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MiR-132 prohibits proliferation, invasion, migration, and metastasis in breast cancer by targeting HN1

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ABSTRACT

Accumulating evidence indicates that miRNAs play critical roles in tumorigenesis and cancer progression. This study aims to investigate the role and the underlying mechanism of miR-132 in breast cancer. Here, we report that miR-132 is significantly down-regulated in breast cancer tissues and cancer cell lines. Additional study identifies HN1 as a novel direct target of miR-132. MiR-132 down-regulates HN1 expression by binding to the 3′ UTR of HN1 transcript, thereby, suppressing multiple oncogenic traits such as cancer cell proliferation, invasion, migration and metastasis *in vivo* and *in vitro*. Overexpression of HN1 restores miR-132-suppressed malignancy. Importantly, higher HN1 expression is significantly associated with worse overall survival of breast cancer patients. Taken together, our data demonstrate a critical role of miR-132 in prohibiting cell proliferation, invasion, migration and metastasis in breast cancer through direct suppression of HN1, supporting the potential utility of miR-132 as a novel therapeutic strategy against breast cancer.

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1. Introduction

Breast cancer is the most frequently diagnosed cancer and leading cause of cancer death in female worldwide, accounting for 23% of the total new cancer cases and 14% of total cancer deaths in 2008 [1]. The precise causes of breast cancer are still unclear. Genetic and epigenetic alterations have long been recognized as two related mechanisms in both initiation and progression of cancer [2]. Besides DNA methylation and histone modification, epigenetics has been extended to microRNAs (miRNAs).

MicroRNAs are a novel class of short (generally 18–24nt) non-coding RNAs that regulate post-transcriptional mRNA expression by binding to 3′ untranslated region (3′-UTR) of the target mRNA sequence resulting in translational repression or degradation of mRNA [3,4]. They act as negative regulators of gene expression and play a crucial role in regulating breast cancer cell proliferation, invasion, migration and metastasis [5–7]. Accumulating data showed a number of miRNAs, such as miR-23 [8], miR-200 [9], miR-378 [10] were reported as key regulators in breast cancer. However, we found that miR-132 had rarely been demonstrated to be involved in breast cancer [11]. So far, it is not clear how miR-132 regulates breast cancer cells.

The hematopoietic- and neurologic-expressed sequence 1 (HN1) gene with an 824 nucleotides 3′ UTR, which encodes a 154aa protein that is highly conserved among species. HN1 was detected as an oncogene in some cancers. Depletion of HN1 in both melanoma and prostate cells results in cell cycle arresting-related proliferation suppression [12,13]. Moreover, *in vitro* and *in vivo* studies demonstrated that overexpressing HN1 promotes wound healing in prostate cancer [14]. Gene Expression Arrays and Recursive Descent Partition Analysis suggested that HN1 could distinguish all tumor sample from normal ovarian surface epithelial cells [15]. Furthermore, people reported that ectopic HN1 expression resulted an increase in the β-catenin degradation leading to loss of E-cadherin interaction, concurrently contributing to actin re-organization, colony formation and migration in prostate cancer cell lines [15]. However, little is known about the regulation of HN1 by miRNAs in breast cancer.

Here, we provided evidence that miR-132 was frequently down-regulated in breast cancer tissues compared with paired para-tumor breast tissues. Overexpression of miR-132 significantly suppressed breast cancer cell proliferation, invasion, migration and metastasis *in vivo* and *in vitro*. Further results revealed that miR-132 was able to repress HN1 translation via its 3′ UTR in breast cancer cells. Moreover, overexpression of HN1 abolished the miR-132-suppressed tumor malignancy in breast cancer cells. Clinical

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data analysis suggested that amplified HN1 expression was correlated with poor overall survival in breast cancer patients. Therefore, these data suggested that miR-132 regulated breast cancer cell malignancy by targeting HN1.

2. Materials and methods

2.1. Human samples and cell culture

A total of 10 paired primary human breast cancer and paratumor breast tissues were collected at the Tongji Hospital, Wuhan. NMuMG, 4TO7, 4T1, MDA-MB-231 and MCF10A cells were purchased from the American Type Tissue Culture Collection. All the cells above were cultured in MEM with 10% fetal bovine serum (Hyclone, Logan, UT, USA).

2.2. Micro-RNAs and plasmids

MiR-132 mimics were purchased from RiboBio Technology (Guangdong, China); Lenti virus based MMIR132-PA-1 was purchased from System Biosciences (Mountain View, CA); HN1 MISSION shRNA plasmids were purchased from Sigma (St. Louis, MO, USA). Sh#1: TRCN0000194141, sh#3: TRCN0000176148; The HN1 cDNA was purchased from Thermo (Pittsburgh, PA, USA) and sub-cloned into pQCXIN Vector, which was used for HN1 over-expression assay. The constructions of plasmids used in Luciferase Reporter Assay were described below.

2.3. Wound healing assay

Cells were grown as a confluent monolayer in six-well plates and transfected with miR-132 mimics or negative control. To initiate migration, the cell layer was scratched using a 100 μl pipette tip. Next, Images of cell morphology were captured at initiation time and 36 or 48 h. The migration abilities were quantified and normalized by relative gap distance. The experiment was performed three times.

2.4. Invasion assay

For cell invasion assay, Transwell chamber (pore size 8 μm) with pre-coated matrigel membrane filter (Corning, NY, USA) was used. 5×10^4 4T1 cells or 1×10^5 MDA-MB-231 cells in DMEM medium containing 0.2% BSA were layered in the upper chamber, medium containing 10% FBS was applied to the lower chamber. The chamber was incubated for 48 h at 37 °C. Then, after the upper surface of the filter was removed, the invaded cells were fixed with 4% paraformaldehyde solution and stained with 0.1% crystal violet. The number of invaded cells was quantified by counting the cell number at 10 random fields.

2.5. Quantitative RT-PCR

Total RNA was extracted using miRNeasy Mini Kit (QIAGENE, Shanghai, China). Assaying of mature miRNAs was performed using the TaqMan miRNA qRT-PCR kit (Life Technologies, Gaithersburg, MD, USA) using U6 as an internal control. To assay mRNAs, 1 μg RNA was reverse-transcriped to cDNA, and qRT-PCR was performed with TaqMan Master Mix (both from Applied Biosystems, NY, USA).

2.6. Western blot assay and antibodies

Cells were lysed in PIRA buffer (Beyongtime, China). After boiled for 5 min, samples were separated on 10% SDS–polyacrylamide gels and then transferred to an Immobilon membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat dry milk in TBS/Tween20 (0.05% v/v) for 1 h followed by incubation at 4 °C overnight with the indicated primary antibodies. The membranes were washed three times with TBST buffer and then incubated for 1 h with HRP-conjugated anti-rabbit or -mouse secondary antibodies. Visualization of protein bands was accomplished using enhanced chemiluminescence (Thermo Scientific, NY, USA). HN1 antibody was purchased from Genetex (GTX106585, Irvine, CA, USA) and β-actin antibody was obtained from Sigma (St. Louis, MO, USA).

2.7. MTT assay

To evaluate the effect of miR-132 on cell proliferation, cells that transfected with miR-132 mimics (50 nM) and negative control-miRNA mimics (50 nM) were seeded at 5000/well in 100 μl medium in 96-well plates. Every 24 h post-transfection, 10 μl MTT reagent (Life science, NY, USA) was added to each well and further incubated for 4 h. After removing the medium, 100 μl DMSO was added to dissolve the formazan and the absorbance was measured at 490 nm.

2.8. Luciferase target assay

The 3′-UTR of HN1, including the binding site for miR-132, was amplified (Forward: 5′-AATTTCTAGACTTCTTGTGTTGGAACTCTG-3′; Reverse: 5′-AATTTCTAGATCAAAAGGTGGGTATTGTTTAC-3′) using cDNA encoding HN1 as a template. The PCR product was then cloned into the reporter plasmid pGL3 (Promega, Madison, WI, USA) downstream of the luciferase reporter gene. QuickChange Site-Directed Mutagenesis kits (Stratagene, La Jolla, CA, USA) were used to contribute base pair mutation in the seed-binding site of HN1 3′ UTR.

Fifty percent confluent HEK293 cells grown in 24-well plates were co-transfected with miR-132 mimics (20 nM) and wild type- or Mut-HN1 3′ UTR Reporter (200 ng). Luciferase activity was measured 48 h later using the Dual-luciferase Reporter Assay System (Promega, Fitchburg, WI, USA). Firefly activity was normalized to Renilla activity to control the transfection efficiency.

2.9. In vivo xenograft assay

5 weeks aged female nude mice were purchased from the Chinese Academy of Medical Sciences Institute of experimental animals. All experiments involving animals were performed according to the guidelines on laboratory animals of the National Institutes of Health guidelines (NIH publication 96-01, 1996 revision) and were approved by the Institute Research Ethics Committee at the Tongji Medical College, HUST (Permit number: SYXK 2010-0057). The 4T1 cells that expressing Luciferase and GFP were harvested by trypsinization, washed twice in PBS, then resuspended (1.6×10^7 cells/ml) in PBS. 50 μl of cells (8×10^5) were injected into tail vein. Luciferase activity was monitored using Xenogen IVIS Imaging System (Caliper Life Sciences, Waltham, MA, USA) once a week. When the ending point (4 weeks after injection) was reached, mice were scarified and lungs were harvested for pathology analysis.

2.10. Statistical analysis

Results are expressed as mean ± SD. Statistical differences between groups were evaluated using Students' *t*-test analysis or one-way ANOVA. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. MiR-132 is down-regulated in breast cancer cells and tissue samples

MiR-132 was rarely reported in cancer research. Here, we detected the miR-132 expression in breast cancer and para-tumor breast tissues from 10 paired samples collected from patients by qRT-PCR. Notably, miR-132 level was significantly decreased in breast cancer tissue compared with para-tumor tissue. The mean miR-132 expression level was 0.27 ± 0.17 in breast cancer tissues, whereas 0.98 ± 0.34 in paired para-tumor tissues (Fig. 1A). Then we performed qRT-PCR assay to analyze miR-132 expression in human and mouse normal breast cell lines and breast cancer cell

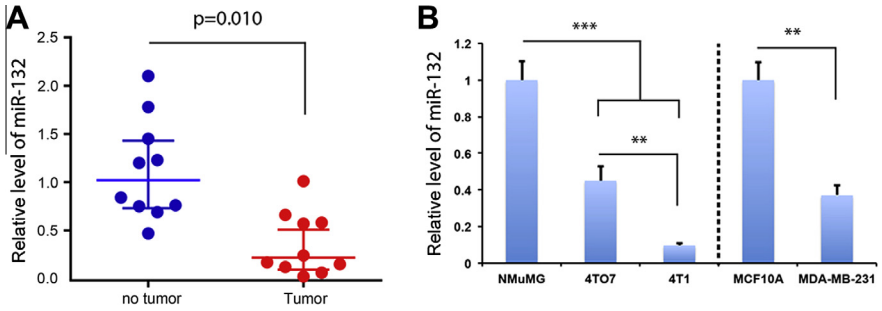

Fig. 1. Expression of miR-132 in breast cancer cells and tissues. (A) Relative expression of miR-132 in 10 cases of breast cancer tissues and paired adjacent breast tissues. The expressions were determined by using qRT-PCR and normalized to endogenous control U6 snRNA. (B) qRT-PCR analysis of miR-132 expression in human and mouse normal breast epithelial cells (MCF10A and NMuMG) and breast cancer cells (MDA-MB-231, 4T1 and 4TO7). Data in the two panels represented the means ± SD and analyzed by student's *t*-test (A and B) or ANOVA (B). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with their respective controls.

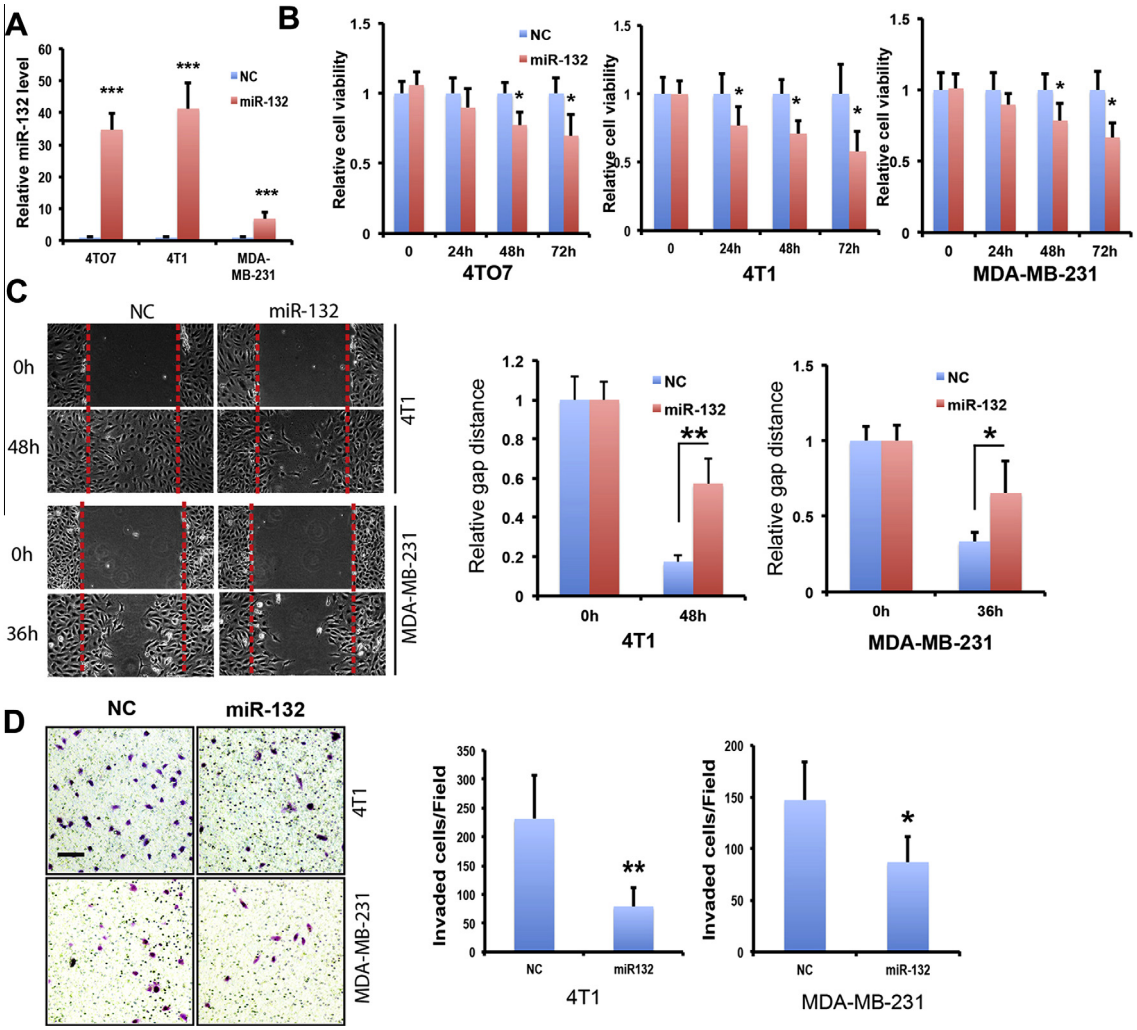

Fig. 2. Effects of miR-132 on proliferation, invasion and migration in breast cancer cell lines. (A) Transient transfection of miR-132 mimics or negative control in mouse breast cancer cell lines (4T1 and 4TO7) and human breast cancer cell line (MDA-MB-231). (B) Relative proliferation rate of three miR-132 mimics transfected cell lines at various time points (0, 24, 48 and 72 h), as detected by MTT assay. (C) Cell migration was evaluated by wound healing assay in 4T1 and MDA-MB-231 cells, which were transfected with miR-132 mimics or negative control. Representative images were obtained at time point 0 and 48 h (4T1) or 36 h (MDA-MB-231). Migration ability was quantified by measuring gap distance. (D) Cell invasion ability was evaluated with Matrigel Transwell Assay. Representative images were shown at left panel (Scale bar, 200 μm), bar charts were shown in the right panel. All data are showed as means ± SD $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Each experiment was repeated at least three times.

lines. Surprisingly, we found that the levels of miR-132 in 4T1 and 4TO7 were down-regulated compared with mouse normal breast cell line NMuMG. As we known, 4T1 usually performed more malignant than 4TO7 cells. In our study, miR-132 expression level in 4T1 cells was lower than 4TO7 cells. Furthermore, human breast cancer cell line MDA-MB-231 cells displayed lower miR-132 level than human normal breast cell line MCF-10A (Fig. 1B). Together, the data demonstrated that miR-132 was down-regulated in breast cancer cells and tissue samples, suggesting miR-132 as a tumor suppressor in breast cancer.

3.2. Overexpression of miR-132 decreases proliferation, invasion and migration of breast cancer cells

Malignant hallmarks of cancer cells involve proliferation, invasion, migration and metastasis. The metastatic process highly depends on cancer cells invasion and migration. Accordingly, we performed proliferation assay, invasion assay and migration assay *in vitro*. MiR-132 mimics or negative controls were transiently transfected into 4T1, 4TO7 and MDA-MB-231 cells. At 48 h after transfection of miR-132 mimics, compared with negative-control-transfected cells, miR-132 expression level was increased by 35.2 ± 7.1 times in 4TO7 cells, 41.3 ± 12.3 times in 4T1 cells and 7.1 ± 2.1 times in MDA-MB-231 ($p < 0.001$; Fig. 2A). The MTT assay was then conducted to analyze the cell proliferation at 0, 24, 48 and 72 h after transfection. All three cell lines showed lower proliferation rate after miR-132 overexpression (Fig. 2B). Furthermore, invasion assay and migration assay were performed in 4T1 and MDA-MB-231 cells after transfection with miR-132 mimics or negative control. Obviously, overexpression of miR-132 significantly decreased migration in both cell lines ($p < 0.05$; Fig. 2C). In addition, transwell chamber invasion assay revealed that miR-132 overexpression significantly impaired invasion in both 4T1 and MDA-MB-231 cells *in vitro* ($p < 0.01$; Fig. 2D). Taken together, miR-132 inhibited malignant features in breast cancer cells.

3.3. HN1 is a direct target of miR-132 in breast cancer cells

To investigate the miR-132 target in breast cancer, we utilized five miRNA target predicted databases (TargetScan, Pictar, miRBase, miRDB and miRanda) to scan for the potential target genes. Based on the analysis, 13 genes were selected out (Fig. 3A and Supplementary Table). Three out of 13 genes behaved as the most possible targets due to high Target score and Pictar score, as well as low PITA and miRSUR score. Furthermore, qRT-PCR assay was performed to confirm the prediction. We found that miR-132 overexpression slightly influenced Ep300 transcription and significantly suppressed HN1 transcription but not Paip2 gene (Fig. 3B). To further determine if HN1 was regulated by miR-132, qRT-PCR and Western blot assays were conducted in miR-132-overexpression and control 4T1 cells. Both mRNA and protein levels of HN1 were

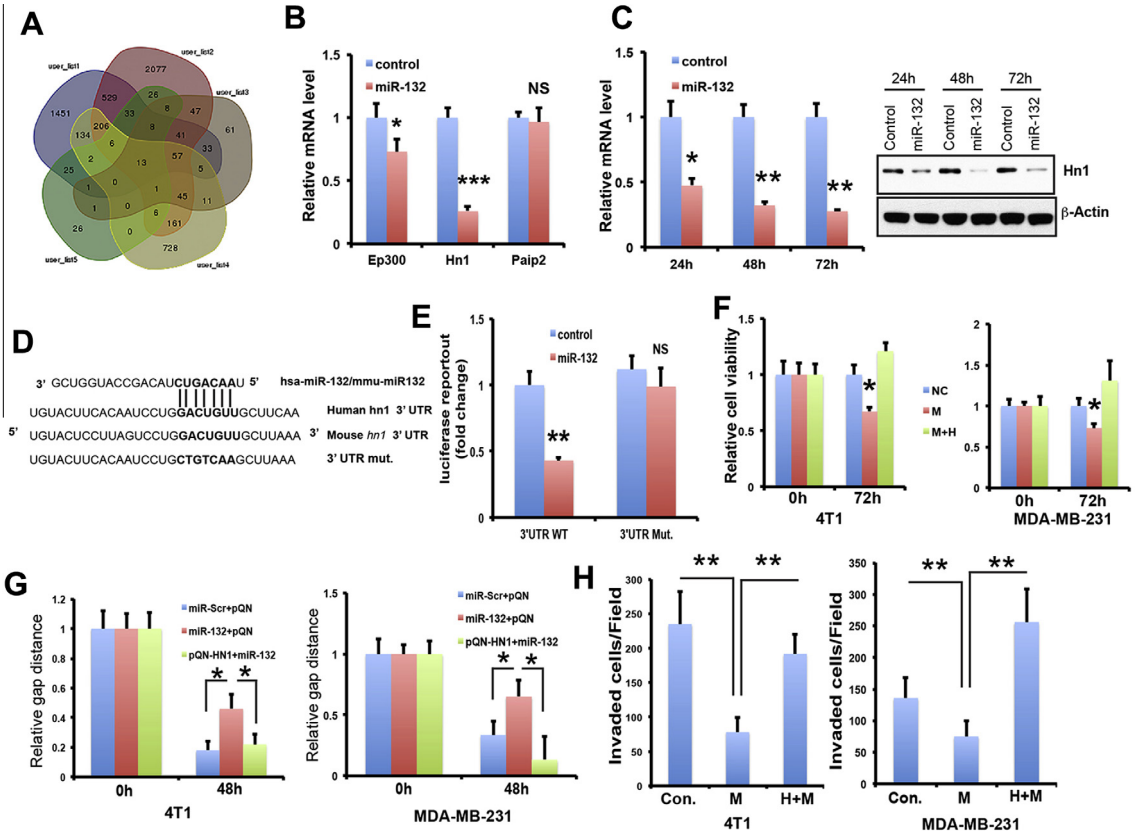

Fig. 3. HN1 is a direct target of miR-132. (A) Schematic showing the predicted targets overlapped from 5 prediction databases. 13 genes were found concurrently in all five target predicted databases (List 1, TargetScan; List 2, miRanda; List 3, PicTar; List 4, miRDB; List 5, miRBase). (B) Three most probable target genes were evaluated by qRT-PCR assay in 4T1 cells, which were transiently transfected with miR-132 mimics or negative control. (C) HN1 mRNA and protein levels were evaluated by qRT-PCR assay and Western blot assay after transient transfection of miR-132 in 4T1 cells. (D) Schematic showing the putative binding sites of miR-132 on HN1 3′ UTR as predicted by 5 databases. HN1-UTR-mut indicates the 3′ UTR of HN1 with the mutation in miR-132 binding sites. The conserved nucleotides in the binding sites of miR-132 were bolded. (E) HEK293 expressing WT or Mutant HN1 3′ UTR luciferase constructs were transfected with control vector (blue bars) or miR-132 mimics (red bars) for 24 h. Values were presented as relative luciferase activity after normalization to Renilla luciferase activity. (F) MTT Assay, (G) Wound Healing Assay, (H) Matrigel Invasion Assay were used for evaluating proliferation, migration and invasion of 4T1 and MDA-MB-231 cells transfected with pQCXIN vector + miR-SCR (NC), pQCXIN + miR-132 mimics (M) or co-transfected with miR-132 and pQCXIN-HN1 (M + H). Each experiment was repeated at least three times, and each sample was assayed in triplicate. Data were shown as mean ± SD from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

down-regulated by increased miR-132 at 24, 48, 72 h post-transfection (Fig. 3C). To further confirm that HN1 was a direct target of miR-132, we used a wild type HN1 3′ UTR luciferase reporter construct and a mutant construct which six nucleotides of potential binding site were replaced (Fig. 3D). MiR-132 overexpression significantly reduced the luciferase signal from WT HN1 3′ UTR luciferase reporter construct but not the mutant, indicating that miR-132 inhibited HN1 by target this sequence (Fig. 3E).

To investigate if HN1 could restore the proliferation, invasion and migration abilities that were decreased by miR-132 overexpression, we transfected 4T1 and MDA-MB-231 cells with miR-132 mimics or co-transfected with pQCXIN-HN1 and miR-132 mimics. MTT assay, invasion assay and migration assay were performed post-transfection within 5 days. We found that the proliferation (Fig. 3F), migration (Fig. 3G) and invasion (Fig. 3H) abilities were reverted by overexpression of HN1. Taken together, HN1 was identified as a target gene of miR-132. Overexpression of HN1 is able to restore the miR-132-suppressed malignant effects in breast cancer.

3.4. MiR-132 decreases breast cancer lung metastasis, whereas HN1 promotes it

To elucidate whether miR-132 could decrease breast cancer metastasis, *in vivo* study was performed by tail vein injection with miR-132 overexpression 4T1 and control cells (Fig. 4A). In this experiment, 4T1 cells were labeled with luciferase, which allowed cancer cells tracking and quantification. We found that miR-132 significantly decreased the 4T1 lung metastasis (Fig. 4B). Alternatively, knocking down HN1 gene in 4T1 cells with shRNAs resulted in lung metastasis decrease *in vivo* (Fig. 4C). Taken together, miR-132 suppressed breast cancer lung metastasis, whereas HN1 increased it.

3.5. HN1 is significantly associated with overall survival in clinical patients

Previous study revealed that five genes include HN1 were associated with disease-specific survival times of 189 patients with resected HCC [16]. To confirm if HN1 overexpression contributed to breast cancer patients' survival time, we analyzed HN1 using BreastMark: http://glados.ucd.ie/BreastMark/index.html. Results revealed that higher expression of HN1 was significantly associated with worse overall survival in breast cancer patients (Fig. 4D).

4. Discussion

Aberrant miRNA expression is correlated with almost all the human cancers, functioning as key regulator in regulating target genes by inhibiting or promoting their translations [17]. Thus miRNA may exhibit different expression levels between normal and cancer cells or tissues. For instance, miR-205 level is significantly lower in breast cancer cells and tissues than in normal breast and corresponding non-tumor breast tissues [18]; MiR-210 is

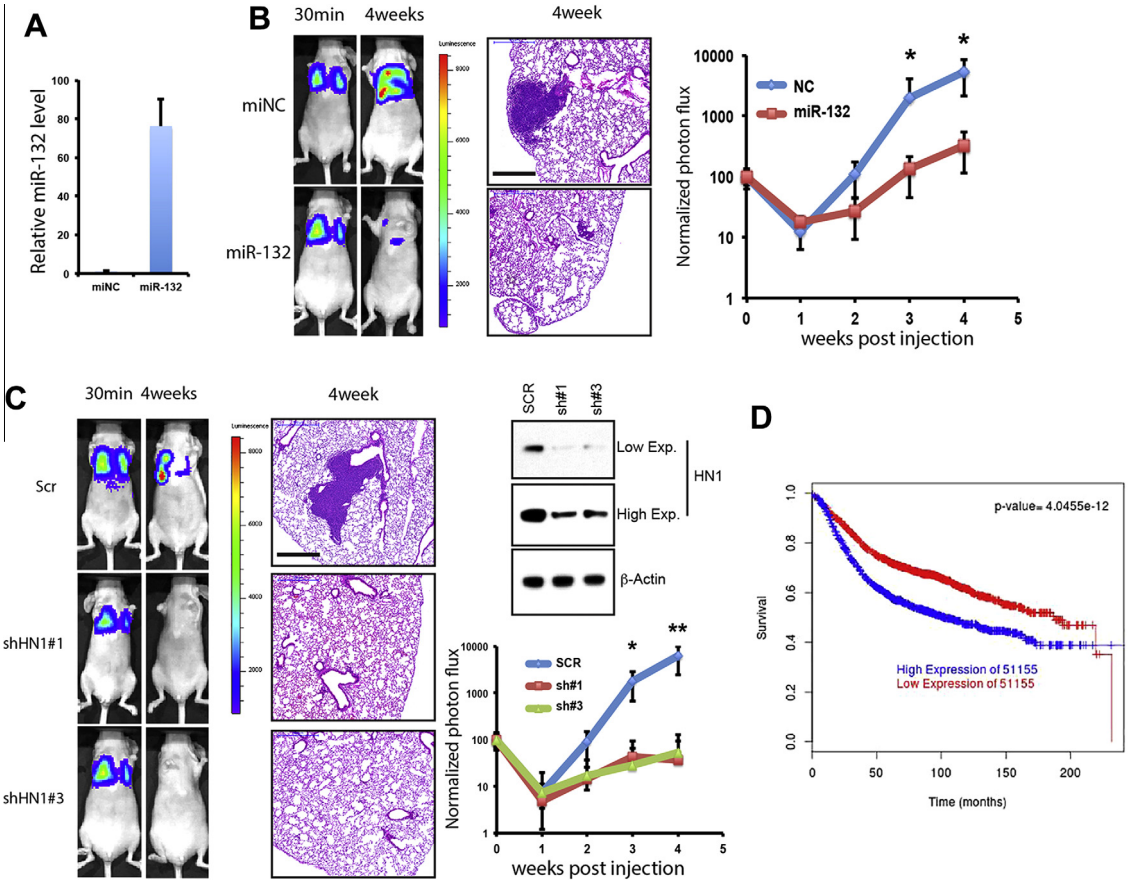

Fig. 4. MiR-132 inhibits lung metastasis *in vivo*. (A) Relative miR-132 level of 4T1 cells that were transfected with Lenti virus based miR-132 plasmid compared to control. (B) Luminescence of nude mice that were tail vein injected with 4T1 cells at the initiation time point and 4 weeks after (left panel); lung sections for H&E staining (middle panel, scaled bar length = 500 μm); relative quantification of luminescence showed in right panel. (C) HN1 expression of 4T1 cells was evaluated by Western blot assay, which were transfected with shRNA or scramble RNA (right upper panel); luminescence of nude mice that were tail vein injected with HN1 silenced 4T1 cells and scramble control cells (left panel); lung sections for H&E staining (middle panel, scale bar length = 500 μm); Relative quantification of luminescence showed in right lower panel. (D) Overall survival curve of 51,155 breast cancer patients with high and low HN1 expression in breast cancer tissues. Data were shown as mean ± SD from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

down-regulated in Hp-infected gastric cancer [19]. Thus, down-regulation of the microRNAs may be an important step in tumor progression. The microRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) was markedly down-regulated in breast cancer cells that had undergone EMT in response to transforming growth factor (TGF)-β [20].

In this study, we determined that miR-132 levels were markedly decreased in breast cancer tissues by comparison with paratumor normal breast tissues (Fig. 1A), suggesting that assaying for miR-132 could be a useful diagnostic biomarker. Moreover, miR-132 overexpression in breast cancer cells reduced cell proliferation, invasion, migration and metastasis *in vivo* and *in vitro* (Figs. 2 and 4). All the evidences from our study and others' revealed that miR-132 was a tumor suppressor in breast cancer progression.

Computational algorithms predicted several genes as potential targets of miR-132, but only a couple of targets were validated, such as hematological and neurological expressed sequence 1 (HN1) and E1A binding protein p300 (Ep300). Here, our results demonstrated that frequently up-regulated miR-132 in breast cancer influence cancer cell behavior by targeting HN1. HN1 expression is high in various carcinomas, while low in benign tumors [13]. In general, HN1 is an interaction partner of the inactive GSK3β/β- catenin/APC complex, which promotes ubiquitin-dependent proteasomal degradation of β-catenin, and contributes to migration in prostate cancer [15]. Conversely, knocking down HN1 suppresses cell proliferation by inducing cell cycle arresting [12]. By qRT-PCR, Western blot, and luciferase reporter assays, we found that HN1 was a direct target of miR-132. Overexpression of HN1 restored the malignancy effects that reduced by miR-132 in breast cancer, including cell proliferation, invasion, migration and metastasis (Fig. 3). Thus, these data proved that HN1 functions as an oncogene in breast cancer, which was regulated by miR-132.

As described above, HN1 contributes to decreased disease-specific survival times of 189 patients with resected HCC [16]. In our study, we demonstrated that high expression level of HN1 was negatively correlated with overall survival time of breast cancer patients. Combination of this clinical evidence with the results of Fig. 1 indicates that decreased miR-132 level directly abolished the HN1 gene suppression, which leaded to a high level of HN1 expression and cancer progression in breast cancer patients.

In this study, we demonstrated the importance of regulation of miR-132 and its target HN1 in breast cancer progression. The results collectively supported a tumor-suppressed role of miR-132 in prohibiting breast cancer cell proliferation, invasion, migration and metastasis through down-regulating HN1 expression. Notably, the newly identified counteracting role of miR-132 and HN1 dictating breast cancer progression should be useful in developing a translational therapeutic strategy in breast cancer. We speculated that therapeutic intervention of miR-132 or HN1 might represent a promising therapeutic strategy for breast cancer in the near future.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.049.

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