delivery platforms, characterized by excellent biocompatibility, low immunogenicity, and effective protection against miRNA degradation, thereby improving miRNA stability and delivery efficiency *in vivo* [63, 64]. Consistent with these findings, previous studies have demonstrated that the black cumin-derived ELNs loaded with miR-152 form an effective miRNA delivery system with significant anticancer effects, further substantiating the promising application of plant-derived vesicles for functional RNA delivery [65].

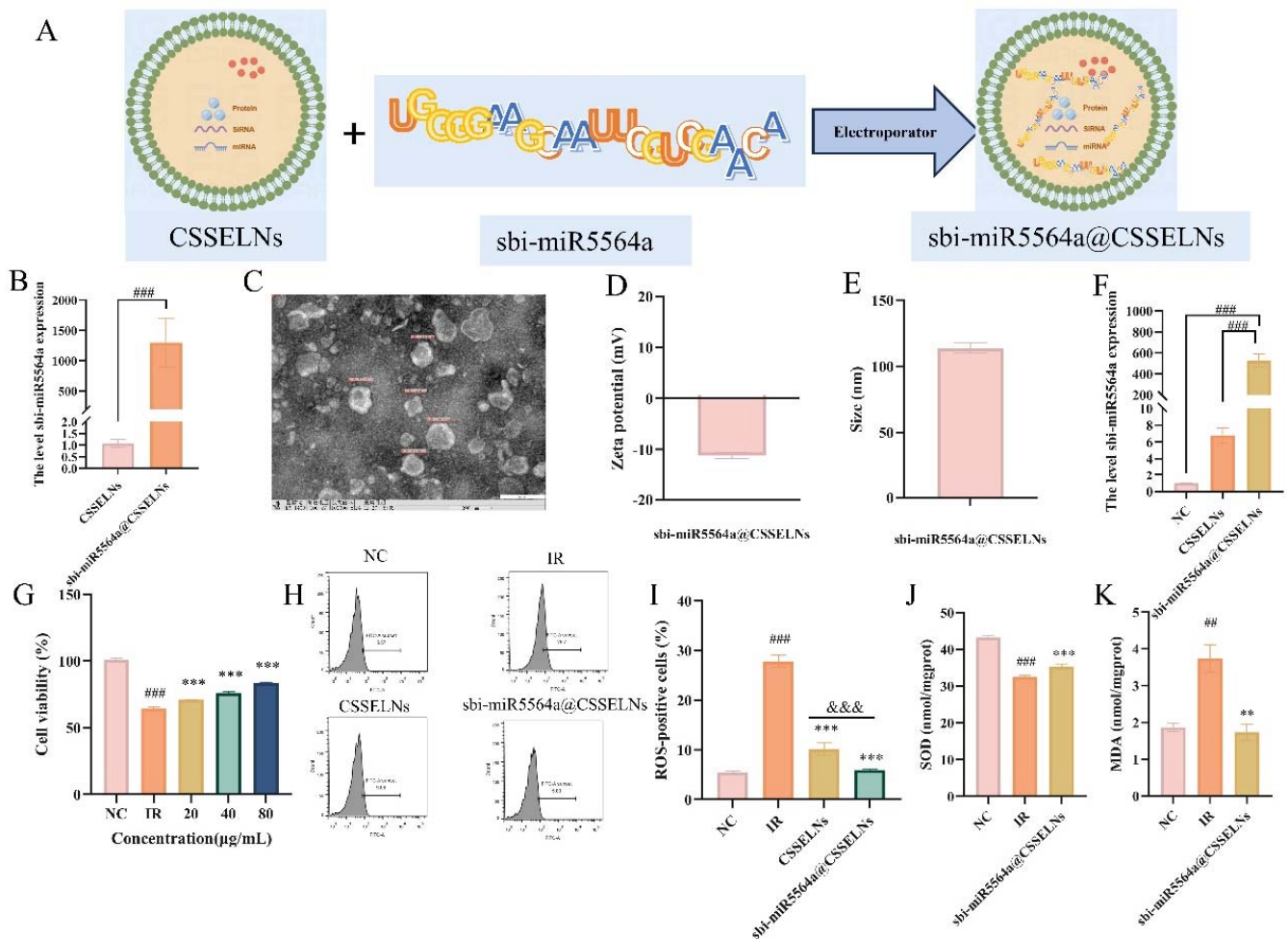
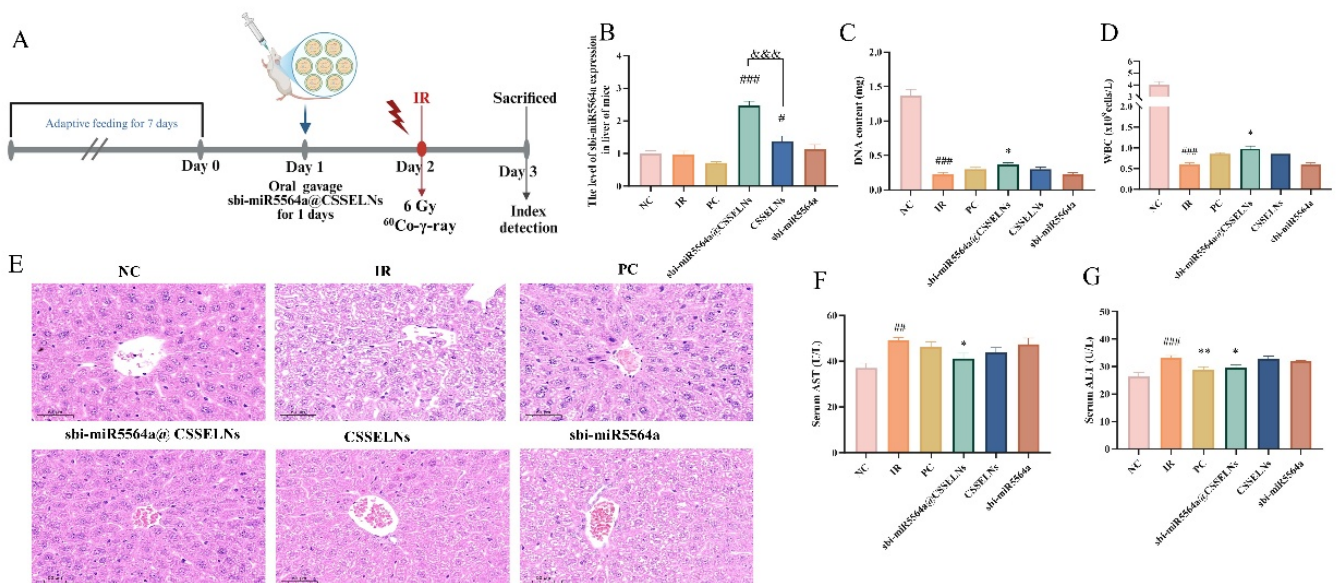


Fig.5 Radioprotective effects of sbi-miR5564a@CSSELNs *in vitro*. (A) Workflow of sbi-miR5564a@CSSELNs from CSSELNs by electroporation-based sbi-miR5564a loading. (B) The expression level of sbi-miR5564a in sbi-miR5564a@CSSELNs (n=3, t-tests). (C) TEM images of sbi-miR5564a@CSSELNs. Scale bar = 200 nm. (D-E) Particle size distribution and the zeta potential of sbi-miR5564a@CSSELNs were measured by DLS (n = 3). (F) The expression level of sbi-miR5564a in THLE-2 cells after treated sbi-miR5564a@CSSELNs for 48 h (n = 3, one-way ANOVA). (G) Effects of sbi-miR5564a@CSSELNs on cell viability in IR-treated THLE-2 cells (n = 5, one-way ANOVA). (H-I) Effects of ROS levels in IR-treated THLE-2 cells with sbi-miR5564a@CSSELNs (n = 3, one-way ANOVA). (J-K) Effects of SOD and MDA levels in IR-treated THLE-2 cells with sbi-miR5564a@CSSELNs (n = 3, one-way ANOVA). ##*P* < 0.01, ###*P* < 0.001 compared with NC group; ***P* < 0.01, ****P* < 0.001 compared with IR group. Data were presented as mean ± SEM.

3.5 Radioprotective mechanism of sbi-miR5564a@CSSELNs *in vivo*

To further investigate the stable delivery of sbi-miR5564a mediated by sbi-miR5564a@CSSELNs and their radioprotective effects *in vivo*, a mouse model of IR-induced was established. Mice received a single oral gavage dose of sbi-miR5564a@CSSELNs loaded with 200 pmol sbi-miR5564a 24 hours prior to IR exposure (**Fig. 6A**). Interestingly, the sbi-miR5564a was detected in the livers of both sbi-miR5564a@CSSELNs and CSSELNs-treated groups, with significantly higher sbi-miR5564a expression in the sbi-miR5564a@CSSELNs group compared to the CSSELNs group (*P* < 0.05) (**Fig. 6B**). Notably, no sbi-miR5564a was undetectable in the oral gavage of sbi-miR5564a group alone, likely due to gastrointestinal degradation of naked miRNAs during oral gavage [66]. Compared with the IR group, a single gavage of sbi-miR5564a@CSSELNs significantly alleviated the IR-induced abnormalities in bone marrow DNA content and WBC levels in mice (**Fig. 6C-D**). Histopathological analysis indicated that the liver tissue structure was disordered and inflammatory infiltration in the IR group, while sbi-miR5564a@CSSELNs could

significantly alleviate the phenomenon of IR-induced liver tissue injury (**Fig. 6E**). Meanwhile, the sbi-miR5564a@CSSELNs group significantly reduced the elevated levels of key liver injury markers, AST and ALT, compared to the IR group, indicating their potential to mitigate radiation-induced liver injury (**Fig. 6F-G**). Additionally, the effects of sbi-miR5564a@CSSELNs and IR on ROS levels in mouse liver were shown in **Fig. 6H-I**. Compared with the NC group, the IR treatment significantly increased ROS levels within mouse liver tissues. Notably, the administration of sbi-miR5564a@CSSELNs, PC, or CSSELNs alone effectively attenuated IR-induced ROS accumulation. Among these treatments, sbi-miR5564a@CSSELNs demonstrated superior efficacy, significantly outperforming the PC group ($P < 0.001$), CSSELNs group ($P < 0.001$), and sbi-miR5564a group ($P < 0.001$) in reducing ROS levels. As shown in **Fig. 6J-O**, compared with the IR group, the oral gavage of sbi-miR5564a@CSSELNs significantly increased SOD activity and GSH content while reducing MDA level in both the liver and serum of IR-treated mice. In contrast, neither a single oral dose of CSSELNs nor free sbi-miR5564a produced any improvement in the aforementioned oxidative stress indicators. Combined with the previous finding that chronic consecutive administration of the Biyang floral mushroom- ELNs administration could exert good radioprotective effects through cumulative effects [8], suggesting that PELNs mediate radioprotection protection function via cumulative action. The temporal efficacy difference, likely attributable to the high enrichment of sbi-miR5564a in sbi-miR5564a@CSSELNs, directly implicates sbi-miR5564a as a critical mediator of radioprotective effects. The above results suggested that at the animal level, sbi-miR5564a@CSSELNs could deliver miRNAs stably into mice to exert resistance to IR-induced oxidative damage in the organism. These findings suggested that the key protective factor sbi-miR5564a in CSSELNs plays a radioprotective role by regulating the level of host oxidative stress.



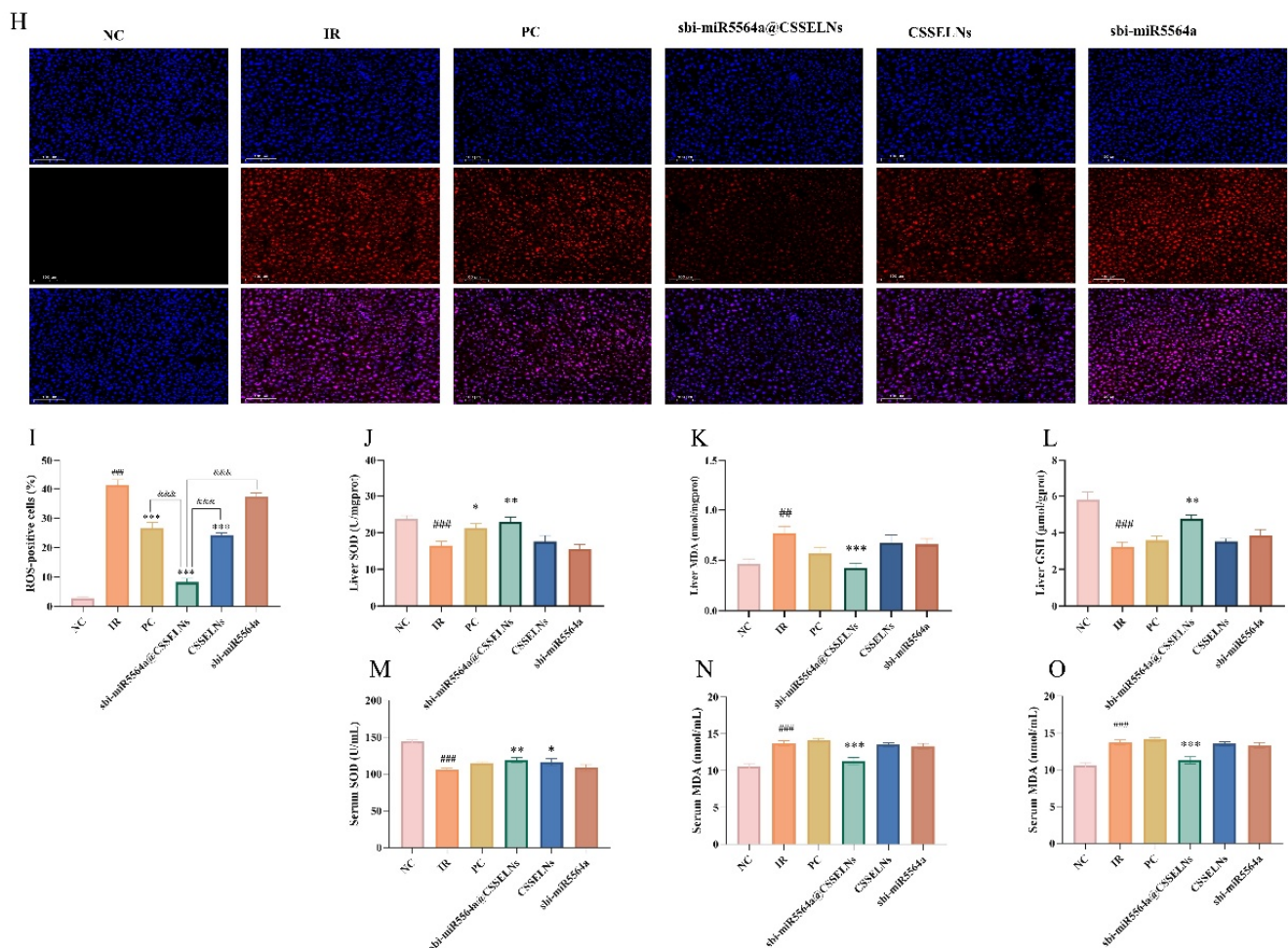


Fig.6 The Radioprotective effects of sbi-miR5564a@CSSELNs *in vivo*. (A) A schematic illustration of IR-induced injury in mice. The experimental group was pretreated with a single oral dose of sbi-miR5564a@CSSELNs 24 h before IR exposure. (B) Expression level of sbi-miR5564a in the livers of mice treated with a single oral gavage sbi-miR5564a alone or CSSELNs or sbi-miR5564a@CSSELNs ($n = 7$, one-way ANOVA). (C-D) Bone marrow DNA content and WBC level in the IR-induced mice ($n = 7$, one-way ANOVA). (E) H&E staining images of liver tissue. (F-G) Levels of AST and ALT in the IR-induced mice ($n = 7$, one-way ANOVA). (H-I) Levels of ROS in the IR-induced mice by immunofluorescence ($n = 7$, one-way ANOVA). (J-L) Levels of SOD, MDA and GSH in the liver of IR-induced mice ($n = 7$, one-way ANOVA). (J-L) Levels of SOD, MDA and GSH in the serum of IR-induced mice ($n = 7$, one-way ANOVA). # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with NC group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with IR group. Significant differences between two groups were marked with && $P < 0.001$. Data were presented as mean $\pm$ SEM.

The above studies demonstrated that CSSELNs-derived sbi-miR5564a provides protection against IR-induced tissue damage. The mechanism of their *in vivo* radioprotection effects was next investigated. As the results were shown in **Fig. 7**, IR treatment significantly upregulated the expression of EP300, p-JNK/JNK and p-FoxO3a/FoxO3a in mouse liver tissues. In contrast, sbi-miR5564a@CSSELNs treatment markedly suppressed the expression levels of EP300, p-JNK/JNK, and p-FoxO3a/FoxO3a. Taken together, these findings were the first to confirm that CSSELNs-derived sbi-miR5564a exerts a radioprotective effect by inhibiting the EP300-regulated JNK/FoxO3a pathway. Nevertheless, it must be noted that our current understanding of the biosynthetic pathways and secretion mechanisms of CSSELNs remains limited. Additionally, the potential synergistic roles of other bioactive constituents, like lipids and proteins, in radioprotection warrant further systematic investigation. Moreover, the biogenesis and endocytic pathways of plant-derived miRNAs, including those within CSSELNs, remain largely unclear. This represents an

important direction for future research, which would significantly facilitate the standardized preparation and functional application of PELNs.

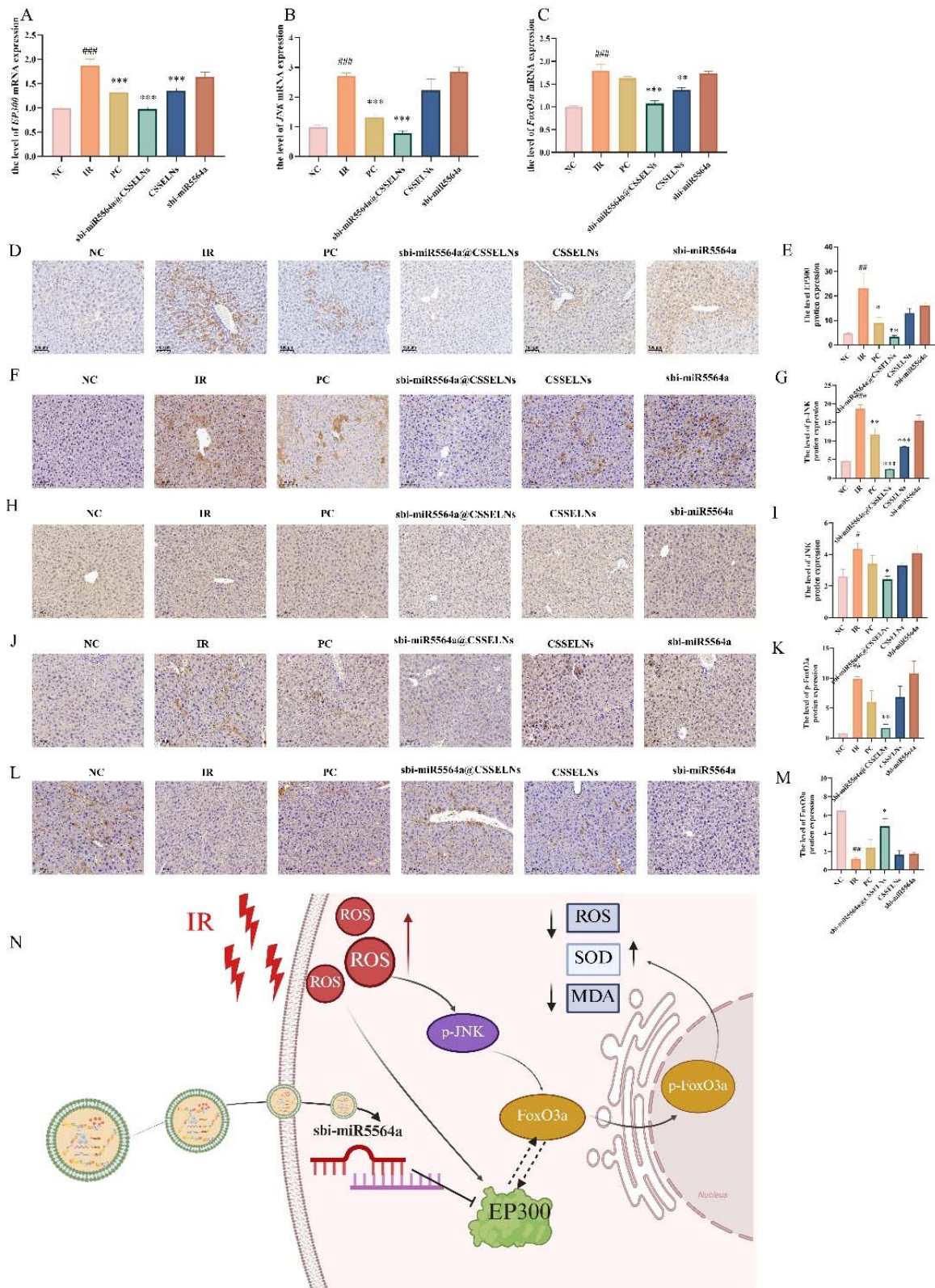


Fig.7 The potential radioprotective mechanism of CSSELN loaded with sbi-miR5564a *in vivo*. (A-C) The mRNA expression levels of EP300, JNK, FoxO3a in the IR-induced mice (n = 7, one-way ANOVA). (D-M) The protein expression levels of EP300, p-JNK, JNK, p-FoxO3a, FoxO3a in the IR-induced mice (n = 3, one-way ANOVA). (N) Schematic illustration of the signaling pathways relevant to the radioprotective effects, activated by CSSELN-derived sbi-miR5564a. [#]*P* < 0.05, ^{##}*P* < 0.01, ^{###}*P* < 0.001 compared with NC group; ^{*}*P* < 0.05, ^{**}*P* < 0.01, ^{***}*P* < 0.001 compared with IR group. Data were presented as mean ± SEM.

4. Conclusion

Our findings reveal that CSSELNs-derived sbi-miR5564a serves as a key radioprotective regulator, mitigating IR-induced oxidative damage by targeting EP300 to modulate the JNK/FOXO3a pathway. Furthermore, a safe sbi-miR5564a-loaded CSSELNs delivery system was developed and demonstrated to highly effective radioprotective effects. These results provide the compelling evidence that the CSSELNs-derived sbi-miR5564a acts as a cross-kingdom communication mediator, while also establishing a novel strategy for utilizing CSSELNs as customizable delivery vehicles through exogenous miRNA loading. The biogenesis and endocytic pathways of PELNs-derived miRNAs remain poorly understood and thus warrant further in-depth investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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