

Long Non-Coding RNA ZEB2-AS1 Promotes Hepatocellular Carcinoma Progression by Regulating The *miR-582-5p*/FOXC1 Axis

Shimin Wu, Ph.D.*, Juan Chen, B.D., Ying Liang, M.D., Qian Luo, B.D., Yaoyao Tong, M.D., Ling Xie, B.D.

Center for Clinical Laboratory, General Hospital of The Yangtze River Shipping, Wuhan Brain Hospital, Wuhan, Hubei, China

*Corresponding Address: Center for Clinical Laboratory, General Hospital of The Yangtze River Shipping, Wuhan Brain Hospital, Wuhan, Hubei, China

Email: youfuyou805@163.com

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Abstract

Objectives: Long non-coding RNAs (lncRNAs) feature prominently in tumors. Reportedly, lncRNA zinc finger E-box-binding homeobox 2 antisense RNA 1 (ZEB2-AS1) is aberrantly expressed in a variety of tumors. The present study was aimed to explore ZEB2-AS1 functions and determine mechanism in hepatocellular carcinoma (HCC) progression.

Materials and Methods: In this experimental study, expressions of ZEB2-AS1, microRNA (*miR*)-582-5p and forkhead box C1 (FOXC1) mRNA in HCC tissues and cell lines were detected via quantitative reverse transcription polymerase chain reaction (qRT-PCR). After establishing gain- and loss-of-functions models, cell counting kit-8, 5-bromo-2'-deoxyuridine (BrdU), Transwell assays and flow cytometry analysis were conducted to examine HCC cell multiplication, migration, invasion and apoptosis, respectively. The targeted relationship between *miR*-582-5p and ZEB2-AS1 was verified via dual-luciferase reporter gene assay. Western blot was utilized for detecting FOXC1 expression in HCC cells after selectively regulating ZEB2-AS1 and *miR*-582-5p.

Results: In HCC tissues and cells, ZEB2-AS1 expression was increased. High ZEB2-AS1 expression was related to relatively large tumor volume, increased tumor-node-metastasis (TNM) stage and positive lymph node metastasis of the patients. ZEB2-AS1 overexpression facilitated HCC cell multiplication, migration, invasion and suppressed apoptosis, while ZEB2-AS1 knock-down caused the opposite effects. It was also confirmed that ZEB2-AS1 could competitively bind with *miR*-582-5p to repress its expression, and indirectly up-regulate FOXC1 expression level in HCC cells.

Conclusion: The current study revealed that ZEB2-AS1 was over-expressed in HCC tissues and cells. It also up-regulated FOXC1, through sponging *miR*-582-5p, to promote HCC progression. This provides new perspectives for elucidating the pathogenesis of HCC.

Keywords: Forkhead Box C1, Hepatocellular Carcinoma, Long Non-Coding RNA, *miR*-582-5p

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Introduction

Globally, known to rank third among the causes of cancer-related deaths, hepatocellular carcinoma (HCC) makes up about 90% of primary liver cancer cases (1, 2), and half of deaths occurred in China (3). Hepatitis C or B virus (HCV or HBV) infection is one of the primary risk factors for HCC tumorigenesis (4). Despite the recent improvements in treatments, such as liver transplantation, hepatectomy, radiotherapy, chemotherapy and targeted therapy, five-year overall survival rate of HCC patients is still very low as a result of metastasis and recurrence (5, 6).

Recognized as a kind of non-coding RNA, long non-coding RNAs (lncRNAs) are with limited or without protein coding ability. They consist of over 200 nucleotides in length (7). lncRNAs regulate diverse biological processes, for instance, cell differentiation, proliferation, embryonic development and tumorigenesis (8, 9). A great deal of research has shown that lncRNAs feature prominently

in cancer biology, regulating tumor cell proliferation, drug resistance and epithelial-mesenchymal transition (EMT) (10-12). Previous studies proved that lncRNA zinc finger E-box-binding homeobox 2 antisense RNA 1 (ZEB2-AS1) was aberrantly expressed in several tumors and was strongly associated with tumorigenesis and cancer progression. For example, ZEB2-AS1 facilitated colorectal carcinoma cell multiplication and repressed apoptosis by enhancing β -catenin protein expression (13). Down-regulated ZEB2-AS1 expression suppressed HCC cell multiplication and metastasis via modulating ZEB2 expression (14). However, the mechanism of ZEB2-AS1 underlying HCC progression needs in-depth investigation.

Known as a type of single-stranded small non-coding RNAs, microRNAs (miRNAs or miRs) bind to mRNA 3'-untranslated region (3'-UTR) to negatively modulate gene expression, inducing the degradation of targeted messenger RNAs (mRNAs) (15). Reportedly, *miR*-582-5p functions as a tumor suppressor in different cancers.

For example, in bladder cancer, *miR-582-5p* represses cell multiplication via reducing human monopolar spindle 1 (HMPS1/TTK) expression (16). *miR-582-5p* was lowly expressed in HCC and it repressed cell multiplication by targeting *CDK1* and *AKT3* (17). Nevertheless, *miR-582-5p* molecular mechanism in HCC needs to be further investigated.

The forkhead box (FOX) transcriptional factor family shared a winged helix-turn-helix DNA binding domain and this domain is crucial in regulating cell differentiation, metabolism, proliferation, migration, invasion and apoptosis (18-20). Reportedly, overexpression of forkhead box C1 (*FOXCI*) induced transactivation of *CXCR1* and *CCL2* and facilitated HCC cell migration and invasion (21).

In the current research, we reported that *ZEB2-AS1* was up-regulated in HCC cell lines and tissues.

Materials and Methods

Tissue samples

Endorsed by the Research Ethics Committee of Wuhan Brain Hospital (Wuhan, China, Approval No. 2019-0517), this study was performed. All patients' informed consent was obtained and the present study enrolled 50 HCC patients who admitted to the hospital. The clinicopathological data of all patients were obtained and none of them underwent chemotherapy or radiotherapy before the surgery. The cancerous and the corresponding adjacent tissues were surgically removed and collected. Additionally, the cancer tissues of 20 breast cancer patients were obtained from our hospital, and then all the tissues were preserved at -196°C in liquid nitrogen.

Cell culture and transfection

In this experimental study, HCC cell lines (BEL7402, HCCLM3, SMMC-7721 and Huh7) and normal liver cell line (MIHA) were obtained from China Center for Type Culture Collection (Wuhan, China). From the American Type Culture Collection (Manassas, USA), we bought human breast cancer cell line MCF-7. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) containing 10% fetal bovine serum (FBS), 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 U/ml penicillin (ThermoFisher Scientific, USA) at 37°C in 5% CO_2 .

Small interfering RNA (siRNA) against *ZEB2-AS1* (si-*ZEB2-AS1-1*, si-*ZEB2-AS1-2* and si-*ZEB2-AS1-3*), siRNA control (si-NC), *miR-582-5p* inhibitors (*miR-582-5p-in*), inhibitors control (miR-in), *miR-582-5p* mimics (*miR-582-5p*), mimics control (miR-NC), *ZEB2-AS1* overexpression vector (*ZEB2-AS1*) and empty vector (Vector) were synthesized by RiboBio (Guangzhou, China). The oligonucleotides and plasmids were transfected into HCC cells using Lipofectamine 3000 (Invitrogen).

Quantitative reverse transcription polymerase chain reaction

TRIzol reagent (Vazyme, China) was utilized for total RNA isolation. PrimeScript RT reagent kit (TaKaRa, China) was applied for complementary DNA (cDNA) synthesis. For miRNAs, the PrimeScript miRNA cDNA Synthesis Kit (TaKaRa) was adopted to carry out reverse transcription. SYBR Premix Ex Taq I was employed to conduct qRT-PCR. *GAPDH* and *U6* acted as internal references for mRNA and miRNA, respectively. The $2^{-\Delta\Delta\text{Ct}}$ method was applied for calculation of the relative expression level. The primers used were as follows:

ZEB2-AS1-

F: 5'-GGCTGGATAGCAAAGGAC-3'
R: 5'-ACACTCTTGGCGAGGT-3'

miR-582-5p-

F: 5'-GCACACATTGAAGAGGACAGAC-3'
R: 5'-TATTGAAGGGGTTCTGGTG-3'

FOXCI-

F: 5'-CAGAACAGCATCCGCCACA-3'
R: 5'-TGTTGTAGGAGTCCGGGTC-3'

U6-

F: 5'-GCTTCGGCAGCACATATACTAAAAT-3'
R: 5'-CGCTTCACGAATTTGCGTGTTCAT-3'

GAPDH-

F: 5'-CACCCACTCCTCCACCTTTG-3'
R: 5'-CCACCACCCTGTTGCTGTAG-3'

Cell counting kit-8 (CCK-8) assay

CCK-8 assay was conducted to evaluate cell proliferation. HCC cells were inoculated at 1×10^3 cells/well in 96-well plates, and they were cultured at 37°C in 5% CO_2 for 24 hours. After culturing cells for 0, 1, 2, 3 and 4 days, 10 μl of CCK-8 reagent (Beyotime Biotechnology, China) was added to each well, followed by cell culture at 37°C in 5% CO_2 for another 2 hours. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad, USA). Four days later, cell proliferation curve was drawn.

5-Bromo-2-deoxyUridine (BrdU) assay

The BrdU method was used to determine DNA synthesis in proliferating cells. BrdU assay was conducted 48 hours after transfection. Briefly, the cells were inoculated in 96-well plates (2×10^3 cells/well) and cultured for 48 hours. Subsequently, the cells were incubated with a final concentration of 10 μM BrdU solution (Wuhan AmyJet Scientific Inc., China) for 4 hours at room temperature, followed by medium removal after the incubation period. The cells were fixed for 30 minutes with paraformaldehyde and then incubated with anti-BrdU antibody (Sigma-Aldrich, China) for 1 hour at room temperature in dark. After that, they were washed with PBS three times and

subsequently incubated with DAPI staining solution at room temperature for 30 minutes in dark. Eventually, the cells were observed under a fluorescence microscope.

Transwell assay

For migration assay, the upper Transwell compartment (BD Biosciences, USA) was loaded with 1×10^5 cells (in 200 μ l of serum-free DMEM). Then, the lower one was loaded with 700 μ l of 10% FBS-containing DMEM. 24 hours later, cells on the membrane underside were stained and then they were counted in three randomly selected high-power fields, under the microscope. For invasion assay, the chambers were precoated with a layer of Matrigel and the same procedures were conducted as described above.

Flow cytometry assay

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Vazyme, China) was adopted to evaluate the apoptosis of HCCLM3 and BEL7402 cells. The cells were harvested 48 hours after transfection and the cell concentration was adjusted to 1×10^6 cells/ml with binding buffer. Subsequently, the cells were incubated overnight with pre-cooled 70% ethanol at 4°C. After being centrifuged, the cells were re-suspended in binding buffer (200 μ l). The re-suspended cells were then stained with PI staining solution (5 μ l) and Annexin V-FITC staining solution (10 μ l) in dark for 15 minutes at room temperature. Afterwards, a MoFlo XDP flow cytometer (Beckman Coulter, USA) was utilized to analyze apoptotic cells. Data were processed by BD FACSDiva™ software (BD Bioscience, USA).

Xenograft model in nude mice

Animal Care and Use Committee of Wuhan Brain Hospital approved all animal experiments. Male BALB/c nude mice were bought from Experimental Animal Center of Wuhan University (Wuhan, China). Twenty nude mice (age: 5 weeks) were randomly divided into two groups, with 10 mice in each group. In the lung metastasis experiment, HCCML3 cells (2×10^6 cells per mouse), overexpressing ZEB2-AS1, or the control cells were injected into the tail vein of nude mice. The mice were euthanized after 3 weeks and the lungs tissues were collected for pathological examination. Lung metastasis was evaluated by hematoxylin-eosin (HE) staining. In brief, after fixation at room temperature for 4 hours, using 4% paraformaldehyde, the lung tissues were dehydrated with ethanol and embedded in paraffin blocks. Then, the tissues were sliced and lung tissue sections with a thickness of 5 μ m were dewaxed in xylene, rehydrated in ethanol of decreasing concentrations and washed with PBS. Then, HE staining solution (Beyotime, China) was employed to stain the sections for 5 minutes at room temperature. Next, the sections were dehydrated, sealed and observed under a light microscope.

Western blotting

To obtain protein samples, RIPA lysis buffer was

employed for lysing the cells and the supernatant was collected after centrifugation. A bicinchoninic acid kit (Abcam, USA) was utilized for determining the concentration of each protein sample. Next, equivalent amounts of proteins were dissolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to the polyvinylidene fluoride membrane (Millipore, USA), which were subsequently blocked with 5% skimmed milk. Then, the membranes were incubated with anti-FOXCI antibody (1:1000; Abcam, USA) and anti-GAPDH antibody (1:1000; Abcam, USA) for 12 hours at 4°C. Then, the membranes, after washing, were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000; Abcam, USA) for 2 hours in room temperature. Subsequently, the enhanced chemiluminescence reagent (Beyotime, China) was employed for developing the protein bands and a ChemiDoc MP system (Bio-Rad, USA) was utilized for the visualization.

Dual-luciferase reporter gene assay

To generate the wild-type (WT) luciferase reporter vector (ZEB2-AS1-WT and FOXCI-WT), the sequences of ZEB2-AS1 or FOXCI 3'-UTR containing the binding site for miR-582-5p were integrated into the pmirGLO luciferase reporter vector (Promega, USA). Meanwhile, the binding sites were mutated to produce the corresponding mutant (MUT) luciferase reporter vectors (ZEB2-AS1-MUT and FOXCI-MUT). The constructed vectors and miR-582-5p or miR-NC were co-transfected into HCCLM3 and BEL7402 cells. Ultimately, 48 hours after transfection, the luciferase reporter detection system (Promega) was utilized to analyze the luciferase activity of the cells.

Statistical treatment

All experiments were conducted in triplicate. All data in the experiments were analyzed by GraphPad Prism 8.0 software (GraphPad Software Inc., USA) and SPSS 22.0 (SPSS Inc., USA). A Chi-square test (χ^2 test) was employed for analyzing the relationship between clinicopathological features and ZEB2-AS1 expression in HCC samples. The measurement data were expressed as mean \pm standard deviation (mean \pm SD). Comparison of data among the multiple groups was performed by one-way analysis of variance, and a t test was performed for the comparison between two groups. $P < 0.05$ denoted that a difference was of statistical significance.

Results

ZEB2-AS1 was highly expressed in HCC and it was related to the patient's poor prognosis

To assess ZEB2-AS1 expression, qRT-PCR was conducted in normal tissues (n=50), HCC tissues (n=50) and breast cancer tissues (n=20). It was indicated that ZEB2-AS1 expression was markedly up-regulated in the HCC tissues against the normal tissues and breast cancer

tissues (Fig.1A). Besides, qRT-PCR was conducted to detect *ZEB2-AS1* expression in the normal liver cell line (MIHA cells), HCC cell lines (SMMC-7721, BEL7402, HCCLM3 and Huh7 cells) and breast cancer cell line, MCF-7. It was unveiled that, in contrast to the MIHA and MCF-7 cells, *ZEB2-AS1* expression in the four types of HCC cell was up-regulated (Fig.1B). Subsequently, the GEPIA database was employed for analyzing the relationship between HCC patients overall survival time and *ZEB2-AS1* expression, and it was discovered that

in comparison with HCC patients with low *ZEB2-AS1* expression, those with the high expression had a lower overall survival rate (Fig.1C). Moreover, the correlation between HCC patient clinicopathological indicators and *ZEB2-AS1* expression was analyzed by chi-square test, and the results suggested that high *ZEB2-AS1* expression was linked to relatively large tumor volume, increased tumor-node-metastasis (TNM) stage and positive lymph node metastasis (Table 1). The aforementioned evidences implied that *ZEB2-AS1* played a cancer-promoting role in HCC.

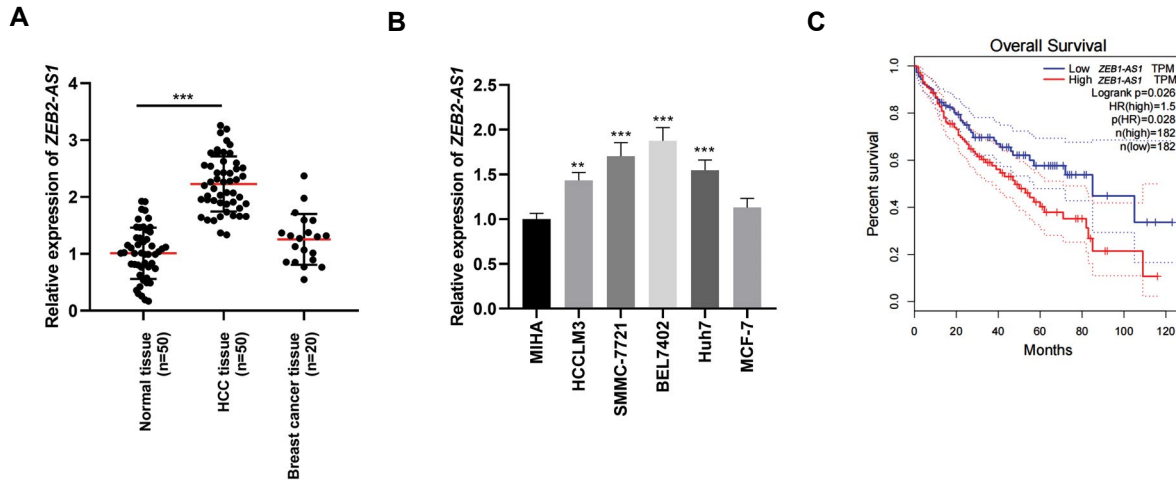


Fig.1: *ZEB2-AS1* is over-expressed in HCC tissues and cells. **A.** Detection by qRT-PCR of *ZEB2-AS1* expression in HCC tissues (n=50), adjacent normal tissues (n=50) and breast cancer tissues (n=20). **B.** Detection by qRT-PCR of *ZEB2-AS1* expression in normal human liver cell line (MIHA cells), HCC cell lines (BEL7402, SMMC-7721, HCCLM3, and Huh7 cells) and human breast cancer cells MCF-7. **C.** GEPIA database was employed for analyzing the relationship between *ZEB2-AS1* expression and HCC patient prognosis. All experiments were repeated 3 times, each in triplicate. **, P<0.01, ***, P<0.001, HCC; Hepatocellular carcinoma, qRT-PCR; Quantitative reverse transcription polymerase chain reaction, and GEPIA; Gene expression profiling interactive analysis.

Table 1: Correlation of *ZEB2-AS1* expression with multiple clinicopathological characteristics in hepatocellular carcinoma (HCC) patients (n=50)

Characteristics	Number	<i>ZEB2-AS1</i> expression		χ^2	P value
		High	Low		
Age (Y)					
≥ 60	28	17	11		
< 60	32	9	13	1.9361	0.1641
Gender					
Male	38	21	17		
Female	12	5	7	0.6755	0.4112
Tumor size (cm)					
≥ 5	32	21	11		
< 5	18	5	13	6.6111	0.0101
Hepatitis					
Negative	30	14	16		
Positive	20	12	8	0.8547	0.3552
TNM stage					
I - II	17	4	13		
III-IV	33	22	11	8.3648	0.0038
Lymph node metastasis					
Negative	21	6	15		
Positive	29	20	9	7.9623	0.0048

ZEB2-AS1 facilitated HCC cell proliferation, migration, invasion and inhibited cell apoptosis

To probe into *ZEB2-AS1* role in HCC progression, we selected HCCLM3 cells with the lowest *ZEB2-AS1* expression and BEL7402 cells with the highest expression to construct *ZEB2-AS1* overexpression or knock-down models, respectively (Fig.2A). Then, CCK-8 and BrdU assays were conducted to detect HCC cell multiplication. Against the control group, overexpression of *ZEB2-AS1* remarkably promoted HCCLM3 cell proliferation, while *ZEB2-AS1* knock-down notably repressed BEL7402 cell proliferation (Fig.2B-D). Additionally, Transwell assays showed that

ZEB2-AS1 overexpression significantly facilitated HCCLM3 cell migration and invasion, while knocking-down *ZEB2-AS1* inhibited BEL7402 cell migration and invasion (Fig.2E-H). Flow cytometry analysis revealed that overexpression of *ZEB2-AS1* suppressed HCCLM3 cell apoptosis and *ZEB2-AS1* knock-down increased BEL7402 cell apoptosis (Fig.2I, J). The results of HE staining revealed that as opposed to the Vector/HCCML3 group, there were significantly more metastasis nodules in the mouse lung tissues of the *ZEB2-AS1*/HCCML3 group (Fig.S1, See Supplementary Online Information www.celljournal.org). The above results indicated that *ZEB2-AS1* facilitated HCC growth and metastasis.

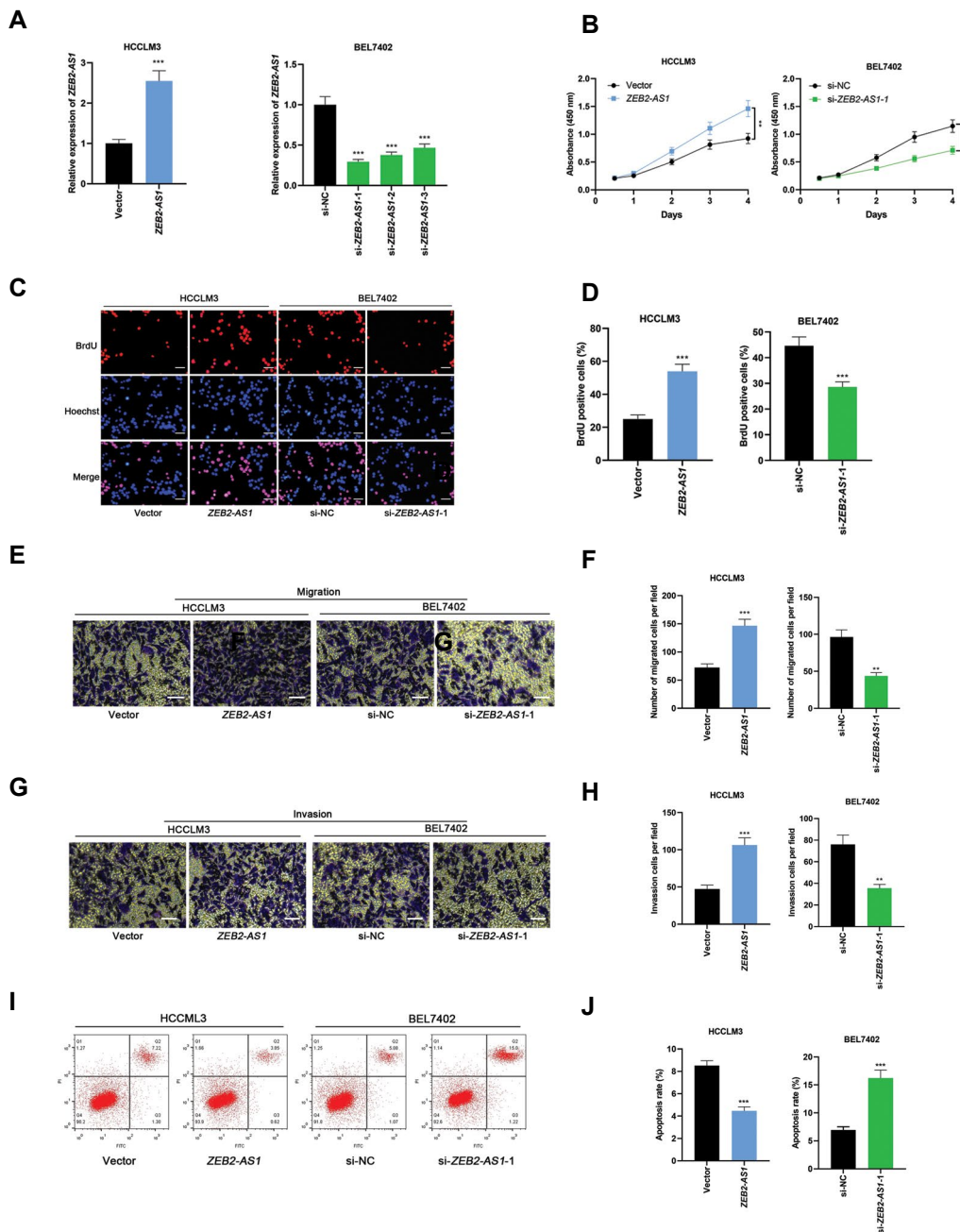


Fig.2: Effects of *ZEB2-AS1* on HCC cell proliferation, migration, invasion and apoptosis. **A.** Detection by qRT-PCR of *ZEB2-AS1* expression in HCCLM3 cells transfected with *ZEB2-AS1* overexpression plasmids and BEL7402 cells transfected with *ZEB2-AS1* siRNAs. **B-D.** CCK-8 and BrdU assays (scale bars: 75 μm) were conducted for detecting HCC cell proliferation after *ZEB2-AS1* overexpression or knock-down. **E-H.** Transwell assay was used to detect HCC cell migration and invasion (scale bars: 250 μm). **I, J.** Flow cytometry was conducted to evaluate apoptosis rate of HCCLM3 and BEL7402 cells after overexpression or knock-down of *ZEB2-AS1*. All experiments were repeated 3 times, each in triplicate. **, P<0.01, ***, P<0.001, HCC; Hepatocellular carcinoma, qRT-PCR; Quantitative reverse transcription PCR, siRNA; Small interfering RNA, CCK-8; Cell counting kit-8, and BrdU; 5-Bromo-2-deoxyUridine.

ZEB2-AS1 directly targeted miR-582-5p

To decipher mechanism of *ZEB2-AS1* in HCC progression, bioinformatics was adopted for predicting miRNAs pairing with *ZEB2-AS1* and it was uncovered that there existed potential binding sites between *ZEB2-AS1* and *miR-582-5p* (Fig.3A). Dual-luciferase reporter gene assay validated that *miR-582-5p* mimics could markedly reduce *ZEB2-AS1*-WT luciferase activity, but exerted no remarkable impact on *ZEB2-AS1*-MUT luciferase activity (Fig.3B). Subsequently, qRT-PCR displayed that *ZEB2-AS1* overexpression significantly inhibited *miR-582-5p* expression, while *ZEB2-AS1* knockdown markedly up-regulated *miR-582-5p* expression (Fig.3C). Additionally, qRT-PCR was conducted for evaluating *miR-582-5p* expression in 50 cases of HCC and para-cancerous tissues, and it was discovered that *miR-582-5p* expression was dramatically down-regulated in HCC tissues in comparison with adjacent normal tissues (Fig.3D). At the same time, we observed that *miR-582-5p* and *ZEB2-AS1* expressions in HCC tissues were inversely related (Fig.3E). The aforementioned evidences suggested that *ZEB2-AS1* directly targets *miR-582-5p* and negatively regulates its expression in HCC.

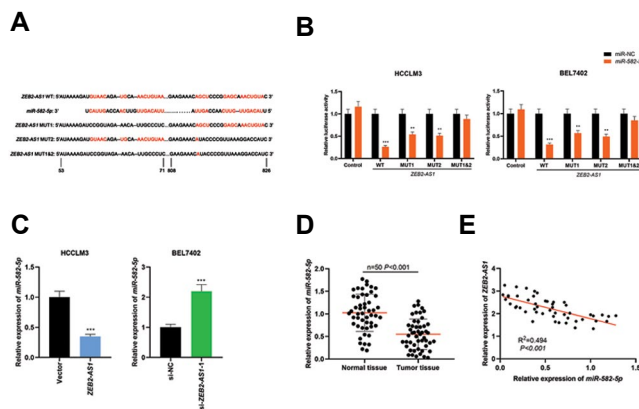


Fig.3: *miR-582-5p* is the target of *ZEB2-AS1* in HCC cells. **A.** Bioinformatics was adopted for predicting binding site between *ZEB2-AS1* and *miR-582-5p*. **B.** Binding relationship between *miR-582-5p* and *ZEB2-AS1* in HCC cells was detected by dual-luciferase reporter gene assay. **C.** Detection via qRT-PCR of *miR-582-5p* expression in HCC cells with overexpression or knock-down of *ZEB2-AS1*. **D.** Detection via qRT-PCR of *miR-582-5p* expression in 50 cases of HCC tissues and adjacent normal tissues. **E.** Detection of the correlation between *ZEB2-AS1* and *miR-582-5p* expressions in HCC tissues via qRT-PCR. All experiments were repeated 3 times, each in triplicate. *, P<0.01, **, P<0.001, HCC; Hepatocellular carcinoma, and qRT-PCR; Quantitative reverse transcription polymerase chain reaction.

miR-582-5p reversed the impact of ZEB2-AS1 on HCC cell multiplication, migration, invasion and apoptosis

To dig deeper into the role of the *ZEB2-AS1/miR-582-5p* axis in HCC, we transfected *ZEB2-AS1* overexpression plasmid, *miR-582-5p* mimic, *ZEB2-AS1* overexpression plasmid+*miR-582-5p* into HCCLM3 cells, respectively and transfected si-*ZEB2-AS1*-1, *miR-582-5p* inhibitors, si-*ZEB2-AS1*-1+*miR-582-5p* inhibitor into BEL7402 cells, respectively. Furthermore, HCC cell multiplication,

migration and invasion were detected through CCK-8, BrdU, Transwell assays and flow cytometry analysis. The results manifested that, as opposed to the control group, *ZEB2-AS1* overexpression markedly facilitated HCC cell proliferation, migration, invasion and inhibited cell apoptosis, while *miR-582-5p* mimics suppressed HCC cell proliferation, migration, invasion and increased cell apoptosis, in addition to weakening the effects of *ZEB2-AS1* overexpression on HCC cells; additionally, knocking-down *ZEB2-AS1* dramatically restrained HCC cell proliferation, migration, invasion and promoted cell apoptosis, while *miR-582-5p* inhibitors facilitated HCC cell proliferation, migration and invasion. It also suppressed cell apoptosis and partially counteracted the inhibiting effects of si-*ZEB2-AS1*-1 on the malignant phenotypes of HCC cells (Fig.4A-E).

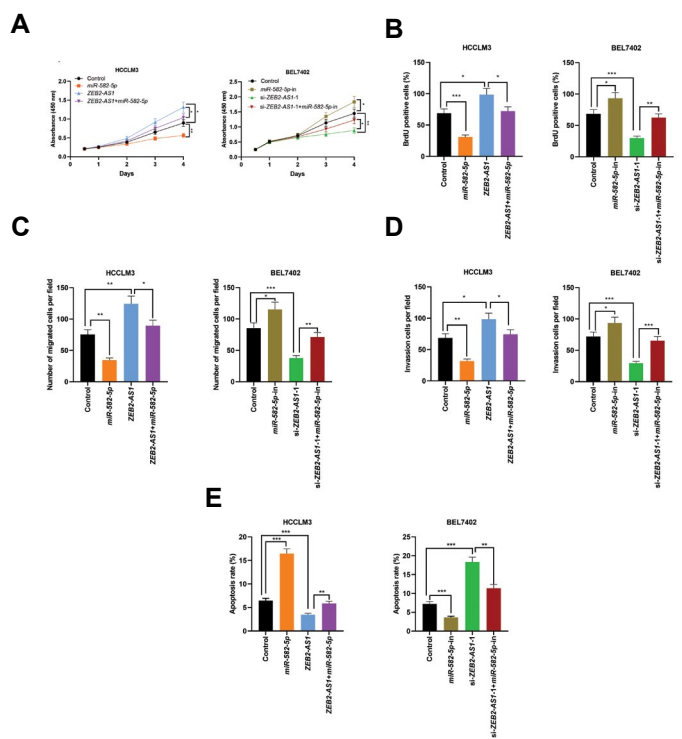


Fig.4: *miR-582-5p* reverses effects of *ZEB2-AS1* on HCC cell proliferation, migration, invasion and apoptosis. *ZEB2-AS1* overexpression plasmid, *miR-582-5p* mimic, *ZEB2-AS1* overexpression plasmid+*miR-582-5p* were transfected into HCCLM3 cells, respectively, and si-*ZEB2-AS1*-1, *miR-582-5p* inhibitors, si-*ZEB2-AS1*-1+*miR-582-5p* inhibitor were transfected into BEL7402 cells, respectively. **A, B.** CCK-8 and BrdU assays were utilized for examining HCCLM3 and BEL7402 cell proliferation. **C, D.** HCCLM3 and BEL7402 cell migration and invasion were detected through Transwell assays. **E.** Flow cytometry analysis was utilized to detect HCCLM3 and BEL7402 cell apoptosis. All experiments were repeated 3 times, each in triplicate. *, P<0.05, **, P<0.01, ***, P<0.001, HCC; Hepatocellular carcinoma, qRT-PCR; Quantitative reverse transcription polymerase chain reaction, CCK-8; Cell counting kit-8, and BrdU; 5-Bromo-2-deoxyUridine.

ZEB2-AS1 elevated FOXC1 expression by adsorbing miR-582-5p

A previous study has shown that *miR-582-5p* targeted and regulated *FOXC1* (22). Consistently, the TargetScan database exhibited that there existed a

binding site between *miR-582-5p* and *FOXC1* (Fig.5A). Subsequently, results of the dual-luciferase reporter gene assay manifested that *miR-582-5p* mimics could significantly inhibit *FOXC1*-WT luciferase activity, but failed to change the luciferase activity of *FOXC1*-MUT (Fig.5B, C). qRT-PCR displayed that *FOXC1* expression was increased in HCC tissues and cells (Fig.5D, E). Additionally, *ZEB2-AS1* overexpression enhanced *FOXC1* expression in HCCLM3 cells, while *miR-582-5p* mimics could down-regulate *FOXC1* expression and weaken the promoting effect of *ZEB2-AS1* overexpression on *FOXC1* expression (Fig.5F, G). On the other hand, knock-down of *ZEB2-AS1* could inhibit *FOXC1* expression, while *miR-582-5p* inhibitors could promote *FOXC1* expression and partially reverse the effect of si-*ZEB2-AS1*-1 on *FOXC1* expression (Fig.5H, I). Moreover, in HCC tissues, *miR-582-5p* and *FOXC1* mRNA expressions were inversely related, while *ZEB2-AS1* and *FOXC1* mRNA expressions were positively correlated (Fig.5J, K). The above-mentioned results suggested that *ZEB2-AS1* played a role in HCC by regulating the *miR-582-5p*/*FOXC1* axis.

Discussion

In recent years, growing evidences showed lncRNAs are strongly associated with the tumorigenesis and progression of a variety of malignancies. Thus, it can be used as specific markers for certain tumors, and feature prominently in regulating cancer cell proliferation and metastasis. For example, lncRNA LOC284454 promoted HCC cell invasion and migration by suppressing *E-cadherin* expression (23). Reportedly, *ZEB2-AS1* expression was increased in lung cancer tissues. It is suggested that *ZEB2-AS1* can facilitate lung cancer cell multiplication and inhibit apoptosis (24). Depletion of *ZEB2-AS1* expression suppressed HCC cell growth and metastasis. In the current study, cancer-promoting role of *ZEB2-AS1* in HCC was also confirmed, which is in line with the previous study (14). It was manifested that *ZEB2-AS1* expression was increased in HCC tissues and cells. High *ZEB2-AS1* expression was related to unfavorable clinicopathologic characteristics. What is more, *ZEB2-AS1* overexpression remarkably boosted HCC cell multiplication, migration, invasion and suppressed cell apoptosis, while *ZEB2-AS1* knock-down had the opposite effects. The aforementioned evidences demonstrated that *ZEB2-AS1* could act as a potential HCC prognostic biomarker and therapeutic target.

miRNAs participate in regulating carcinogenesis and cancer progression (25). It was reported that *miR-582-5p* was aberrantly expressed in various cancers and it could play a cancer-suppressing role. For example, *miR-582-5p* inhibited bone metastasis of prostate cancer cells by inhibiting TGF- β signal transduction (26). The up-regulation of *miR-582-5p* repressed endometrial cancer cell multiplication and promoted apoptosis by targeting *AKT3* (27). This study unveiled that *miR-582-5p* expression was underexpressed in HCC tissues. Moreover, *miR-582-5p* overexpression notably repressed cell multiplication, migration, invasion and promoted cell apoptosis, while *miR-582-5p* inhibition had the opposite effects. These suggested that *miR-582-5p* inhibited HCC progression as a tumor suppressor.

lncRNAs can directly interact with miRNAs and act as competing endogenous RNAs (ceRNAs) to modulate mRNA expression. For example, *ZEB2-AS1* up-regulated *HMGB1* expression via sponging *miR-204* to promote the growth and invasion of pancreatic cancer cells (28). *ZEB2-AS1* boosted laryngeal squamous cell cancer development via modulating *miR-6840-3p*/*PLXNB1* axis (29). In this study, we found, through bioinformatics analysis, that there existed binding sites between *miR-582-5p* and *ZEB2-AS1*. Dual-luciferase reporter gene assay validated that *ZEB2-AS1* could sponge *miR-582-5p*. Additionally, *miR-582-5p* could weaken the effect of *ZEB2-AS1* on HCC cell multiplication, migration, invasion and apoptosis. Therefore, we concluded that *ZEB2-AS1* participated in facilitating HCC cell multiplication, migration, invasion and suppressing apoptosis via targeting *miR-582-5p*.

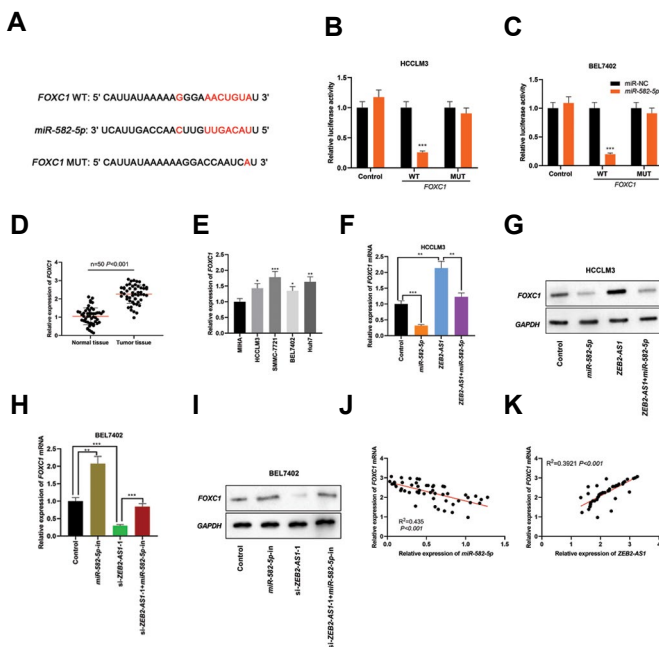


Fig.5: Regulation of *FOXC1* expression by the *ZEB2-AS1*/*miR-582-5p* axis. **A.** Bioinformatics was used for predicting binding site between *miR-582-5p* and *FOXC1*. **B, C.** Binding relationship between *miR-582-5p* and *FOXC1* in HCC cells was detected via dual-luciferase reporter gene assay. **D, E.** Detection of *FOXC1* expression by qRT-PCR in 50 cases of the HCC tissues and adjacent normal tissues, as well as normal human liver cell line (MIHA cells) and HCC cell lines (BEL7402, SMMC-7721, HCCLM3, and Huh7 cells). **F-I.** qRT-PCR and Western blot were used to detect regulatory effect of *miR-582-5p* or *ZEB2-AS1* on *FOXC1* protein expression. Original blots are shown in Supplementary material. **J, K.** qRT-PCR was employed for analyzing the correlation between *FOXC1* mRNA and *miR-582-5p* as well as *ZEB2-AS1* expression in HCC tissues. All experiments were repeated 3 times, each in triplicate. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, HCC; Hepatocellular carcinoma, and qRT-PCR; Quantitative reverse transcription polymerase chain reaction.

FOXC1 gene is located on chromosome 6p25, and it functions as a transcription factor (30). The FOX family partakes in various biological processes, for instance, embryonic development, cell cycle regulation, metabolic control, stem cell niche maintenance and signal transduction (31). *FOXC1* takes part in the progression of various tumors, and highly expressed *FOXC1* is strongly associated with the cancer patient poor prognosis (32). *FOXC1* expression was increased in lung cancer and *FOXC1* facilitated lung cancer cell proliferation and invasion, and its high expression was related to the low survival rate of lung cancer patients (33). In colorectal cancer, *FOXC1* contributed to chemoresistance and it facilitated tumor growth by regulating the *miR-31-5p/LATS2* pathway (34). A previous study showed that dysregulation of *miR-582-5p/FOXC1* axis could inhibit migration and invasion of salivary adenoid cystic cancer cells (22). In this study, with the TargetScan database, it was revealed that there existed a binding site between *miR-582-5p* and *FOXC1* 3'UTR. It was further validated by dual-luciferase reporter gene assay that *miR-582-5p* could bind to *FOXC1* 3'-UTR. Furthermore, overexpression of *ZEB2-AS1* or knock-down of *miR-582-5p* was able to remarkably up-regulate *FOXC1* expression. Therefore, we concluded that *ZEB2-AS1* was able to participate in regulating HCC progression via targeting *miR-582-5p/FOXC1* axis. Our work also partly explained the mechanism of *FOXC1* dysregulation in HCC.

Conclusion

Collectively, *ZEB2-AS1* promoted HCC cell proliferation, migration and invasion via modulating the *miR-582-5p/FOXC1* axis. Our study helps elucidate the mechanism underlying HCC development, in addition to presenting potential treatment targets and biomarkers for HCC.

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Authors' Contributions

SM.W., J.C.; Conceived and designed the experiments. Y.L., Y.Y.T.; Performed the experiments. Q.L.; Supervised the experiments. L.X.; Analysed the data. J.C.; Wrote the manuscript, which was revised by SM.W. All authors have read and approved the final manuscript.

References

1. Wang D, Bai T, Chen G, Liu J, Chen M, Zhao Y, et al. Upregulation of long non-coding RNA FOXP4-AS1 and its regulatory network in hepatocellular carcinoma. *Onco Targets Ther.* 2019; 12: 7025-7038.
2. Liu J, Li W, Zhang J, Ma Z, Wu X, Tang L. Identification of key genes and long non-coding RNA associated ceRNA networks in hepatocellular carcinoma. *PeerJ.* 2019; 7: e8021.
3. Hartke J, Johnson M, Ghabril M. The diagnosis and treatment of hepatocellular carcinoma. *Semin Diagn Pathol.* 2017; 34(2): 153-159.
4. Tang X, Feng D, Li M, Zhou J, Li X, Zhao D, et al. Transcriptomic analysis of mRNA-lncRNA-miRNA interactions in hepatocellular carcinoma. *Sci Rep.* 2019; 9: 16096.
5. Tong MJ, Rosinski AA, Huynh CT, Raman SS, Lu DS. Long-term survival after surveillance and treatment in patients with chronic viral hepatitis and hepatocellular carcinoma. *Hepatol Commun.* 2017; 1(7): 595-608.
6. Su Y, Lv X, Yin W, Zhou L, Hu Y, Zhou A, et al. CircRNA Cdr1as functions as a competitive endogenous RNA to promote hepatocellular carcinoma progression. *Aging (Albany NY).* 2019; 11(9): 8182-8203.
7. Tiansheng G, Junming H, Xiaoyun W, Peixi C, Shaoshan D, Qianping C. LncRNA metastasis-associated lung adenocarcinoma transcript 1 promotes proliferation and invasion of non-Small cell lung cancer cells via down-regulating miR-202 expression. *Cell J.* 2020; 22(3): 375-385.
8. Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet.* 2016; 17(1): 47-62.
9. Peng WX, Koirala P, Mo YY. LncRNA-mediated regulation of cell signaling in cancer. *Oncogene.* 2017; 36(41): 5661-5667.
10. Liu F, Yuan JH, Huang JF, Yang F, Wang TT, Ma JZ, et al. Long noncoding RNA FTX inhibits hepatocellular carcinoma proliferation and metastasis by binding MCM2 and miR-374a. *Oncogene.* 2016; 35(41): 5422-5434.
11. Tang X, Zhang W, Ye Y, Li H, Cheng L, Zhang M, et al. LncRNA HOTAIR contributes to sorafenib resistance through suppressing miR-217 in hepatic carcinoma. *Biomed Res Int.* 2020; 2020: 9515071.
12. Kou JT, Ma J, Zhu JQ, Xu WL, Liu Z, Zhang XX, et al. LncRNA NEAT1 regulates proliferation, apoptosis and invasion of liver cancer. *Eur Rev Med Pharmacol Sci.* 2020; 24(8): 4152-4160.
13. Guo X, Jing YM, Lou HZ, Lou QA. Effect and mechanism of long non-coding RNA ZEB2-AS1 in the occurrence and development of colon cancer. *Math Biosci Eng.* 2019; 16(6): 8109-8120.
14. Lan T, Chang L, Wu L, Yuan Y. Downregulation of ZEB2-AS1 decreased tumor growth and metastasis in hepatocellular carcinoma. *Mol Med Rep.* 2016; 14(5): 4606-4612.
15. Stavast CJ, Erkeland SJ. The non-canonical aspects of microRNAs: many roads to gene regulation. *Cells.* 2019; 8(11): 1465.
16. Tian Y, Guan Y, Su Y, Luo W, Yang G, Zhang Y. MiR-582-5p inhibits bladder cancer-genes by suppressing TTK expression. *Cancer Manag Res.* 2020; 12: 11933-11944.
17. Zhang Y, Huang W, Ran Y, Xiong Y, Zhong Z, Fan X, et al. miR-582-5p inhibits proliferation of hepatocellular carcinoma by targeting CDK1 and AKT3. *Tumour Biol.* 2015; 36(11): 8309-8316.
18. Dai W, Meng X, Mo S, Xiang W, Xu Y, Zhang L, et al. FOXE1 represses cell proliferation and Warburg effect by inhibiting HK2 in colorectal cancer. *Cell Commun Signal.* 2020; 18(1): 7.
19. Lee YH, Lee HT, Chen CL, Chang CH, Hsu CY, Shyu WC. Role of FOXC1 in regulating APSCs self-renewal via STI-1/PrPC signaling. *Theranostics.* 2019; 9(22): 6443-6465.
20. Lin YJ, Shyu WC, Chang CW, Wang CC, Wu CP, Lee HT, et al. Tumor hypoxia regulates forkhead box C1 to promote lung cancer progression. *Theranostics.* 2017; 7(5): 1177-1191.
21. Huang W, Chen Z, Zhang L, Tian D, Wang D, Fan D, et al. Interleukin-8 induces expression of FOXC1 to promote transactivation of CXCR1 and CCL2 in hepatocellular carcinoma cell lines and formation of metastases in mice. *Gastroenterology.* 2015; 149(4):1053-1067.
22. Wang WW, Chen B, Lei CB, Liu GX, Wang YG, Yi C, et al. miR-582-5p inhibits invasion and migration of salivary adenoid cystic carcinoma cells by targeting FOXC1. *Jpn J Clin Oncol.* 2017; 47(8): 690-698.
23. Wang YL, Liu JY, Yang JE, Yu XM, Chen ZL, Chen YJ, et al. Lnc-UCID promotes G1/S transition and hepatoma growth by preventing DHX9-mediated CDK6 down-regulation. *Hepatology.* 2019; 70(1): 259-275.
24. Guo Y, Hu Y, Hu M, He J, Li B. Long non-coding RNA ZEB2-AS1 promotes proliferation and inhibits apoptosis in human lung cancer cells. *Oncol Lett.* 2018; 15(4): 5220-5226.
25. Qian Y, Wu X, Wang H, Hou G, Han X, Song W. MicroRNA-4290 suppresses PDK1-mediated glycolysis to enhance the sensitivity of gastric cancer cell to cisplatin. *Braz J Med Biol Res.* 2020; 53(5): e9330.
26. Huang S, Zou C, Tang Y, Wa Q, Peng X, Chen X, et al. miR-582-

- 3p and miR-582-5p suppress prostate cancer metastasis to bone by repressing TGF- β signaling. *Mol Ther Nucleic Acids*. 2019; 16: 91-104.
27. Li L, Ma L. Upregulation of miR-582-5p regulates cell proliferation and apoptosis by targeting AKT3 in human endometrial carcinoma. *Saudi J Biol Sci*. 2018; 25(5): 965-970.
 28. Gao H, Gong N, Ma Z, Miao X, Chen J, Cao Y, et al. LncRNA ZEB2-AS1 promotes pancreatic cancer cell growth and invasion through regulating the miR-204/HMGB1 axis. *Int J Biol Macromol*. 2018; 116: 545-551.
 29. Xu Q, Liu H, Yu B, Chen W, Zhai L, Li X, et al. Long noncoding RNA ZEB2-AS1 facilitates laryngeal squamous cell carcinoma progression by miR-6840-3p/PLXNB1 axis. *Onco Targets Ther*. 2019; 12: 7337-7345.
 30. Elian FA, Yan E, Walter MA. FOXC1, the new player in the cancer sandbox. *Oncotarget*. 2017; 9(8): 8165-8178.
 31. Quintero-Ronderos P, Laissue P. The multisystemic functions of FOXC1 in development and disease. *J Mol Med (Berl)*. 2018; 96(8): 725-739.
 32. Yang Z, Jiang S, Cheng Y, Li T, Hu W, Ma Z, et al. FOXC1 in cancer development and therapy: deciphering its emerging and divergent roles. *Ther Adv Med Oncol*. 2017; 9(12): 797-816.
 33. Bai X, Shao J, Zhou S, Zhao Z, Li F, Xiang R, et al. Inhibition of lung cancer growth and metastasis by DHA and its metabolite, RvD1, through miR-138-5p/FOXC1 pathway. *J Exp Clin Cancer Res*. 2019; 38(1): 479.
 34. Hsu HH, Kuo WW, Shih HN, Cheng SF, Yang CK, Chen MC, et al. FOXC1 regulation of miR-31-5p confers oxaliplatin resistance by targeting LATS2 in colorectal cancer. *Cancers (Basel)*. 2019; 11(10): 1576.
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