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Original Article

MiR-130a regulates the proliferation and metastasis of HCC cells through targeting ZEB1/2

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Abstract: Accumulating evidence indicates that microRNA-130a (miR-130a) function as a tumor suppressor, involved in many biological processes including tumor initiation, development and metastasis. However, the function and underlying molecular mechanism of miR-130a in hepatocellular carcinoma (HCC) still remains to be further elucidated. During to the resistant to chemo-/radio-therapy and high recurrence/metastatic rate after surgery, five-year survival rate of patients diagnosed with HCC is still unsatisfied. Thus, exploring the underlying mechanism of HCC and finding new treatment targets is essential for improving the survival rate of HCC patients. In the present study, we measured the dysregulated level of miR-130a in clinical HCC tissues and HCC cells, and to investigate its function and underlying mechanisms in the initial and progression of HCC. The current study revealed that miR-130a expression was significantly downregulated in HCC tissues and cell lines. Gain-of-function and loss-of-function assays indicated that forced expression of miR-130a in HCC cells inhibited cell proliferation and migration/invasion. Bioinformatics and luciferase reporter assays confirmed that ZEB1/2 was targets gene of miR-130a. The results of the present study indicated that miR-130a could be a potential target for treating HCC.

Keywords: HCC, miR-130a, proliferation, metastasis, ZEB1/2

Introduction

Liver cancer is the second most common cause of cancer-associated death worldwide [1]. As a subtype of liver cancer, hepatocellular carcinoma occupies 70-90% of primary liver cancer. During to the resistant to chemo-/radio-therapy and high recurrence/metastatic rate after surgery, five-year survival rate of patients diagnosed with HCC is still unsatisfied [2, 3]. Therefore, exploring the underlying mechanism of HCC and finding new treatment targets is essential for improving the survival rate of HCC patients.

MicroRNAs (miRNAs), a group of small noncoding RNAs, have been reported as critical regulators involving in a variety of physiological and pathological processes through binding to the 3'-untranslated region (UTR) of the target Mrna [4]. MiR-130a has been shown to both as oncogene and anti-oncogene in multiple cancers [5-8]. Li B et al. has reported that miR-130a down-regulated in hepatocellular carcinoma and associates with poor prognosis, however, the underlying mechanism has not been completely elucidated [9]. Thus, the function of miR-130a in HCC needs to be further illustrated.

In the current study, we investigate the expression of miR-130a in HCC tissues and HCC cells. We also identified zinc finger E-box-binding homeobox 1 and 2 (ZEB1 and ZEB2) as target gene of miR-130a involved in the function of miR-130a exerted in the proliferation and metastasis of HCC cells. We demonstrate that miR-130a acts as a tumor suppressor in HCC, and targeting miR-130a may be a novel pathway for HCC treatment.

Method and materials

Patients

HCC tissues (n=36) and pair-matched noncancerous tissues were obtained from patients diagnosed with HCC at Shanghai Tongren Hospital. Informed consent was obtained from patients.

Cell lines

Three HCC cell lines (SMMC-7721, HepG2, Hep3B), and a normal liver epithelium cell line (L02) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in DMEM (GIBCO-BRL) medium supplemented with 10% fetal bovine serum (10% FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified air at 37°C with 5% CO_2.

Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA from tissues and cells was isolated with Trizol reagent (Invitrogen, CA, USA) under the manufacturer's instructions. Reverse transcription was performed with PrimeScript RT reagent Kit (Takara, Japan) according to the manufacturer's protocol. qRT-PCR was performed with SYBR Prime Script RT-PCR Kits (Takara, Japan) based on the manufacturer's instructions. The miR-130a and EZH1/2 level was calculated with the $2^{-\Delta\Delta Ct}$ method, which was normalized to u6 and GAPDH, respectively. All assays were performed in triplicate. The expression levels were relative to the fold change of the corresponding controls, which were defined as 1.0.

Cell viability

Cell viability was assessed via 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-trtrazolium bromide (MTT) assay. 5×10^3 cells/well were seeded in a 96-well flat-bottomed plate for 24 h, then transfected with indicated vectors and cultured in normal medium. At 0, 24, 48, 72 h and 96 h after transfection, the MTT solution (5 mg/ml, 20 μl) was added to each well. Following incubation for 4 h, the media was removed and 100 μl DMSO were added to each well. The relative number of surviving cells was assessed by measuring the optical density (O.D.) of cell lysates at 560 nm. All assays were performed in triplicate.

Colony formation assay

Cells (500 cells/well) were plated in 6-well plates and incubated in normal medium at 37°C. After two weeks, the cells were fixed and stained with 0.1% crystal violet. The number of visible colonies was counted manually.

Wound healing assays

Cell migration capacity was calculated by wound healing assay. 2×10^5 cells with or without transfection were plated into 12-well plates and incubated in DMEM with 10% FBS at 37°C. After reaching 100% confluence, cells were wounded by scraping with a 200 μl tip, following washed 3 times in serum-free medium and incubated in regular medium. Wounds were observed at 0 and 48 h. The cell migration distance was calculated by subtracting the wound width at each time point from the wound width at the 0 h time point. Three independent assays were assayed.

Cell migration and invasion assays

Cell migration/invasion assays were detected by transwell chamber (8 um pore size, Corning). 48 h after transfection, cells in serum-free media were placed into the upper chamber, while medium containing 10% FBS was added into the lower chamber. After 48 h incubation, cells remaining in upper membrane were wiped off, and cells that migrated were fixed in methanol, and stained with 0.1% crystal violet. Cells were counted under a microscope. Three independent experiments were carried out.

Cell attachment and detachment assay

For attachment assay, cells were seeded in 24-well plates at 5×10^4 cells per well. Unattached cells were removed after 1 h incubation, and the attached cells were counted after trypsinization. The data were presented as a percentage of the attached cells compared to total cells. For cell detachment assay, after 24 h incubation, the cells were incubated with 0.05% trypsin for 3 min to detach the cells. Then, the culture medium was added to inactivate the trypsin and the detached cells were collected. The remaining cells were incubated with 0.25% trypsin to detach and counted. The data were presented as a percentage of the detached cells to total cells. Three independent experiments were carried out.

Cell transfection

PcDNA3.1/ZEB1/2 (pcDNA3.1 as control) and miR-130a mimics and inhibitor were purchased from Applied Biological Materials (ABM, Canada). Transfections were performed using

the Lipofectamine 2000 kit (Invitrogen) according to the manufacturer's instructions.

Dual luciferase reporter assay

pmirGLO-ZEB1wt and pmirGLO-ZEB1-mut (miR-130a) (or pmirGLO-ZEB2wt and pmirGLO-ZEB2-mut (miR-130a) was co-transfected with miR-130a mimics or miRNA NC into HEK 293 cells by Lipofectamine-mediated gene transfer. The relative luciferase activity was normalized to Renilla luciferase activity 48 h after transfection. The data were relative to the fold change of the corresponding control groups defined as 1.0.

Western bolt analysis and antibodies

Total protein lysates were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were electrophoretically transferred to polyvinylidene difluoride membranes (Roche). Protein loading was estimated using mouse anti-GAPDH monoclonal antibody. The membranes were blotted with 10% non-fat milk in TBST for 2 h at room temperature, washed and then probed with the rabbit anti-human ZEB1 (1:2000 dilution), ZEB2 (1:2000 dilution), and GAPDH (1:3000 dilution), overnight at 4°C, followed by treatment with secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. The proteins were detected using an enhanced chemiluminescence system and exposed to X-ray film. All antibodies were purchased from Abcam (USA).

Statistical analysis

Data are shown as the means ± standard error of at least three independent experiments. The SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Two group comparisons were performed with a Student t test. Multiple group comparisons were analyzed with one-way ANOVA. Statistically significant positive correlation between miR-130a and ZEB1/2 expression levels in 36 cases of HCC tissues was analyzed by Spearman's correlation analysis. Statistically significant negative correlation between miR-130a and ZEB1/2 expression levels in 36 cases of HCC tissues was analyzed by Spearman's correlation analysis. All tests performed were two-sided. $P<0.05$ was considered statistically significant.

Results

Expression of miRNA-130a, ZEB1, and ZEB2 in HCC tissues and HCC cell lines

To investigate the role of miR-130a in HCC, we collected 36 pairs HCC tissues and adjacent normal tissues, and performed qRT-PCR to measure the expression of miR-130a. As shown in **Figure 1A**, the average expression level of miR-130a was significantly downregulated in HCC tissue samples compared with adjacent controls. Additionally, we also analyzed the putative targets of ZEB1, and ZEB2 in HCC tissues and corresponding normal tissues. The mRNA levels of ZEB1 and ZEB2 were significantly upregulated in HCC tissues compared with the adjacent tissues (**Figure 1B**). Moreover, the similar results were observed in the HCC cell lines. We examined the level of miR-130a and ZEB1/2 in three HCC cell lines (HepG2, Hep3B, SMMC-7721), and a normal liver epithelium cell line L02. As is shown in **Figure 1C**, the lowest level of miR-130a (and ZEB1/2) was observed in SMMC-7721 (and HepG2) cells, while the highest level of miR-130a (and ZEB1/2) was obtained in HepG2 (and SMMC-7721) cells. These results indicated that miR-130a and ZEB1/2 might be involved in the progression of HCC.

ZEB1/2 was regulated by miRNA-130a both in transcription and translation level

Two putative targets of miR-130a screened by two bioinformatic analysis websites (http://www.targetscan.org/cgi-bin/targetscan/vert_61/view_gene.cgi?taxid=9606&rs=NM_001083588&members=&showcnc=0&shownc=0&showncf=), and (http://mirdb.org/cgi-bin/target_detail.cgi?targetID=1804872) and dual luciferase reporter assay was performed to make further confirm, as present in **Figure 2A** (top), the 3'UTR region of both ZEB1 and ZEB2 contains miR-130a binding site, and **Figure 2A** (bottom) showed that miR-130a mimics reduced the luciferase activity of wild-type (WT) ZEB1 and ZEB2 reporter vector but not that of mutant reporter vector (MUT). To further confirm the putative targets of miR-130a, we transfected miR-130a mimics or miR-130a inhibitor

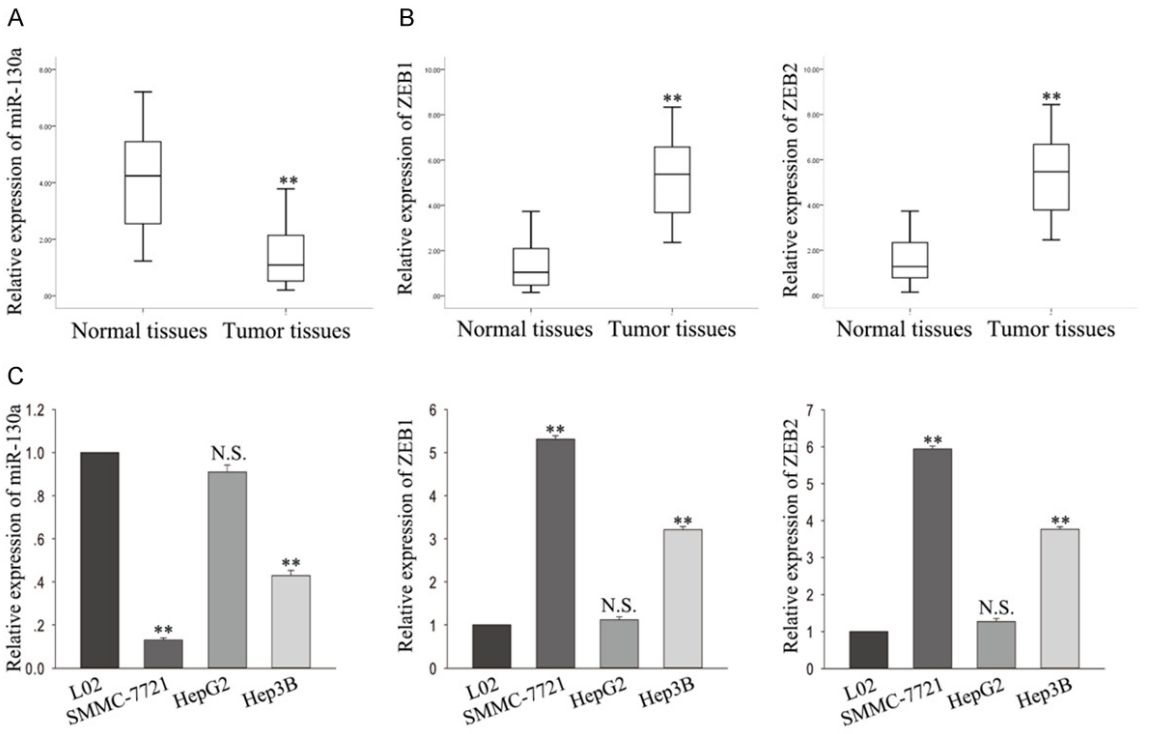

Figure 1. Expression of miRNA-130a, ZEB1, and ZEB2 in HCC tissues and HCC cell lines. A, B. qRT-PCR was performed to measure the miR-130a and ZEB1/2 expression in 36 pairs of HCC tissues and corresponding histologically normal tissues. C. The level of miR-130a in four HCC cell lines (HepG2, Hep3B, SMMC-7721), and a normal liver epithelium cell line L02. Error bars represent the mean ± SEM of at least three independent experiments. N.S.: no significant *P<0.05, **P<0.01 vs. control group.

into SMMC-7721 cells and HepG2 cells to overexpress or delete the expression of miR-130a (**Figure 2B**). After miR-130a mimics or inhibitor transfection, we analyzed the mRNA and protein expression level of ZEB1 and ZEB2. As shown in **Figure 2C**, the mRNA and protein levels of ZEB1 and ZEB2 were markedly decreased by miR-130a mimic transfection and obviously increased by miR-130a inhibitor transfection compared with the negative control, respectively. These results determined that miR-130a directly targeted ZEB1 and ZEB2 and regulates their expression at transcriptional and translational levels.

The effects of miRNA-130a on cell proliferation, metastasis of HCC cells

Then we explore the function of miR-130a on proliferation and metastasis of HCC cells. Cell proliferation was measured by using MTT and colony formation assays. As shown in **Figure 3A**, MTT revealed that overexpression of miR-130a decrease cells viability, while downregulation of miR-130a increased cell viability. Consistent with MTT, colony formation showed that forced expression of miR-130a significantly reduced the proliferation ability of HCC cells, and vice vasa in cells knockdown miR-130a (**Figure 3B**). Then, results from wound healing assay, transwell and attachment/detachment assays showed that forced expression of miR-130a significantly inhibited HCC cells metastasis, while deletion of miR-130a obviously facilitated cells metastasis (**Figure 3C-E**). These results together indicate that miR-130a could suppress the proliferation and metastasis of HCC cells.

The effect of miRNA-130a is dependent on ZEB1/2

To further confirm that the role of miR-130a played in HCC is mediated by ZEB1/2, rescue assays were performed. SMMC-7721 cells were transfected with empty vector, miR-130a

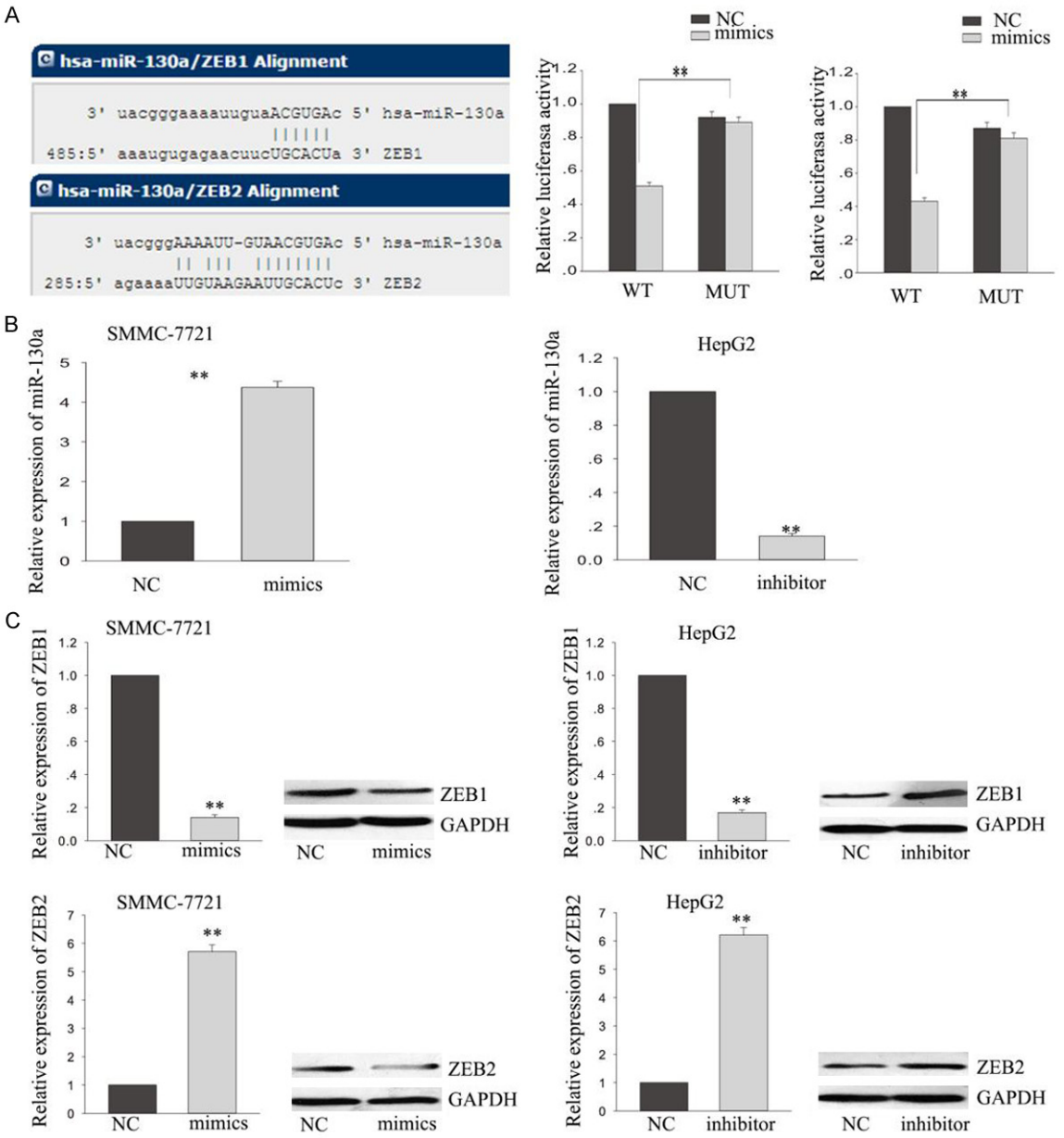

Figure 2. ZEB1/2 was regulated by miRNA-130a both in transcription and translation level. A. The binding site between miR-130a and ZEB1/2 (top), luciferase reporter assays (bottom), luciferase reporter assays (bottom). B. Satisfactory transfection efficiency of miR-130a mimics and inhibitor. C. The level of ZEB1/2 both in mRNA and protein level in response to the level of miR-130a. Error bars represent the mean ± SEM of at least three independent experiments. N.S.: no significant *P<0.05, **P<0.01 vs. control group.

mimics and pcDNA3.1/ZEB1 or ZEB2. As shown in **Figure 4A** and **4B**, MTT and colony formation assays revealed that single used of ZEB1 or ZEB2 can't obviously reverse the anti-proliferation function of miR-130a, while co-transfected with ZEB1/2 and miR-130a could significantly abolished anti-proliferation function of miR-130a. Additionally, wound healing assays, transwell assays, and attachment/detachment assays described that single transfected with ZEB1 or ZEB2 can't markedly reverse the anti-metastasis effect of miR-130a, while co-transfected with ZEB1/2 and miR-130a could significantly reversed anti-metastasis function of miR-130a (**Figure 4C-E**). These findings suggest that miR-130a could significantly inhibit the proliferation and metastasis of HCC cells in a ZEB1/2-dependent way.

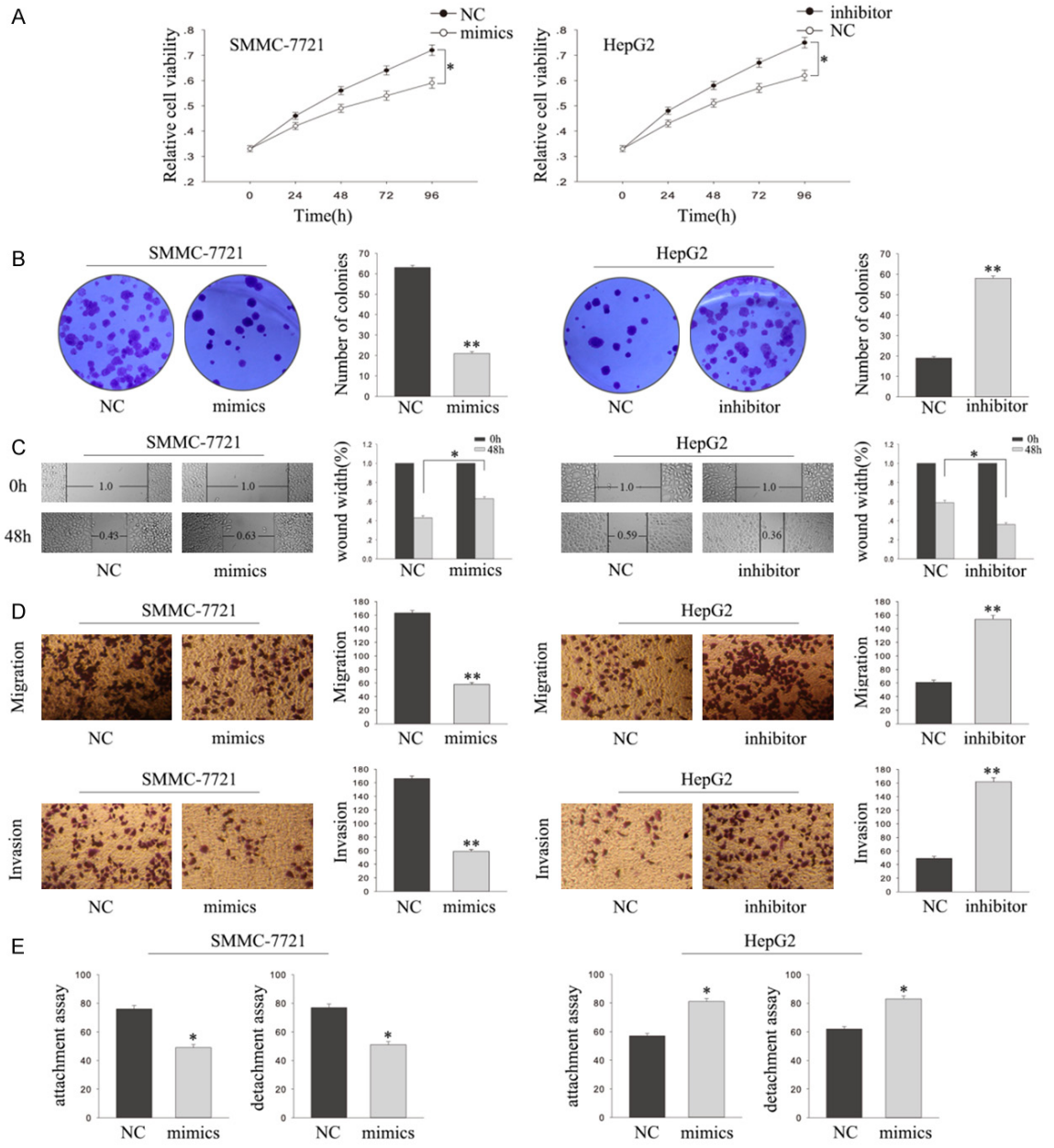

Figure 3. The effects of miRNA-130a on cell proliferation, metastasis of HCC cells. A, B. MTT and colony formation assays were performed to measure the effect of miR-130a on HCC cells proliferation. C-E. Wound healing assays, transwell assays and attachment/detachment assays were performed to detect the function of miR-130a on HCC cells migration capacity. Error bars represent the mean ± SEM of at least three independent experiments. N.S.: no significant *P<0.05, **P<0.01 vs. control group.

ZEB1/2 was negatively correlated with miR-130a

Finally, we analyzed the expression relationship between miR-130a and ZEB1/2. The results showed that the level of ZEB1 and ZEB2 was negative correlation with the level of miR-130a expression level, respectively (2-tailed Spearman's correlation, r=-0.884 and r=-0.899, P<0.01; **Figure 5A**). Collectively, these findings indicated a regulatory signal pathway in which miR-130a regulated ZEB1/2, eventually causing decreased proliferation ability and metastasis capacity in HCC cells (**Figure 5B**).

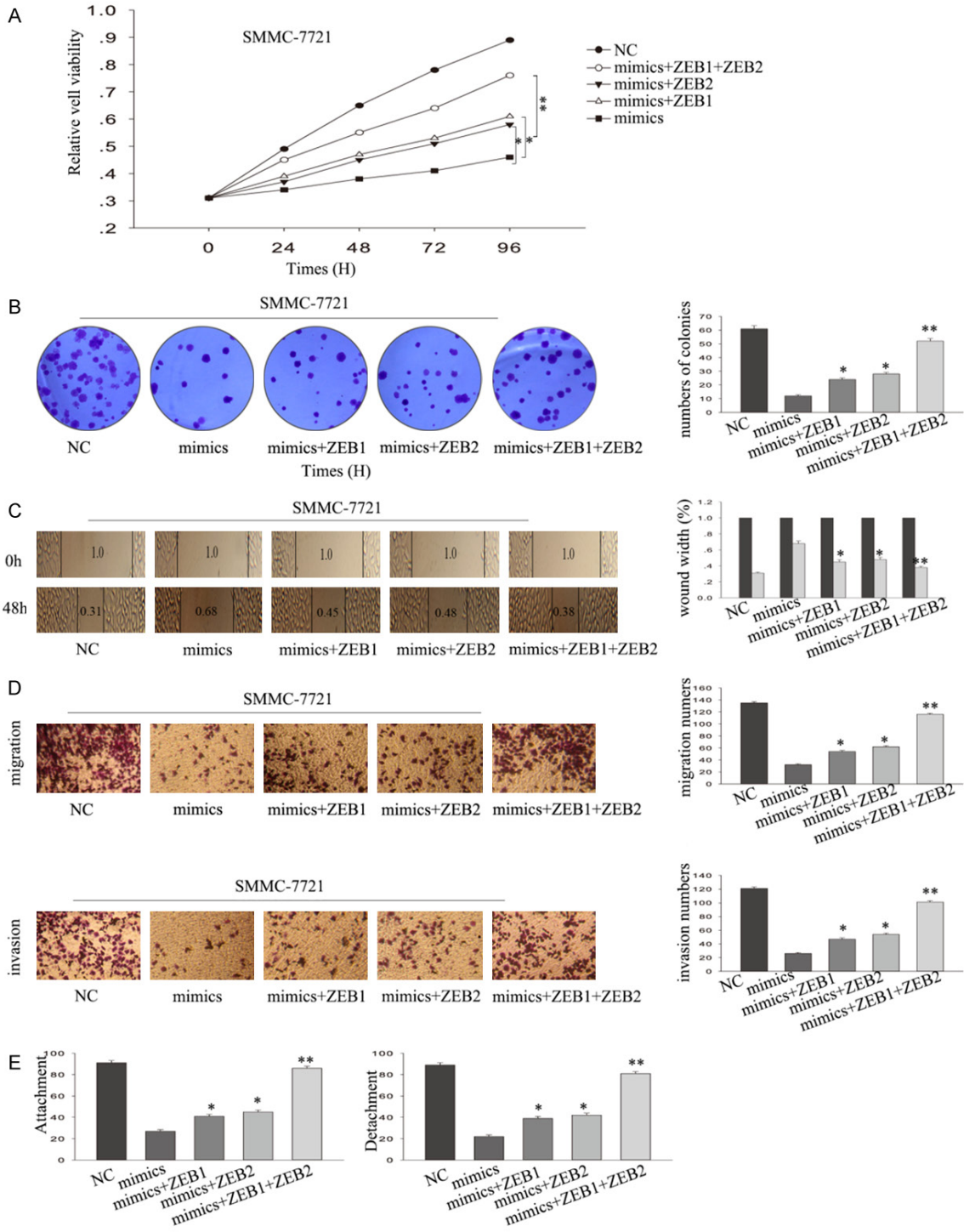

Figure 4. The effect of miRNA-130a is dependent on ZEB1/2. A, B. MTT and colony formation rescue assays. C-E. Wound healing, transwell, and attachment/detachment rescue assays. Error bars represent the mean ± SEM of at least three independent experiments. N.S.: no significant *P<0.05, **P<0.01 vs. control group.

Discussion

In the present study, we demonstrated that the decreased miR-130a was critical for the proliferation and metastasis of HCC cells. Also, restoration of miR-130a could suppress the proliferation ability and metastasis capacity in HCC cells via targeting ZEB1/2.

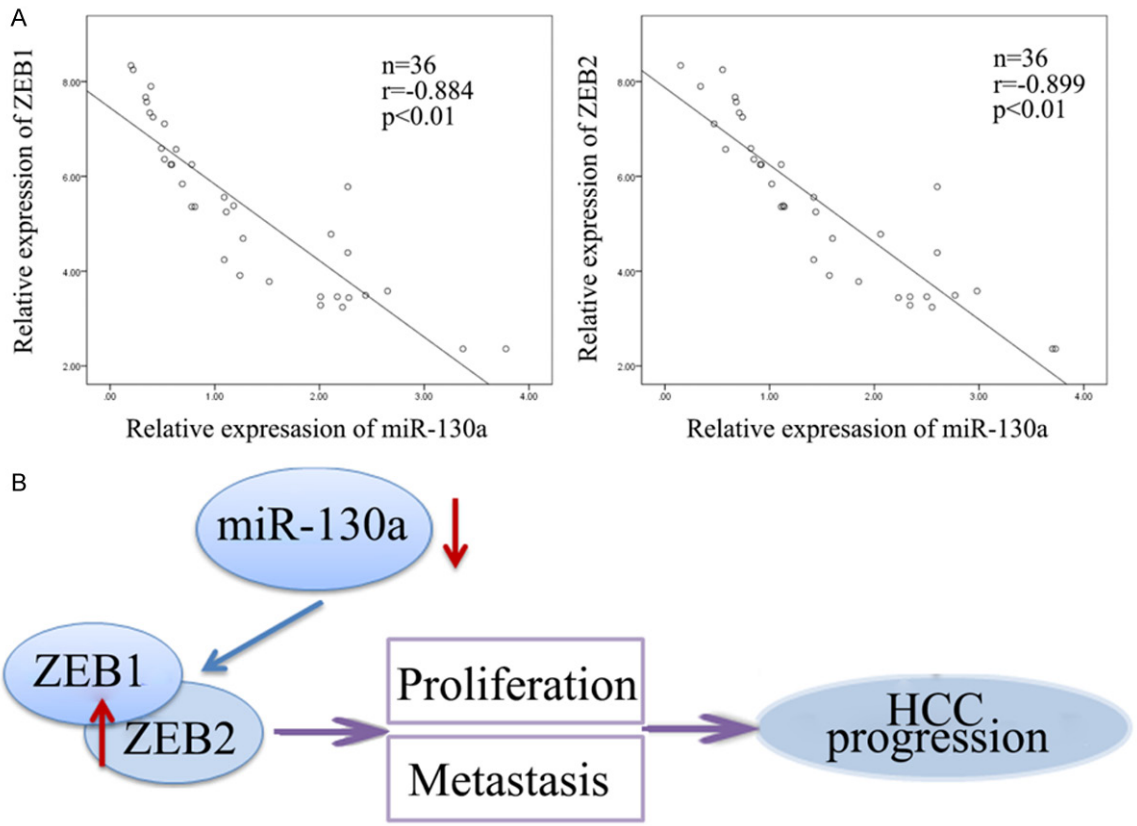

Figure 5. ZEB1/2 was negatively correlated with miR-130a. A. The level of ZEB1/2 was negative correlation with the level of miR-130a expression level (Spearman's correlation analysis, r=-0.884 and r=-899, P<0.01). B. Schematic overview of miR-130 regulatory signaling. Error bars represent the mean ± SEM of at least three independent experiments. *P<0.05, **P<0.01 vs. control group.

Hepatocellular carcinoma (HCC) is the third leading cause of cancer associated mortality. Although the improvement of mechanism study and clinical treatment has been made, the survival of patients with HCC still remains unsatisfied. Accumulating evidence has shown that microRNAs (miRNAs) function as critical factors for tumor recurrence and metastasis. MiR-130a has been identified down-regulated in hepatocellular carcinoma and associates with poor prognosis; however, the underlying mechanism has not been completely elucidated. To investigate the function of miR-130 in HCC, we measure the level of miR-130 in HCC tissues and HCC cells. Results from qRT-PCR showed that miR-130a was significantly down-regulated in HCC tissues and HCC cells. MiR-130 has been reported in many cancers and function both as oncogene and tumor suppressor [5, 10-16]. In 2016, Quan Li et al. revealed that miRNA-130a regulates cell malignancy by targeting RECK in chronic myeloid leukemia [17]. Shun Meng et al. demonstrated that downregulation of miRNA-130a contributes to endothelial progenitor cell dysfunction in diabetic patients via targeting Runx3 [18]. What's more, miRNA-130a was identified by Ningwei Li et al. as novel regulators of cisplatin resistance in human ovarian cancer A2780 cells in 2015 [19]. In our study, we found that ectopic of miR-130a could inhibitor HCC cell proliferation and metastasis. Meanwhile, deletion of miR-130a could significantly promote the proliferation ability and metastatic potential of HCC cells. Additionally, we found that the 3'-UTR of ZEB1 and ZEB2 gene contained binding sites for miR-130a by utilizing TargetScan, and miRBase. ZEB1 and ZEB2, two members of the ZEB family of transcription factors, are characterized by the presence of two zinc finger clusters and a centrally located homeodomain [20]. The expression of ZEB1/2 is regulated by multiple signaling pathways, including WNT, transforming growth factor beta, and miRNAs [21]. We

revealed that miR-130a can directly target to 3'-UTR of ZEB1 and ZEB2 and regulate their expression both at transcriptional and translational levels. In HCC cells, miR-130a interacts with the oncogene ZEB1/ZEB2 at their 3'-UTR, and silenced the level of miR-130 results in high level of ZEB1/ZEB2 expression, while ectopic expression of miR-130a can inhibit the progression of HCC cells.

In summary, our study was the first to investigate the role of miR-130a/ZEB1/ZEB2 in HCC cells. Our results showed that miR-130a was down-expression in HCC tissues and HCC cells and these results might provide a strong rationale for its potential use as a therapeutic target for treating HCC.

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Disclosure of conflict of interest

None.

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