

E2F1-induced microRNA-224-5p expression is associated with hepatocellular carcinoma cell migration, invasion and epithelial-mesenchymal transition via MREG

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Abstract. MicroRNA (miR)-224-5p has been reported to be associated with multiple types of cancer. However, its biological role and underlying mechanism in hepatocellular carcinoma (HCC) has yet to be fully elucidated. The aim of the present study was to investigate whether miR-224-5p mRNA expression level was increased in hepatocellular carcinoma and whether it was associated with poor prognosis. Decreased mRNA expression level of miR-224-5p was shown to suppress liver cancer cell migration, invasion and epithelial-mesenchymal transition (EMT). Mechanistically, E2F1 was found to regulate miR-224-5p expression by binding to its promoter region. Melanoregulin (MREG) was identified as the direct target of miR-224-5p by searching the TargetScan, miRDB and StarBase databases. Overexpression of MREG could attenuate liver cancer cell migration, invasion and EMT. Rescue experiments further confirmed that MREG was associated with the regulation of miR-224-5p in liver cancer. In addition, the E2F1/miR-224-5p axis was shown to promote liver cancer cell migration, invasion and EMT by regulating MREG expression. These results suggested that E2F1-induced upregulation of miR-224-5p may serve an important role in MREG-induced liver cancer cell migration, invasion and EMT, and highlights the regulatory function of miR-224-5p in liver cancer. Therefore, the E2F1/miR-224-5p/MREG axis

may provide a theoretical basis for the clinical treatment of hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma is one of the most common tumors and was the fourth leading cause of cancer-related mortality globally in 2019 (1-4). Despite the advances in diagnosis and treatment (5), the recurrence rate of hepatocellular carcinoma remains high (6,7), and its prognosis is dismal (8-11). Therefore, elucidating the mechanism underlying the initial development and progression of hepatocellular carcinoma is important for investigating new therapeutic targets.

MicroRNAs (miRNAs/miRs) have been associated with diverse biological processes, such as cell growth, cell cycle control, apoptosis and differentiation, by binding to the 3'-untranslated region (3'-UTR) of their target genes (12). Dysregulation of miRNAs was found to be associated with the initial development and progression of multiple types of cancer, including colorectal, breast, ovarian and non-small cell lung cancers, and hepatocellular carcinoma (5,13-18). MiR-224-5p has been found to play an important role in a variety of diseases in mice, rats and humans. For example, miR-224-5p upregulation relieved allergic rhinitis in mice via the toll-like receptor 4/myeloid differentiation primary response 88/NF- κ B signaling pathway (19). In rats, rno-miR-224-5p was shown to regulate deiodinase-mediated thyroxine metabolism (20) and was found to prevent dental pulp stem cell apoptosis by regulating Rac1 (21). MiR-224-5p dysregulation has also been found to be associated with multiple types of human tumor. Studies have shown that miR-224-5p acted as an oncogene in papillary thyroid carcinoma (22), pancreatic mucinous cystadenocarcinoma (23), renal cell carcinoma (24) and breast cancer (25), whereas miR-224-5p expression level was found to be decreased in uveal melanoma (26), prostate cancer (27) and glioma (28). Of note, the function of miR-224-5p in hepatocellular carcinoma has also been reported in previous studies (29-32), suggesting that miR-224-5p is important in hepatocellular carcinoma. However, the role of miR-224-5p in the prognosis and epithelial-mesenchymal transition (EMT) of hepatocellular carcinoma, and the underlying mechanisms have yet to be extensively investigated.

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Abbreviations: RT-qPCR, reverse transcription-quantitative PCR; 3'-UTR, 3'-untranslated region; ChIP, chromatin immunoprecipitation; inh, inhibitor; HCC, Hepatocellular carcinoma

Key words: microRNA-224-5p, melanoregulin, E2F1, hepatocellular carcinoma, cell migration, cell invasion, epithelial-to-mesenchymal transition

Melanoregulin (MREG) is encoded by the MREG^{dsu} gene, and it is a highly charged small protein consisting of 214 amino acids (28 kDa) (19). It was previously demonstrated that MREG suppressed the coat color of dilute mice in a MYO5A-independent manner (19). MREG was also reported to be required for lysosome maturation in pigment epithelial cells (33). Furthermore, MREG was found to be associated with the centripetal movement of melanosomes (34). Subsequently, studies revealed that the MREG protein level was decreased in thyroid cancer and inhibited thyroid cancer cell proliferation, and invasion (35), indicating that reduced MREG expression level may be associated with tumorigenesis. However, the function of MREG and the association between MREG, and miR-224-5p in hepatocellular carcinoma remains unknown.

The present study was undertaken to investigate whether miR-224-5p mRNA expression level was increased in hepatocellular carcinoma and whether it served as a predictor of poor prognosis. The effects of miR-224-5p overexpression on liver cancer cell migration, invasion and EMT were also examined. Lastly, it was investigated whether MREG was a target of miR-224-5 and whether miR-224-5p could be directly transcriptionally regulated by E2F1.

Materials and methods

Data analysis. The expression level of miR-224-5p and E2F1 in hepatocellular carcinoma was analyzed using R software (version 3.6.3) ggplot2 (version 3.3.3) package by downloading The Cancer Genome Atlas-Liver Hepatocellular Carcinoma level 3 BCGSC miRNA Profiling (<https://github.com/bcgsc/mirna>) miRNAseq dataset. Characteristics of patients with hepatocellular carcinoma divided by miR-224-5p or MREG expression level were analyzed using basic R package. The prognostic analysis of hepatocellular carcinoma patients with miR-224-5p and MREG expression was analyzed using the Kaplan-Meier plotter online database (https://kmplot.com/analysis/index.php?p=service&cancer=liver_rnaseq) based on data stratified according to the best cut-off. For the 5-year overall survival rate of hepatocellular carcinoma patients with MREG expression, alcohol consumption was excluded as a risk factor. The targets of miR-224-5p were predicted using the TargetScan (<http://www.targetscan.org/>), miRDB (<http://www.mirdb.org/>) and StarBase (<http://starbase.sysu.edu.cn/index.php>) databases. The correlation between miR-224-5p and MREG expression levels in hepatocellular carcinoma samples was analyzed using StarBase v3.0 online tool (<http://starbase.sysu.edu.cn/index.php>). The binding sites between E2F1 and the promoter region of miR-224-5p were predicted using the JASPAR online tool (jaspar.genereg.net/) (36).

Cell culture. The human THLE-2 fetal liver cell line (Fuheng Biology) and two human liver cancer cell lines, HepG2 and Huh7 (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences), which were authenticated by short tandem repeat profiling, were cultured in DMEM (HyClone; Cytiva), supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin-streptomycin, and incubated at 37°C with 5% CO₂.

Reverse transcription-quantitative (RT-qPCR). TRIzol[®] (Invitrogen; Thermo Fisher Scientific, Inc.) was used to harvest the total RNA from cells according to the manufacturer's instructions. cDNA synthesis was performed using PrimeScript RT-polymerase (Takara Biotechnology Co., Ltd.) for 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C, with a hold at 4°C. The mRNA expression level of miR-224-5p was determined using a SYBR Premix Ex Taq miRNA kit (Takara Biotechnology Co., Ltd.). The relative expression level of MREG was measured using SYBR Premix Ex Taq[™] II kit (Takara Biotechnology Co., Ltd.). U6 small nuclear RNA and GAPDH were used as internal references for miRNA and mRNA, respectively. The thermocycling conditions were 95°C for 10 min, followed by a total of 40 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 45 sec, the last cycle was 72°C for 10 min. The relative expression level of miR-224-5p and MREG was evaluated using the 2^{-ΔΔC_q} method (37). The primers used are listed in Table I.

Plasmid construction and cell transfection. The full-length MREG sequence was synthesized and subcloned into the pCDNA3.1 vector (Youbio). The interference oligonucleotides (oligos) targeting MREG and E2F1 (si-MREG and si-E2F1) were purchased from Shanghai GenePharma Co., Ltd. Plasmids and oligos were transfected using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. miR-224-5p mimics and inhibitor were purchased from Guangzhou RiboBio Co., Ltd. Untransfected cells, cells transfected with miR-mimic-NC or miR-inhibitor-NC were used as a control group. HepG2 and Huh7 cells were plated in 12-well dishes at a density of 2x10⁵ cells per well. The next day, the cells were transfected with 25 nM miRNA mimic, 25 nM miRNA inhibitor, 100 nM siRNAs (100 nM si-E2F1, 100 nM si-E2F1 and 100 nM si-MREG) or 1 μg plasmid DNA (1 μg each E2F1, 1 μg E2F1 and 1 μg MREG). The transfection above mentioned (plasmids, siRNAs or oligos) and the transfection reagent (Lipofectamine[®] 2000) were mixed and incubated together for 20 min at 37°C and then added into the medium. The group of E2F1 and E2F1/MREG was examined after 48 h. The group of si-E2F1 and si-E2F1/si-MREG was examined at transfection after 72 h. Sequences of siRNAs and miRNA mimic/inhibitor are listed in Table SI.

Transwell and Matrigel assays. The HepG2 and Huh7 cell lines were seeded into 24-well plates, at a density of 5,000 cells/well and cultured at 37°C overnight. The Transwell inserts were incubated with Matrigel (BD Biosciences) at 37°C for 2 h for invasion assays. In the top chamber, the cells were plated in medium without serum and the lower chamber was filled with medium, supplemented 10% FBS. The cells remaining on the upper surface of the membrane were removed with a cotton swab after incubation at 37°C for 48 h. The cells that had migrated/invaded through the membrane were fixed with 4% formaldehyde for 30 min and stained with DAPI for 20 min at room temperature, then images were captured by an ME21 digital microscope (Olympus) and the number of cells were counted.

Table I. Primer sequences used in reverse transcription-quantitative PCR.

Name	Sequence
miR-224-5p	Forward: 5'-GGTCCTAAGTCACTAGTGGTTCCGTT-3' Reverse: 5'-CCAGTGCAGGGTCCGAGGT-3'
U6	Forward: 5'-CTCGCTTCGGCAGCAC-3' Reverse: 5'-AACGCTTCACGAATTTGCGT-3'
MREG	Forward: 5'-CCCTTGGCATTATCTGGA-3' Reverse: 5'-AAGCTGCATTACAGCATTG-3'
GAPDH	Forward: 5'-GCACCGTCAAGGCTGAGAAC-3' Reverse: 5'-GGATCTCGCTCCTGGAAGATG-3'

miR, microRNA; MREG, melanoregulin.

Western blot analysis. Total protein was extracted using lysis buffer (Beyotime Institute of Biotechnology) and quantified using a BCA protein assay kit (Beyotime Institute of Biotechnology). Protein lysates (150 μ g/sample) were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes (MilliporeSigma). After blocking with 5% skimmed milk for 1 h at room temperature, the membranes were incubated with anti-MREG (Santa Cruz Biotechnology, Inc.; catalog no. sc-374216; 1:500), anti-ZEB1 (Abcam; catalog no. ab203829; 1:500), anti-E-cadherin (Abcam; catalog no. ab1416; 1:50), anti-Slug (Abcam; catalog no. ab27568; 1:500), anti-Vimentin (Abcam; catalog no. ab92547; 1:1,000) and GAPDH (Abcam; catalog no. ab8245; 1:1,000) antibodies overnight at 4°C. After being washed with 1X PBS, the membranes were incubated with horseradish peroxidase-labeled goat anti rabbit (Abcam; catalog no. ab6721; 1:2,000) or mouse IgG (Abcam; catalog no. ab6728; 1:2,000) at room temperature for 2 h. Signal detection was performed using an enhanced chemiluminescence system (Amersham; Cytiva).

If the size of the target proteins was similar to the loading control, the samples were divided equally into two parts: One part was used for detecting the target proteins and the other was used for the internal loading control.

Luciferase reporter assay. The HepG2 and Huh7 cell lines, seeded in the 24-well plates (5x10³ cells/well), were co-transfected with MREG 3'-UTR wild-type (WT), MREG 3'-UTR WT + miR-224-5p mimic, or MREG 3'-UTR mutant (MUT; constructed by replacing the binding site with its complementary sequence) + miR-224-5p mimic using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 48 h at 37°C after transfection, the HepG2 and Huh7 cell lines were washed twice with PBS, lysed with Passive Lysis Buffer (Promega Corporation) and harvested. The luciferase activities were detected using a Dual-Luciferase Reporter assay system (Promega Corporation). The luciferase activities were analyzed as relative activity of firefly to *Renilla*.

Chromatin immunoprecipitation (ChIP) assay. A ChIP assay was performed using the SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling Technology, Inc.). In brief, the liver cancer cells were crosslinked with formaldehyde, lysed with SDS

buffer, followed by ultrasonication (25% power, 4.5 sec shock, 9 sec interval, 14 times), and incubated with 2 μ g/ml anti-E2F1 antibody (cat. no. ab179445; Abcam) or 2 μ g/ml rabbit IgG control (cat. no. ab205718; Abcam) immobilized with 10 μ l protein G magnetic beads at 4°C overnight. After washing with high- and low-salt wash solution, DNA was eluted de-cross-linked, and enrichment was assessed using qPCR and 1% gel electrophoresis containing ethidium bromide (1:10,000), DNA bands were viewed under UV light (orange fluorescence). The primers are shown in Table I. qPCR was performed using SYBR Green Mix (Takara Biotechnology, Co., Ltd.) according to the manufacturer's protocol. Thermocycling conditions were 94°C for 7 min, 94°C for 1 min, 58°C for 1 min and 72°C for 1 min (33 cycles), and then 72°C for 10 min. GAPDH was the internal control.

Statistical analysis. All the data analyses were performed using SPSS 17.0 software (SPSS Inc.) and GraphPad Prism 8. The data are shown as the mean \pm SD. The expression profile, ROC analysis and the overall survival analysis were performed using R software (version 3.6.3). The correlation analysis was performed using ENCORI (<http://starbase.sysu.edu.cn/index.php>). The intersection was assessed by Venny 2.1.0 (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>). Student's t-test was performed for comparisons between paired tumor and adjacent normal tissues or the two groups of cells. Mann-Whitney U was used for comparisons between clinical samples and unpaired normal tissues. One-way ANOVA followed by Tukey's post hoc test was used for comparisons between multiple groups. Comparisons of categorical data were performed using χ^2 or Fisher's exact or Wilcoxon rank sum test. Each experiment was repeated three times. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-224-5p mRNA expression level is increased and predicts poor prognosis in hepatocellular carcinoma. miR-224-5p mRNA expression level was found to be increased in HCC tissues compared with unpaired normal tissues (Fig. 1A, left) or the paired adjacent normal tissues (Fig. 1A, right). The clinical characteristics of hepatocellular carcinoma patients with miR-224-5p are shown in Table II. The receiver operating characteristics (ROC) curve

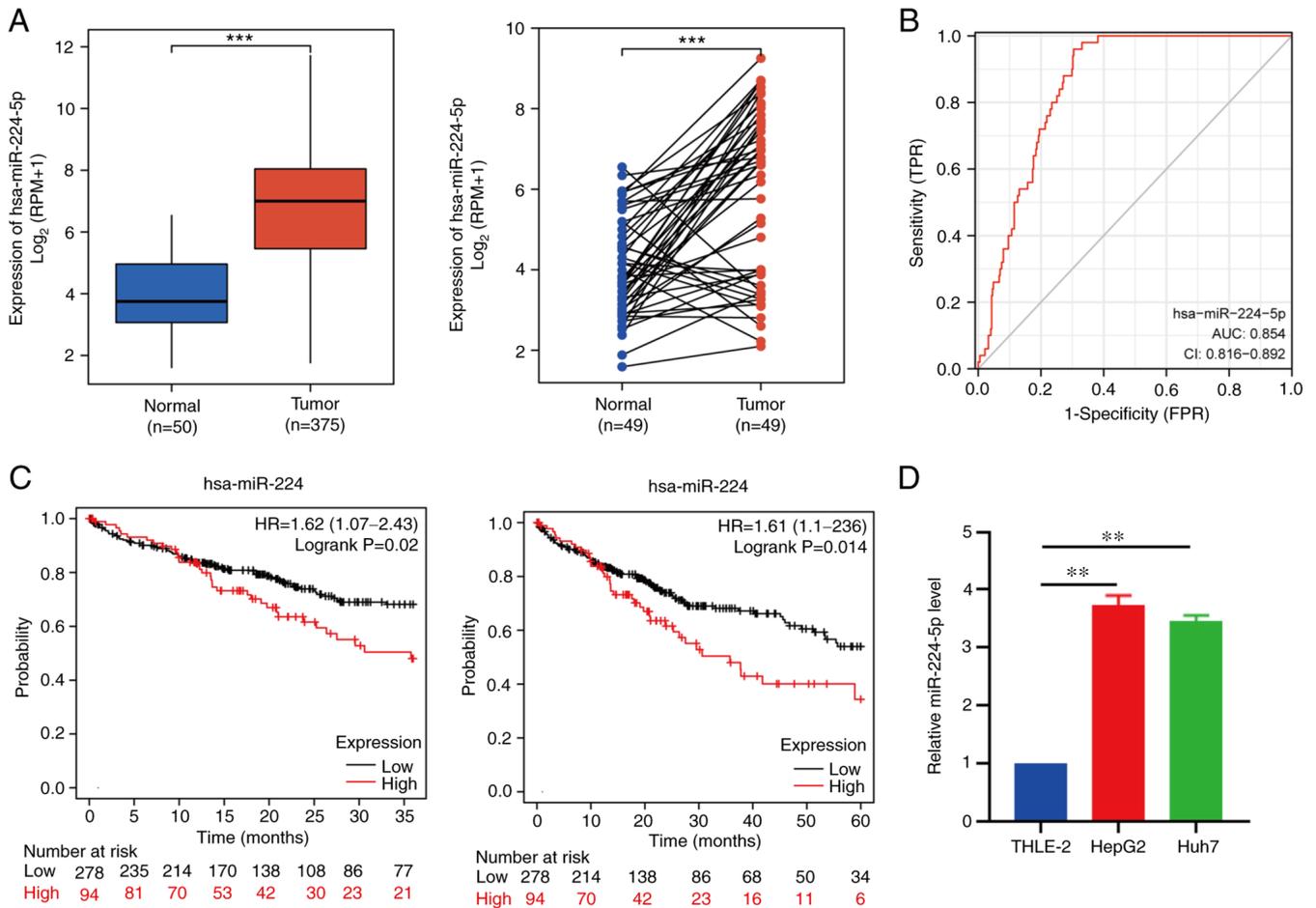


Figure 1. miR-224-5p expression level is upregulated in patients with HCC and predicts poor prognosis. (A) miR-224-5p expression level was analyzed in unpaired ($N_{\text{normal}}=50$, $N_{\text{tumor}}=375$) and paired ($n=49$) tumor samples using TCGA-LIHC level 3 BCGSC miRNA data. (B) The receiver operating characteristics curve of miR-224-5p in HCC was analyzed using TCGA-LIHC miRNA data. (C) The 3- and 5-year overall survival time in patients with divided by miR-224-5p expression level was analyzed using the Kaplan-Meier plotter database. (D) Relative miR-224-5p expression level was analyzed using reverse transcription-quantitative PCR in the THLE-2 cells and in the liver cancer cell lines, HepG2 and Huh7. Data are presented as the mean \pm SD. ** $P<0.01$ and *** $P<0.001$. miR, microRNA; TCGA-LIHC, The Cancer Genome Atlas-Liver Hepatocellular Carcinoma; AUC, area under the curve; HR, hazard ratio.

analysis indicated that miR-224-5p had a diagnostic accuracy for HCC (Fig. 1B). Furthermore, patients with HCC exhibiting high expression levels of miR-224-5p had shorter 3- and 5-year overall survival times (Fig. 1C). In addition, miR-224-5p expression was found to be highly expressed in the two liver cancer cell lines (HepG2 and Huh7) compared with that in the human normal THLE-2 liver cell line (Fig. 1D). Taken together, these results suggest that miR-224-5p expression level is increased in liver cancer and may be of diagnostic, and prognostic value in patients with HCC.

miR-224-5p increases liver cancer cell migration, invasion and EMT in vitro. To evaluate the biological role of miR-224-5p on liver cancer cell migration and invasion, miR-224-5p expression was knocked down in the HepG2 and Huh7 cells using transfection with a miR-224-5p inhibitor. miR-224-5p expression was significantly decreased in the HepG2 and Huh7 cells, as shown in Fig. 2A. The Transwell and Matrigel assays revealed that decreased miR-224-5p expression level suppressed the migratory and invasive abilities of the HepG2 and Huh7 cells, respectively (Fig. 2B and C). Furthermore, the expression level of EMT-related proteins, including

E-cadherin, vimentin, ZEB1 and SLUG, was also detected. It was found that there was an increase in E-cadherin expression level and a decrease in vimentin, ZEB1 and SLUG expression level in both the HepG2 and Huh7 cell lines, following transfection with miR-224-5p inhibitor (Fig. 2D). The results demonstrated that knockdown of miR-224-5p expression inhibited liver cancer cell migration, invasion and EMT.

MREG is a direct target of miR-224-5p. To further investigate the mechanism of miR-224-5p in HCC, the targets of miR-224-5p were predicted using the TargetScan, miRDB and StarBase databases. A total of 36 targets were obtained from the intersection of these three prediction tools using Venny 2.1.0 (<https://bioinfo.gp.cnb.csic.es/tools/venny/index.html>) (Table SII), of which MREG was predicted as the potential target with the highest score (Fig. 3A). A conserved sequence was found in the 3'-UTR of MREG mRNA that matched to the seed sites of miR-224-5p (Fig. 3B). To identify whether miR-224-5p directly targeted MREG in the liver cancer cell lines, a luciferase reporter assay was conducted using WT and MUT MREG 3'-UTR plasmids. The expression level of miR-224-5p was significantly increased in miR-224-5p

Table II. Characteristics of patients with HCC divided by miR-244-5p expression level.

Characteristic	Low expression of hsa-miR-224-5p	High expression of hsa-miR-224-5p	P-value	Statistical value	Method
n	187	188			
T stage, n (%)			0.790	1.05	χ^2 test
T1	94 (25.3)	90 (24.2)			
T2	49 (13.2)	46 (12.4)			
T3	36 (9.7)	44 (11.8)			
T4	6 (1.6)	7 (1.9)			
N stage, n (%)			1.000		Fisher's test
N0	130 (50)	126 (48.5)			
N1	2 (0.8)	2 (0.8)			
M stage, n (%)			0.123		Fisher's test
M0	134 (48.7)	137 (49.8)			
M1	0 (0)	4 (1.5)			
Age in years, median (IQR)	61 (51,69)	61 (52,69)	0.657	17019.5	Wilcoxon

Table III. Characteristics of patients with HCC divided by MREG expression level.

Characteristic	Low expression of MREG	High expression of MREG	P-value	Statistical value	Method
n	187	187			
T stage, n (%)			0.907	0.55	χ^2 test
T1	95 (25.6)	88 (23.7)			
T2	47 (12.7)	48 (12.9)			
T3	38 (10.2)	42 (11.3)			
T4	6 (1.6)	7 (1.9)			
N stage, n (%)			1.000		Fisher's test
N0	126 (48.8)	128 (49.6)			
N1	2 (0.8)	2 (0.8)			
M stage, n (%)			1.000		Fisher's test
M0	136 (50)	132 (48.5)			
M1	2 (0.7)	2 (0.7)			
Age in years, median (IQR)	63 (53,70)	60 (51,68)	0.053	19408	Wilcoxon

MREG, melanoregulin; IQR, interquartile range.

mimic-transfected cells (Fig. S1A). As shown in Fig. 3C, overexpression of miR-224-5p significantly repressed WT MREG 3'-UTR luciferase activity, whereas it had no effect on MUT MREG 3'-UTR luciferase activity in the HepG2 and Huh7 cells. Furthermore, MREG expression, at the mRNA and protein level, markedly increased with the inhibition of miR-224-5p compared to miR inhibitor-NC group, while MREG expression level was decreased with the overexpression of miR-224-5p (Fig. 3D and E). Taken together, the results indicated that MREG was a direct target of miR-224-5p.

MREG inhibits liver cancer cell migration, invasion and EMT in vitro. Subsequently, the effect of MREG expression in HCC was investigated. It was found that high expression level of

MREG in HCC was associated with a prolonged 3- and 5-year overall survival time (Fig. 4A). The characteristics of patients with HCC divided by MREG expression level is shown in Table III. The ROC curve analysis indicated that MREG was accurate in the diagnosis of HCC (Fig. 4B). To investigate its role in HCC, MREG overexpression plasmids were constructed by cloning the full length MREG sequence into the pcDNA3.1 plasmid. The results demonstrated that MREG overexpression markedly inhibited the migratory and invasive abilities of the HepG2 and Huh7 cells (Fig. 4C and D). In addition, increased protein expression level of E-cadherin and decreased protein expression level of vimentin, ZEB1 and SLUG were observed in MREG-overexpressing cells (Fig. 4E). These results suggested that MREG suppressed liver cancer cell migration, invasion

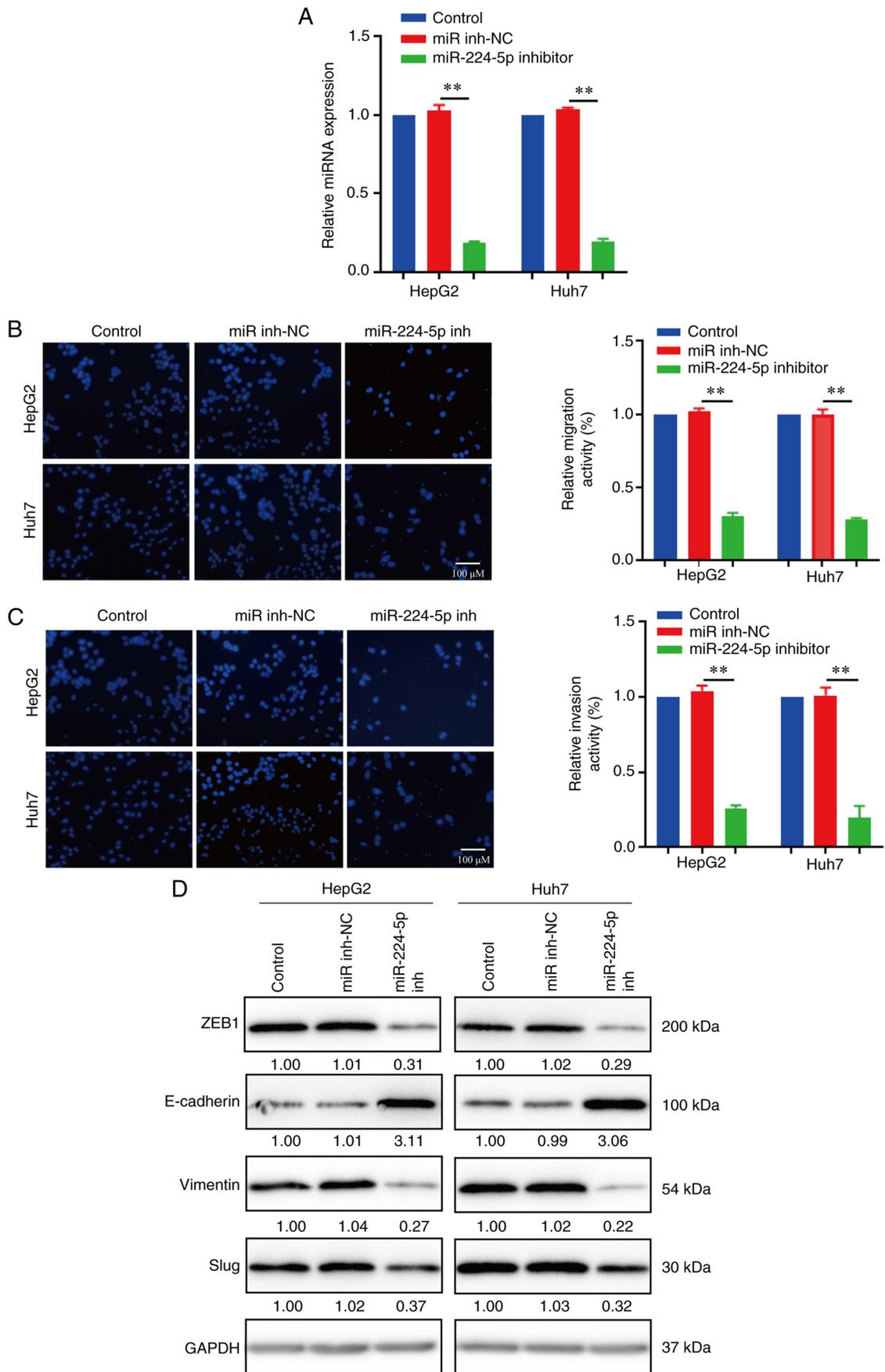


Figure 2. miR-224-5p promotes liver cancer cell migration and invasion *in vitro*. (A) Relative miR-224-5p expression level was determined using reverse transcription-quantitative PCR in the HepG2 and Huh7 cells transfected with miR-224-5p inhibitor or the inhibitor NC. (B) Migration and (C) invasion ability was detected in the HepG2 and Huh7 cells transfected with miR-224-5p inhibitor or mimics. (D) Expression level of epithelial-to-mesenchymal transition-related proteins was detected in the HepG2 and Huh7 cells transfected with miR-224-5p inhibitor or mimics. ** $P < 0.01$. miR, microRNA; NC, negative control; inh, inhibitor.

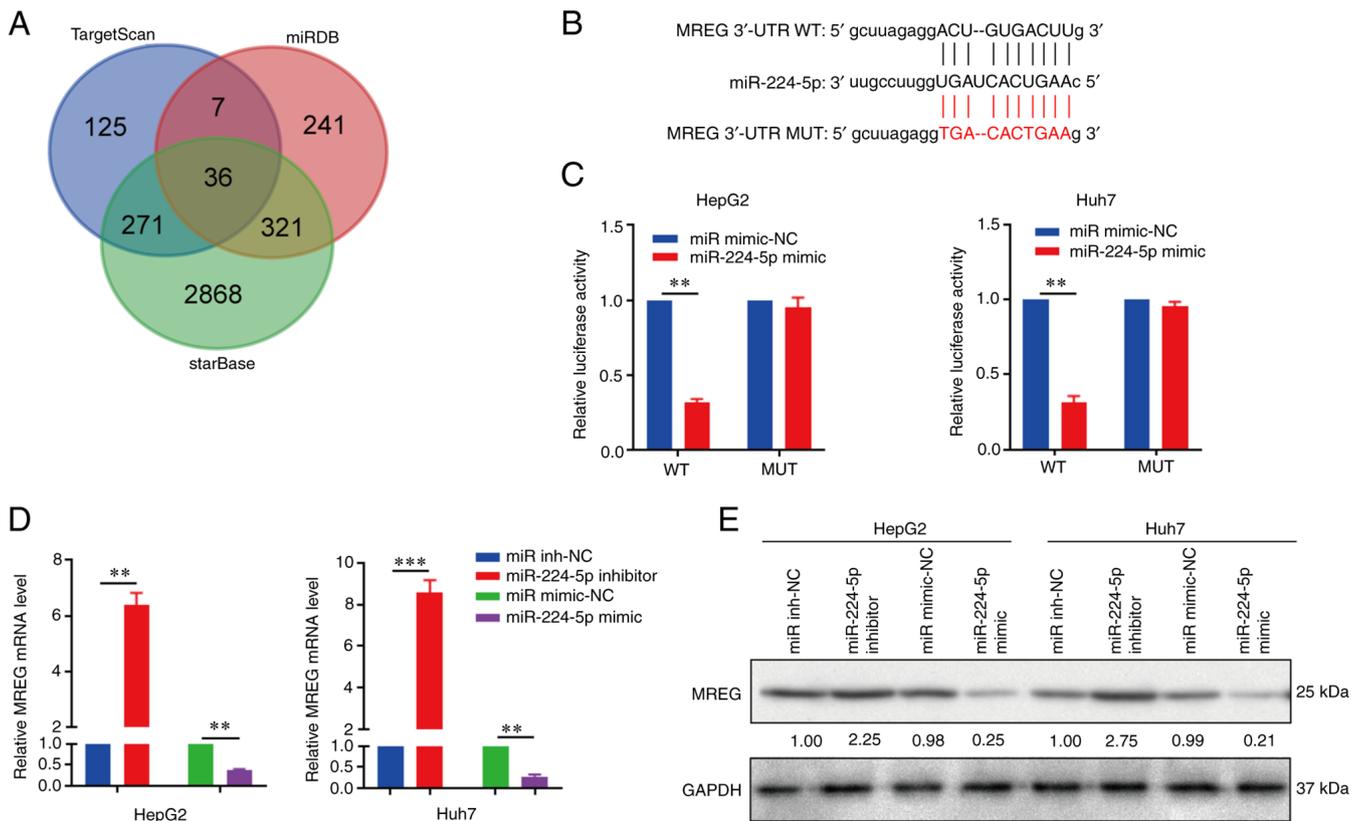


Figure 3. MREG is a target of miR-224-5p. (A) miR-224-5p targets were predicted using the TargetScan, miRDB and StarBase databases. (B) Binding sites between MREG and miR-224-5p. (C) Relative luciferase activity was detected in the HepG2 and Huh7 cells transfected with miR-224-5p mimic. (D) MREG mRNA and (E) protein expression level was determined in miR-224-5p inhibitor-transfected or miR-224-5p mimic-transfected liver cancer cells using reverse transcription-quantitative PCR and western blot analysis, respectively. **P<0.01 and ***P<0.001. miR, microRNA; MREG, melanoregulin; UTR, untranslated region; WT, wild-type; MUT, mutant; NC, negative control.

and EMT, and has a diagnostic and prognostic significance in patients with HCC.

miR-224-5p promotes liver cancer cell migration, invasion and EMT via MREG. MREG was identified as a direct target gene of miR-224-5p in the aforementioned experiments, and overexpression of MREG suppressed liver cancer cell migration, invasion and EMT. Furthermore, rescue experiments revealed that MREG expression was increased in cells transfected with miR-224-5p inhibitor, but the effects were reversed by co-transfection with small interfering (si)RNA (si-MREG) (Fig. 5A). The knockdown efficiency of si-MREG was validated using western blot analysis, as shown in Fig. S1B. Functionally, the inhibitory effects on cell migration and invasion induced by miR-224-5p knockdown could be rescued by transfecting with si-MREG in miR-224-5p-knockdown cells (Fig. 5B and C). In addition, the increase in the protein expression level of E-cadherin induced by decreased miR-224-5p expression could be restored by MREG knockdown. Furthermore, the decrease in the protein expression level of vimentin, ZEB1 and SLUG resulting from miR-224-5p inhibition could also be rescued by MREG knockdown (Fig. 5D). Lastly, miR-224-5p expression was negatively associated with MREG expression levels in HCC samples according to the StarBase v3.0 database ($r=-0.21$; $P<0.001$; Fig. 5E). Thus, the aforementioned results revealed that miR-224-5p may promote liver cancer cell migration, invasion and EMT by downregulating MREG expression.

E2F1 regulates miR-224-5p expression by binding to its promoter. A previous study reported that E2F1 could bind to the putative miR-224-5p promoter motif in melanoma cells; therefore, regulating miR-224-5p expression at the transcriptional level (38). E2F1 is known to play a pivotal role in HCC development (39). Thus, it was investigated whether miR-224-5p was transcriptionally activated by E2F1 in HCC. It was found that E2F1 expression was also increased in HCC tumor tissues (Fig. 6A). The binding sites between E2F1 and the promoter region of miR-224-5p were predicted using the JASPAR online tool (Fig. 6B). The miR-224-5p promoter activity was detected in E2F1-knockdown liver cancer cells using a luciferase reporter assay. The results demonstrated that E2F1 knockdown significantly inhibited the promoter activity of miR-224-5p in both HepG2 and Huh7 cells compared with that in cells transfected with the negative control (Fig. 6C). siRNA targeting E2F1 was successfully transfected into the liver cancer cells and reduced the protein expression level of E2F1 (Fig. 6D), which was also accompanied by decreased mRNA expression level of miR-224-5p (Fig. 6E). Furthermore, a ChIP assay was performed with anti-E2F1 antibody to determine whether E2F1 could directly bind to the miR-224-5p promoter region. The result revealed that there was an interaction between E2F1 and the miR-224-5p promoter region (Fig. 6F). In addition, E2F1 mRNA expression level was positively associated with miR-224-5p mRNA expression level in HCC samples according to the results from the StarBase v3.0 database ($r=0.258$; $P<0.001$; Fig. 6G). Taken together, the results demonstrated that E2F1 could

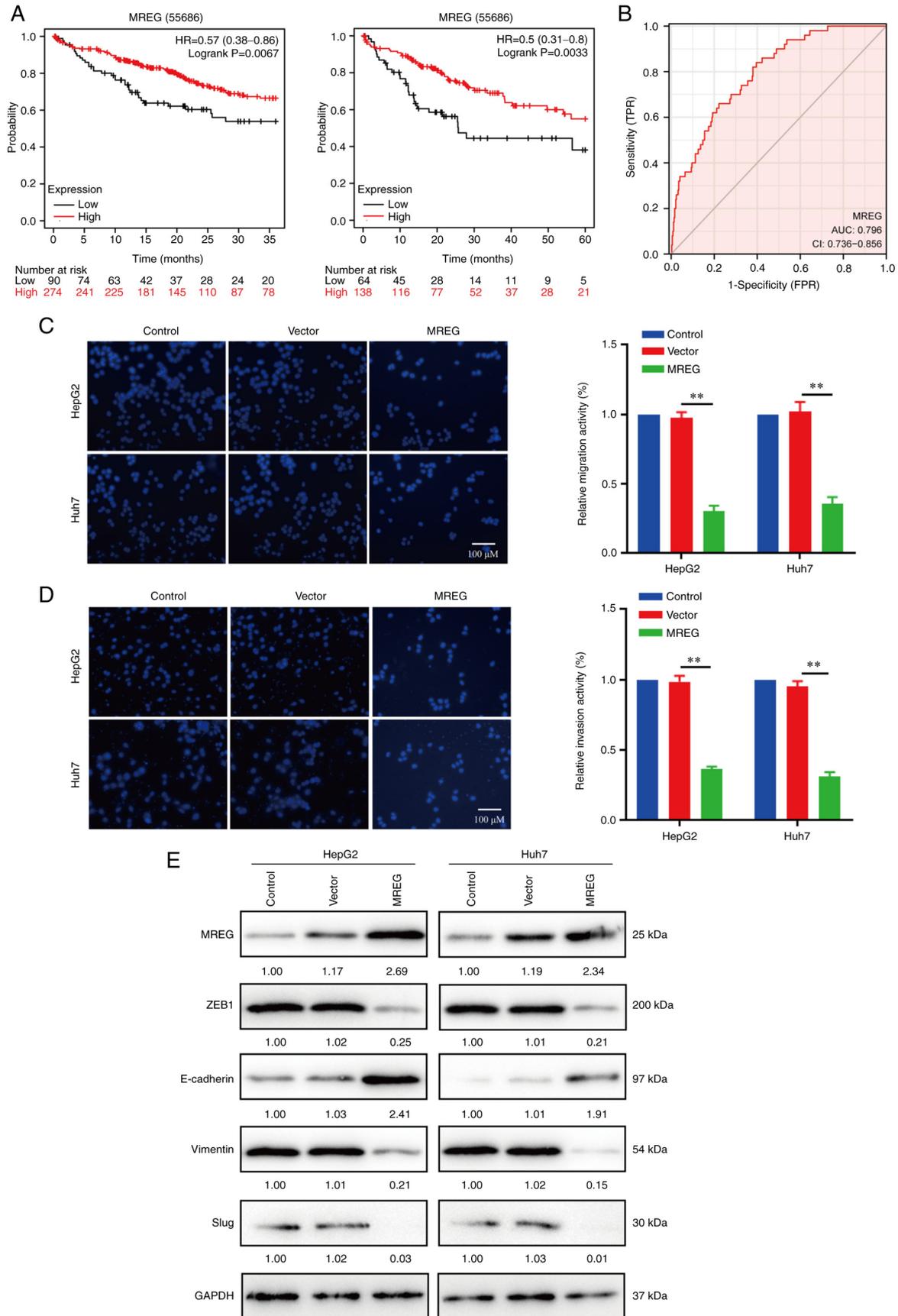


Figure 4. MREG overexpression inhibits liver cancer cell migration and invasion *in vitro*. (A) The 3- and 5-year overall survival time in patients divided by MREG expression level was analyzed using the Kaplan-Meier plotter database. (B) The receiver operating characteristics curve of MREG was analyzed using The Cancer Genome Atlas-Liver Hepatocellular Carcinoma microRNA data. (C) Migration and (D) invasion ability was detected in HepG2 and Huh7 cells following transfection with MREG overexpression plasmid. (E) Expression of epithelial-mesenchymal transition-related proteins in the HepG2 and Huh7 following transfection with MREG overexpression plasmid was measured using western blot analysis. ** $P < 0.01$. MREG, melanoregulin; AUC, area under the curve; HR, hazard ratio.

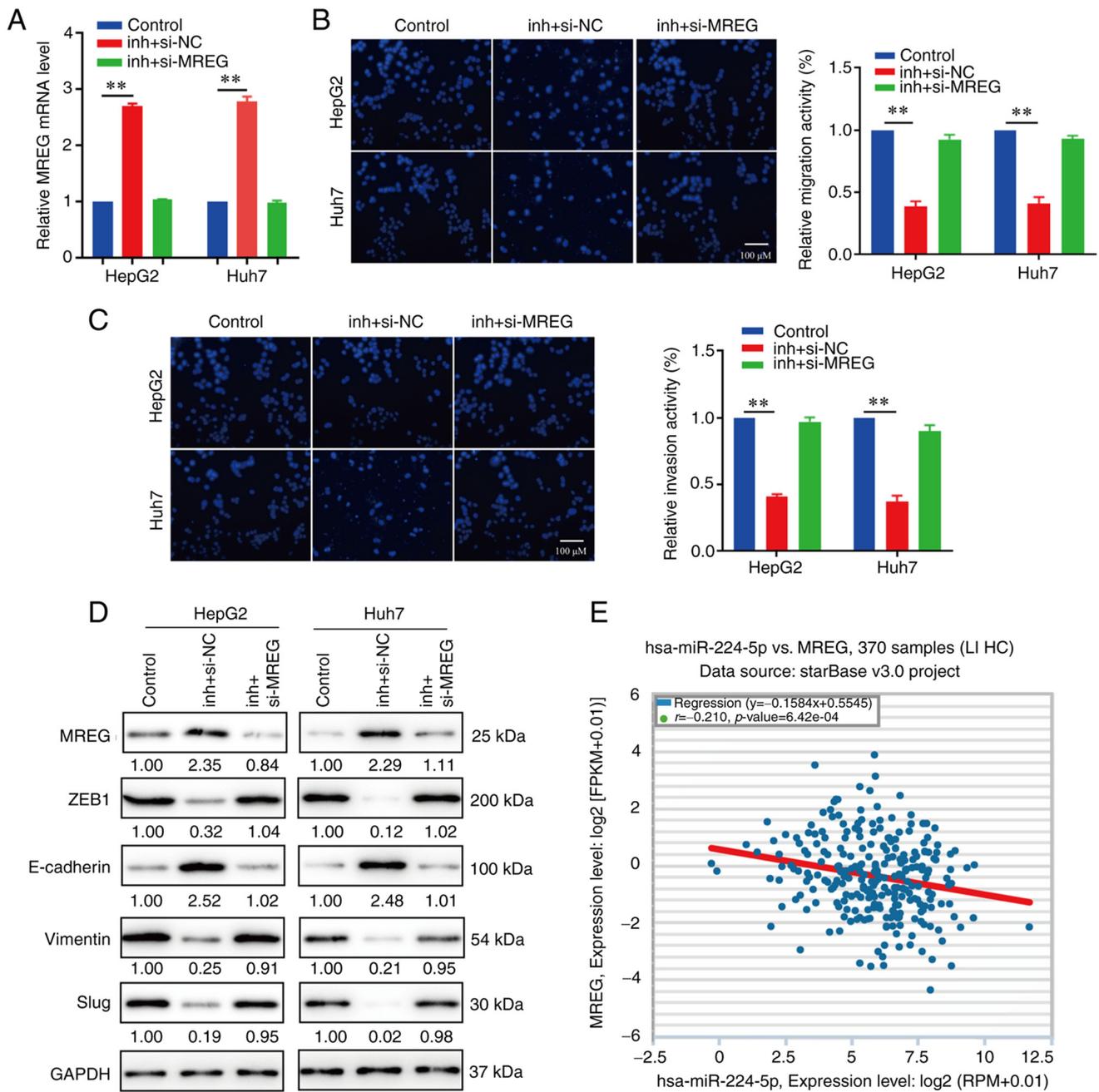


Figure 5. miR-224-5p promotes liver cancer cell migration and invasion by targeting MREG *in vitro*. (A) MREG mRNA expression level was measured using reverse transcription-quantitative PCR in the HepG2 and Huh7 cells following transfection with miR-224-5p inhibitor alone or in combination with si-MREG, or control. (B) Migration and (C) invasion abilities were measured in the HepG2 and Huh7 cells transfected with miR-224-5p inhibitor alone or in combination with si-MREG, or control. (D) Expression of epithelial-mesenchymal transition-related proteins was detected in the HepG2 and Huh7 cells transfected with miR-224-5p inhibitor alone or in combination with si-MREG, or control. (E) Correlation between miR-224-5p and MREG expression level in HCC tumor tissues. **P<0.01. miR, microRNA; MREG, melanoregulin; si, small inhibiting; NC, negative control; inh, inhibitor.

transcriptionally regulate the expression level of miR -224-5p in HCC.

E2F1-induced miR-224-5p upregulation promotes liver cancer cell migration, invasion and EMT via MREG. Based on the aforementioned results indicating that MREG was the direct target of miR-224-5p, it was examined whether MREG downregulation could promote liver cancer cell migration and invasion via the E2F1/miR-224-5p axis. miR-224-5p expression was shown to be significantly increased in both E2F1-overexpressing and E2F1/MREG-overexpressing cells, whereas it was decreased

in both E2F1-knockdown and E2F1/MREG-knockdown cells compared with that in the cells transfected with negative control (Fig. 7A). Furthermore, the western blot results demonstrated that the decreased protein expression level of E-cadherin in cells transfected with E2F1 overexpression vector was restored following co-overexpression of MREG, while the increase in ZEB1 and vimentin protein expression level of E2F1 was rescued by co-overexpression of MREG. Consistently, the increased expression level of E-cadherin in cells transfected with siE2F1 was reversed by MREG knockdown and the decreased protein expression level of ZEB1 and vimentin was

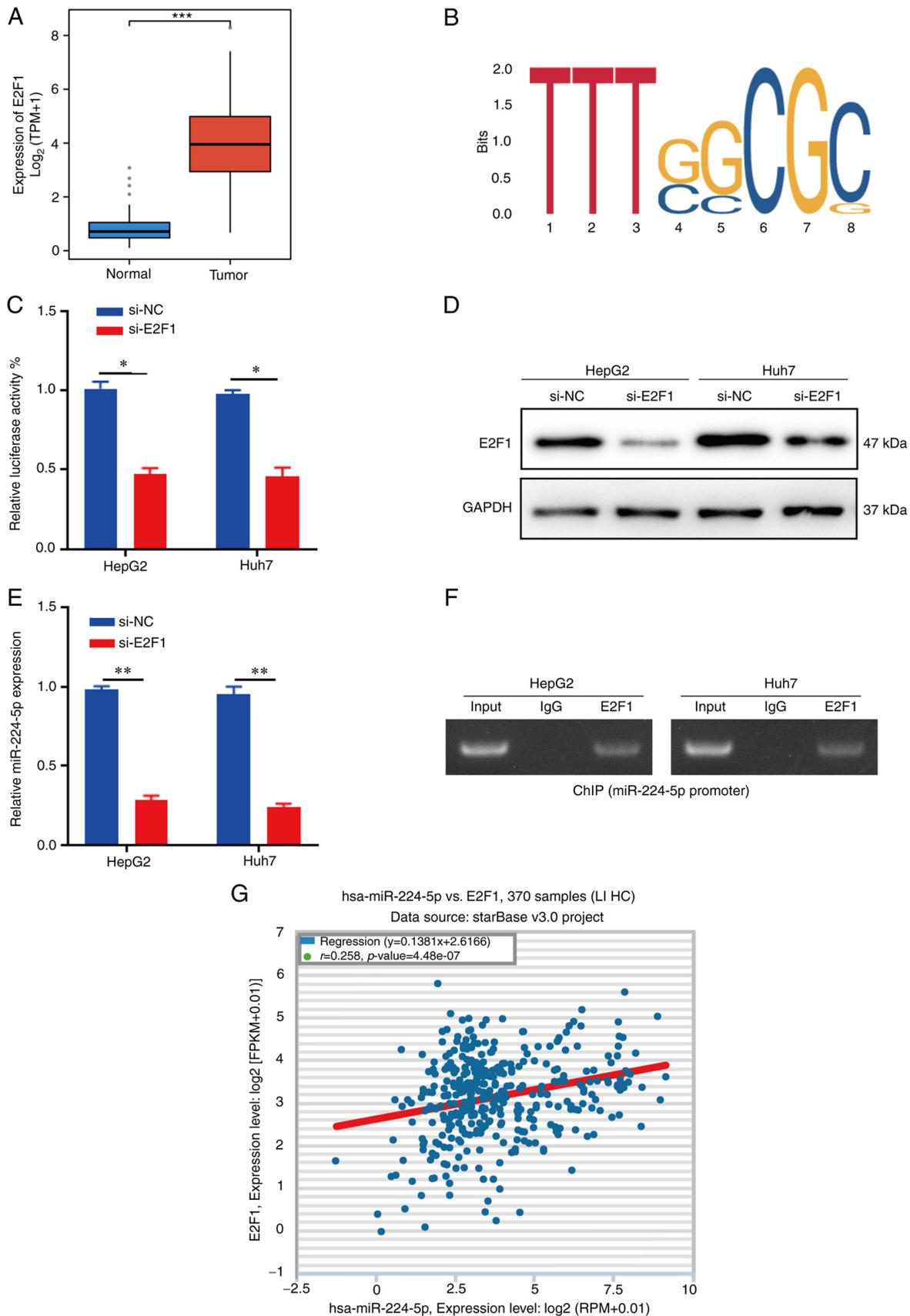


Figure 6. E2F1 regulates miR-224-5p transcription. (A) E2F1 mRNA expression level was analyzed using The Cancer Genome Atlas-Liver Hepatocellular Carcinoma dataset. (B) Binding sites between E2F1 and miR-224-5p on the promoter region. (C) Luciferase reporter assay was performed in the HepG2 and Huh7 cells following si-E2F1 transfection. (D) The knockdown efficiency of si-E2F1 was confirmed in the HepG2 and Huh7 cells using western blot analysis. (E) miR-224-5p expression level was measured in the HepG2 and Huh7 cells following si-E2F1 transfection. (F) The interaction between the E2F1 and miR-224-5p promoter region was determined using a chromatin immunoprecipitation assay. The specific band indicated the input DNA and the E2F1 antibody (anti-E2F1)-precipitated DNA. (G) The correlation between E2F1 and miR-224-5p was analyzed using StarBase v3.0. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. miR, microRNA; si, small inhibiting; NC, negative control.

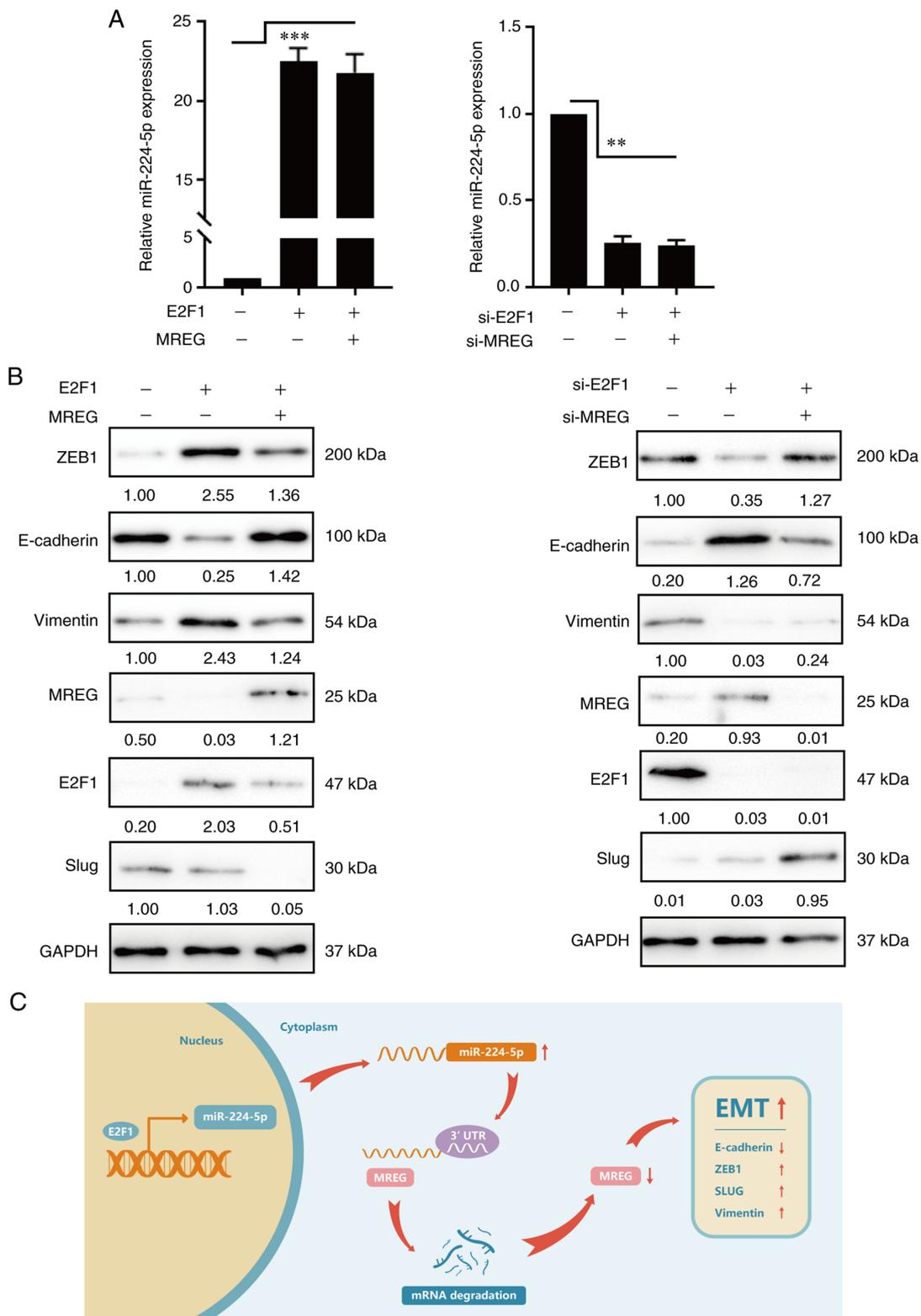


Figure 7. E2F1-induced miR-224-5p expression promotes EMT by downregulating MREG expression in HCC. (A) Relative expression level of miR-224-5p was measured using reverse transcription-quantitative PCR. The HepG2 cells were transfected with E2F1 and/or MREG overexpression plasmids, or si-E2F1 and/or si-MREG. (B) EMT-related protein expression was examined using western blot analysis. The HepG2 cells were transfected with E2F1 and/or MREG overexpression plasmid, or si-E2F1 and/or si-MREG. (C) Schematic regulatory network of the E2F1/miR-224-5p/MREG axis, in which liver cancer cell migration, invasion and EMT is regulated. **P<0.01 and ***P<0.001. miR, microRNA; EMT, epithelial-to-mesenchymal transition; MREG, melanoregulin; si, small inhibiting.

reversed by MREG knockdown (Fig. 7B). These data suggested that the E2F1/miR-224-5p axis may regulate liver cancer cell migration, invasion and EMT by regulating MREG expression.

In summary, it was demonstrated that miR-224-5p is upregulated in liver cancer and transcriptionally regulated by E2F1, and that E2F1-induced miR-224-5p upregulation

may promote liver cancer cell migration, invasion and EMT by targeting MREG. A summary illustration presenting the regulatory network is shown in Fig. 7C.

Discussion

Accumulating evidence has revealed that miRNAs have been associated with multiple biological functions in human cancer and may play a key role in carcinogenesis (40). However, the mechanisms of action and effects of miRNAs on HCC tumorigenesis, and progression remain largely unknown to date. miR-224-5p has been found to play different roles in diverse types of tumor (21-27), or play opposite roles in the same tumor. For example, one study found that miR-224-5p downregulation inhibited gastric cancer (GC) progression (30), as miR-224-5p acted as an oncogene in GC. On the contrary, other studies reported that miR-224-5p downregulation promoted GC cell proliferation, migration and invasion (30), suggesting that it may act as a tumor suppressor in GC. Based on the multifaceted role of miR-224-5p in GC and its important role in HCC indicated by previous research (29-32), the role of miR-224-5p was investigated as the aim of the present study.

Several studies have demonstrated the importance of miR-224-5p in HCC. For example, one of the first studies investigating the role of miR-224-5p in HCC (32) showed that miR-224 promoted liver cancer cell migration and invasion by regulating PPP2R1B. Another study presented one possible regulatory role of miR-224 in HCC, by regulating the downstream target, CPEB3 (29-32). miR-224 was also found to have an early diagnostic value in HCC (30). A recent study focused on the regulatory mechanism of MaFf in HCC, of which the upstream target was circular RNA-ITCH/miR-224-5p (30). These studies suggest that miR-224 plays an important role in the occurrence and development of HCC. According to the aforementioned studies, the present study identified the following innovations and highlights. Firstly, it was found that miR-224-5p expression level had a prognostic value in patients with HCC. Secondly, the upstream mechanism of miRNA regulation was investigated. E2F1 regulated miR-224-5p expression by binding to its promoter region. Thirdly, miR-224-5p had a critical role in EMT of liver cancer, as well as with cell migration and invasion, of which the E2F1/miR-224/MREG axis was associated. Based on the prognosis value of miR-224 and MREG in HCC, the E2F1/miR-224/MREG axis might be a potential therapeutic strategy in HCC. Taken together, the results from the present study advances the understanding and significance of the research topic.

In the present study, miR-224-5p was found to be upregulated and had a diagnostic value in HCC, which was consistent with the finding of a previous study (30). Furthermore, miR-224-5p overexpression facilitated HCC cell migration and invasion, which was also in accordance with the findings of a previous study (32). In addition, the present study also uncovered that high expression level of miR-224-5p could promote EMT in liver cancer cells and was associated with poor overall survival time in patients with HCC. These findings not only confirmed the oncogenic role of miR-224-5p in HCC, but may also expand its function and clinical guidance significance in HCC.

It was also discovered that MREG is a direct target of miR-224-5p and that miR-224-5p is transcriptionally regulated

by E2F1. Overexpression of MREG suppressed liver cancer cell migration, invasion and EMT via the E2F1/miR-224-5p axis. These findings indicated that miR-224-5p may function as an oncogene favoring HCC progression.

As miRNAs may serve as valuable diagnostic and therapeutic targets in cancer (41), it is crucial to fully elucidate the underlying mechanisms to provide the theoretical basis for the design of effective RNA-based antitumor strategies. Therefore, the potential mechanism in which miR-224-5p was associated with HCC tumorigenesis was further investigated. In the present study, the miRNA targets were predicted using the intersection of the three online tools (TargetScan, miRDB and StarBase) (41) and a luciferase reporter assay verified that MREG was the target of miR-224-5p. Consistently, the expression level of MREG at the mRNA and protein levels was negatively regulated by miR-224-5p.

Previous research has demonstrated that MREG was essential for maintaining retinal health (32) and lysosomal function (32). MREG was reported to regulate thyroid cancer cell proliferation and invasion by inhibiting the Akt/mTOR signaling pathway (32), thereby acting as a tumor suppressor in thyroid cancer. The findings of the present study suggested that miR-224-5p may promote HCC cell migration, invasion and EMT by targeting MREG. However, the expression and function of MREG in HCC remains unclear. The present study discovered that MREG may be of prognostic and diagnostic value in HCC. Overexpression of MREG suppressed migration and invasion in liver cancer cell lines, indicating that MREG may function as a tumor suppressor in HCC. The rescue experiments revealed that MREG could reverse the effects of miR-224-5p on liver cancer cell migration, invasion and EMT, suggesting that miR-224-5p could mediate HCC carcinogenesis by regulating MREG.

E2F1 is a well-known transcription factor that plays a key role in diverse tumor types, including HCC (32). E2F1 has been shown to be associated with biological processes via multiple pathways, such as its transcription activities. For example, it was reported that E2F1 transcriptional activity was inhibited in calcium/calmodulin-dependent protein kinase II inhibitor 1-mediated HCC growth (32). A previous study demonstrated that IQ motif-containing GTPase activating protein 3 promoted HCC progression, which was transactivated by E2F1 (32). In the present study, a promoter-binding motif for miR-224-5p was identified in the E2F1 gene sequence. It was confirmed that E2F1 regulated miR-224-5p expression at the transcriptional level from luciferase reporter and ChIP assays. The results confirmed that E2F1 was associated with miR-224-5p regulation via its transcriptional activity.

To verify the role of the E2F1/miR-224-5p/MREG regulatory network in HCC, knockdown and overexpression rescue experiments were performed. The results demonstrated that miR-224-5p expression promoted liver cancer cell migration, invasion and EMT by decreasing the expression level of MREG. Next, E2F1 was shown to activate miR-224-5p expression at the transcriptional level in HCC. Furthermore, MREG expression was regulated by the E2F1/miR-224-5p axis, thereby affecting liver cancer cell migration, invasion and EMT (Fig. 7). However, there were certain limitations to the present study. First, clinical samples were not collected and analyzed, and only data was from TCGA was analyzed. Second, no *in vivo* animal studies were conducted to validate the function of the E2F1/miR-224-5p/MREG regulatory

network. Third, the EMT-associated signaling pathways regulated by E2F1/miR-224-5p/MREG were also not investigated further. These issues will be addressed in future studies.

In summary, it was demonstrated that miR-224-5p mRNA expression level was associated with poor prognosis and exerted an oncogenic effect in HCC. Mechanistically, E2F1 positively regulated miR-224-5p expression level by binding to its promoter region. MREG was identified as the direct target of miR-224-5p. Rescue experiments confirmed that E2F1-induced miR-224-5p increased expression promoted liver cancer cell migration, invasion and EMT by decreasing the expression level of MREG. Thus, the E2F1/miR-224-4p/MREG axis contributes to liver cancer cell migration, invasion and EMT, and may hold promise as a clinical application prospect in patients with liver cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AL and JS designed the project. AL and NW performed the experiments and analyzed the data. AL wrote the manuscript. JS revised the manuscript. AL, JS and NW confirm the authenticity of all the raw data. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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