

Knockdown of SLC34A2 Inhibits Hepatocellular Carcinoma Cell Proliferation and Invasion

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The gene solute carrier family 34 (sodium phosphate), member 2 (*SLC34A2*), is a member of the SLC34 family. Increasing evidence suggests that *SLC34A2* is involved in the development of many human carcinomas. However, its role in hepatocellular carcinoma (HCC) is still unclear. Therefore, in this study we investigated the role of *SLC34A2* in HCC and explored the underlying mechanism. We found that the expression of *SLC34A2* is upregulated in HCC cell lines. Knockdown of *SLC34A2* obviously inhibited HCC cell proliferation, migration/invasion, and the epithelial–mesenchymal transition (EMT) phenotype. Furthermore, knockdown of *SLC34A2* significantly inhibited the expression of phosphorylated PI3K and AKT in HCC cells. Taken together, these results suggest that knockdown of *SLC34A2* inhibits proliferation and migration by suppressing activation of the PI3K/AKT signaling pathway in HCC cells, and *SLC34A2* may be a potential therapeutic target for the treatment of HCC.

Key words: Hepatocellular carcinoma (HCC); Epithelial–mesenchymal transition (EMT); PI3K/AKT pathway

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies and leading causes of cancer-related deaths in the world (1). There are about 600,000 new-onset patients with this disease every year (2). Despite the fact that various treatments for HCC have been improved recently, the 5-year survival rate of HCC patients remains low (3–5). To date, the molecular mechanism of HCC oncogenesis remains unclear. Thus, there is an urgent need to further understand the molecular mechanism underlying HCC.

SLC34A2, a member of the SLC34 family, was initially isolated from a human small intestine in 1999 (6). *SLC34A2* is a multipass membrane protein and encodes a type 2b sodium-dependent phosphate transporter, NaPi-IIb. It is known that *SLC34A2* can mediate transporting inorganic phosphate into epithelial cells via sodium ion cotransport (7). A vast array of studies has been conducted to understand the role of *SLC34A2* in various malignancies such as papillary thyroid carcinoma, breast carcinoma, and lung adenocarcinoma (8–10). Yang et al. indicated that *SLC34A2* was significantly downregulated in human non-small cell lung cancer tissues and

cell lines. *SLC34A2* also strongly inhibited tumor growth and metastasis ability in an A549 subcutaneous tumor model and lung metastasis model (11). However, at present, the expression pattern and the biological functions of *SLC34A2* in HCC cells remain unknown. The purpose of this study was to estimate the expression pattern and biological functions of *SLC34A2* in the progression of HCC. To the best of our knowledge, the present study revealed for the first time that *SLC34A2* was highly expressed in HCC cell lines, and *SLC34A2* may play an important role in regulating HCC proliferation and invasion.

MATERIALS AND METHODS

Cell Culture

The HCC cell lines HepG2, 97H, Hep3B, and HCCLM3 and a hepatocyte cell line HL-7702 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in 5% CO₂ at 37°C.

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Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from HCC cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and 2 µg of RNA of each sample was reverse transcribed using SuperScript RT kit (Invitrogen). PCR was performed using the Bio-Rad iQ5 Quantitative PCR System (Takara, Dalian, P.R. China). The primer sequences used for PCR amplification were SLC34A2, 5'-GAGAACATCGCCAAATGC-3' (forward) and 5'-GCAACCACAGAGGACCAG-3' (reverse); β-actin was used as an internal standard, and the primers were 5'-GTCCACCGCAAATGCTTCTA-3' (forward) and 5'-TGCTGTACCTTCACCGTTC-3' (reverse). For analysis, the expression of target genes was normalized by the gene β-actin. On the basis of the ΔΔCt method, relative amounts of mRNA were expressed as $2^{-\Delta\Delta Ct}$.

Western Blot Analysis

HCC cells were washed twice with ice-cold phosphate-buffered saline (PBS) and prepared with RIPA buffer. For Western blot analysis, 30 µg of protein per lane was separated by 12% SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Amersham, Little Chalfont, UK). The blot was blocked with 5% nonfat milk for 3 h at room temperature, incubated with appropriate polyclonal primary antibodies (SLC34A2, E-cadherin, N-cadherin, vimentin, p-PI3K, PI3K, p-AKT, AK, and GAPDH; from Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST (100 mM Tris-HCl, pH 7.5, 0.9% NaCl, 0.1% Tween 20) overnight at 4°C. The membranes were washed and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. The target protein was visualized by enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA), and the fluorescence was scanned using a Typhoon scanner (Amersham Biosciences, Piscataway, NJ, USA).

Stable Transfection of HCC Cells With siRNA-SLC34A2

The human SLC34A2 siRNA expression vector (si-SLC34A2) and control siRNA (vector) were chemically synthesized (GenePharma Co. Ltd.). HCC cells were transfected with si-SLC34A2 or vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell Proliferation Assay

Cell proliferation was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. In brief, HCC cells transfected with si-SLC34A2 or vector were seeded into a 96-well plate at a density of 1×10^4 cells/well. After 1–4 days, MTT reagent (diluted from a 4 mg/ml solution in PBS) (Sigma-Aldrich) was added to all the wells at a final concentration of 0.8 mg/

ml, and the cells were further incubated for an additional 4 h. The reaction was terminated by adding 200 µl/well dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The absorbance was recorded at 490 nm with a microplate reader.

In Vitro Migration and Invasion Assays

In vitro migration and invasion assays were performed in a 24-well Boyden chamber with 8-µm pore size polycarbonate membrane (Millipore, Boston, MA, USA). In brief, HCC cells transfected with si-SLC34A2 or vector were seeded in the upper chamber, and 500 µl of DMEM with 10% FBS was added into the lower chamber. After incubation at 37°C for 24 h, the cells on the upper surface of the filters were removed with a cotton swab, and cells that had migrated to the lower surface were fixed, stained, and counted using a microscope (Olympus, Tokyo, Japan). Cell invasion assay was done by the same procedure, except that the membrane was coated with Matrigel to form a matrix barrier.

Statistical Analysis

Statistical analyses were performed with SPSS 13.0 software (SPSS, Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance or paired *t*-tests. A value of $p < 0.05$ was considered significant.

RESULTS

Both mRNA and Protein Levels of SLC34A2 Were Increased in HCC Cell Lines

RT-qPCR was performed to evaluate the mRNA level of SLC34A2 in HCC cell lines and normal live cell line. As shown in Figure 1A, the mRNA level of SLC34A2 was elevated in HCC cell lines (HepG2, 97H, Hep3B, and HCCLM3). To further study the protein level of SLC34A2 in HCC cell lines, Western blot analysis was performed, and upregulation of SLC34A2 was observed in HCC cell lines compared with the hepatocyte cell line HL-7702 (Fig. 1B).

Knockdown of SLC34A2 Suppresses Proliferation in HCC Cells

To investigate the biological function of SLC34A2 in HCC, HepG2 and HCCLM3 cells were transfected with si-SLC34A2 or vector, respectively. Forty-eight hours after transfection, the expression of SLC34A2 was significantly reduced in si-SLC34A2-transfected HepG2 (Fig. 2A) and HCCLM3 cells (Fig. 2B). Furthermore, we observed the effects of SLC34A2 on cell proliferation by MTT assay. We found that knockdown of SLC34A2 significantly reduced the proliferative capability of the HepG2 (Fig. 2C) and HCCLM3 cells (Fig. 2D), compared with the vector group.

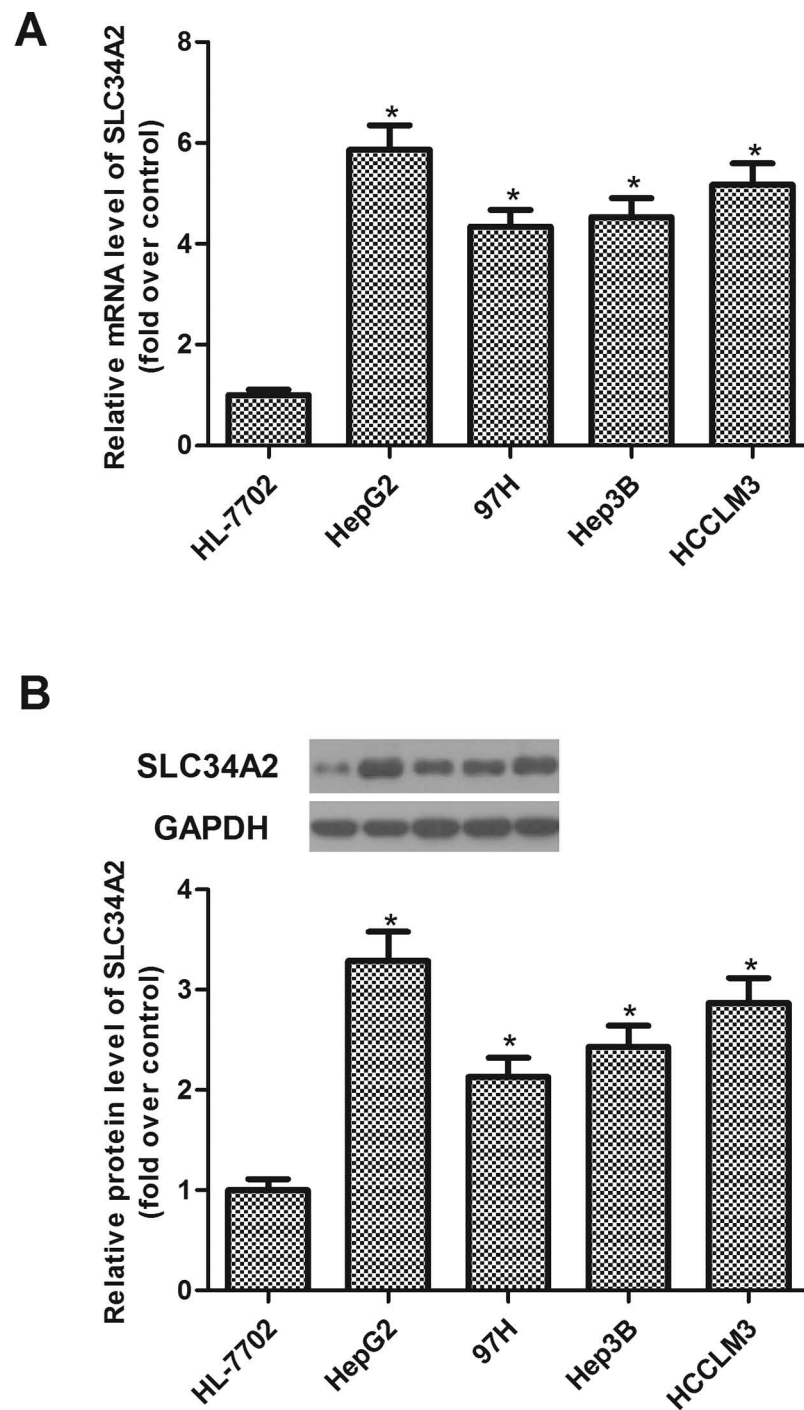


Figure 1. Both mRNA and protein levels of SLC34A2 were increased in HCC cell lines (A). The mRNA expression of SLC34A2 in HCC cell lines HepG2, 97H, Hep3B, and HCCLM3 and a hepatocyte cell line HL-7702 was detected by qRT-PCR (B). The protein expression of SLC34A2 in HCC cell lines HepG2, 97H, Hep3B, and HCCLM3, and a hepatocyte cell line HL-7702 was detected by Western blot. Results presented are mean \pm SD. *Differences from the HL-7702 group at $p < 0.05$.

Knockdown of SLC34A2 Suppresses Migration and Invasion in HCC Cells

Because cell migration and invasion are important steps in the development of cancer, we performed Transwell assays and a Matrigel invasion assay to assess the effects of SLC34A2 on cell migration and invasion. We found that knockdown of SLC34A2 reduced the number of HepG2 and HCCLM3 cells that migrated to the lower chamber, with a 55.4% reduction in HepG2 cells (Fig. 3A) and a 51.4% reduction in HCCLM3 cells (Fig. 3C). In addition, the results of the Matrigel invasion assay also showed

that the invasion of HepG2 (Fig. 3B) and HCCLM3 cells (Fig. 3D) was suppressed after infection with si-SLC34A2, respectively, compared with the vector group.

Knockdown of SLC34A2 Suppresses the EMT Phenotype in HCC Cells

Next, we investigated the effect of SLC34A2 on EMT phenotype in HCC cells. The results of the Western blot analysis indicated that knockdown of SLC34A2 significantly increased the expression of the E-cadherin protein and decreased the expression of N-cadherin and

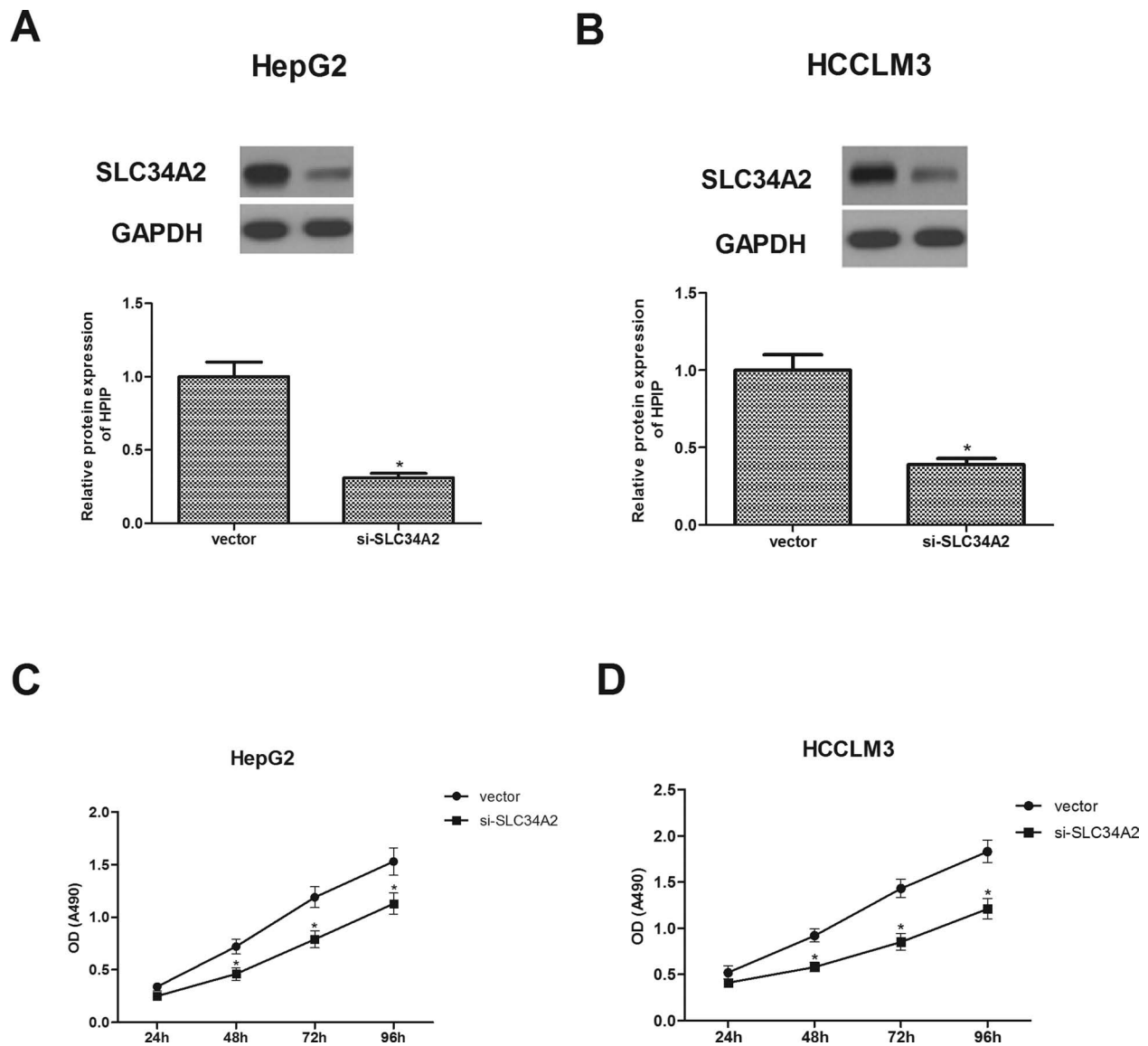


Figure 2. Knockdown of SLC34A2 suppresses proliferation in HCC cells. (A, B) HepG2 and HCCLM3 cells were transfected with si-SLC34A2 or vector for 48 h. The transfection efficiency was evaluated by Western blot. (C, D) Cell proliferation of HepG2 and HCCLM3 cells was assessed by MTT assay. Results presented are mean \pm SD. *Differences from the vector group at $p < 0.05$.

vimentin proteins in HepG2 (Fig. 4A) and HCCLM3 cells (Fig. 4B), compared with the vector group.

Knockdown of SLC34A2 Suppresses the Activation of PI3K/AKT Signaling Pathway in HCC Cells

The PI3K/AKT signaling pathway is involved in cell proliferation and invasion. To further illuminate the molecular mechanism by which SLC34A2 affects HCC proliferation and invasion, Western blot was used to detect the changes of PI3K/AKT signaling molecules in HepG2 cells before and after SLC34A2 knockdown. As shown in Figure 5A, knockdown of SLC34A2 significantly decreased the phosphorylation of PI3K and AKT in HepG2 cells, compared with the vector group.

To further confirm the involvement of PI3K/AKT signaling, an AKT inhibitor (MK-2206) was used. Cell proliferation and invasion assays showed that the inhibition effects of si-SLC34A2 on cell proliferation and invasion were enhanced by MK-2206 exposure (Fig. 5D and E).

DISCUSSION

The majority of HCC deaths are caused by metastasis; thus, novel metastasis-related genes urgently need to be uncovered in order to shed new light on the molecular mechanisms driving HCC metastasis. In the present study, we observed the upregulation of SLC34A2 in human HCC cell lines. We discovered that knockdown

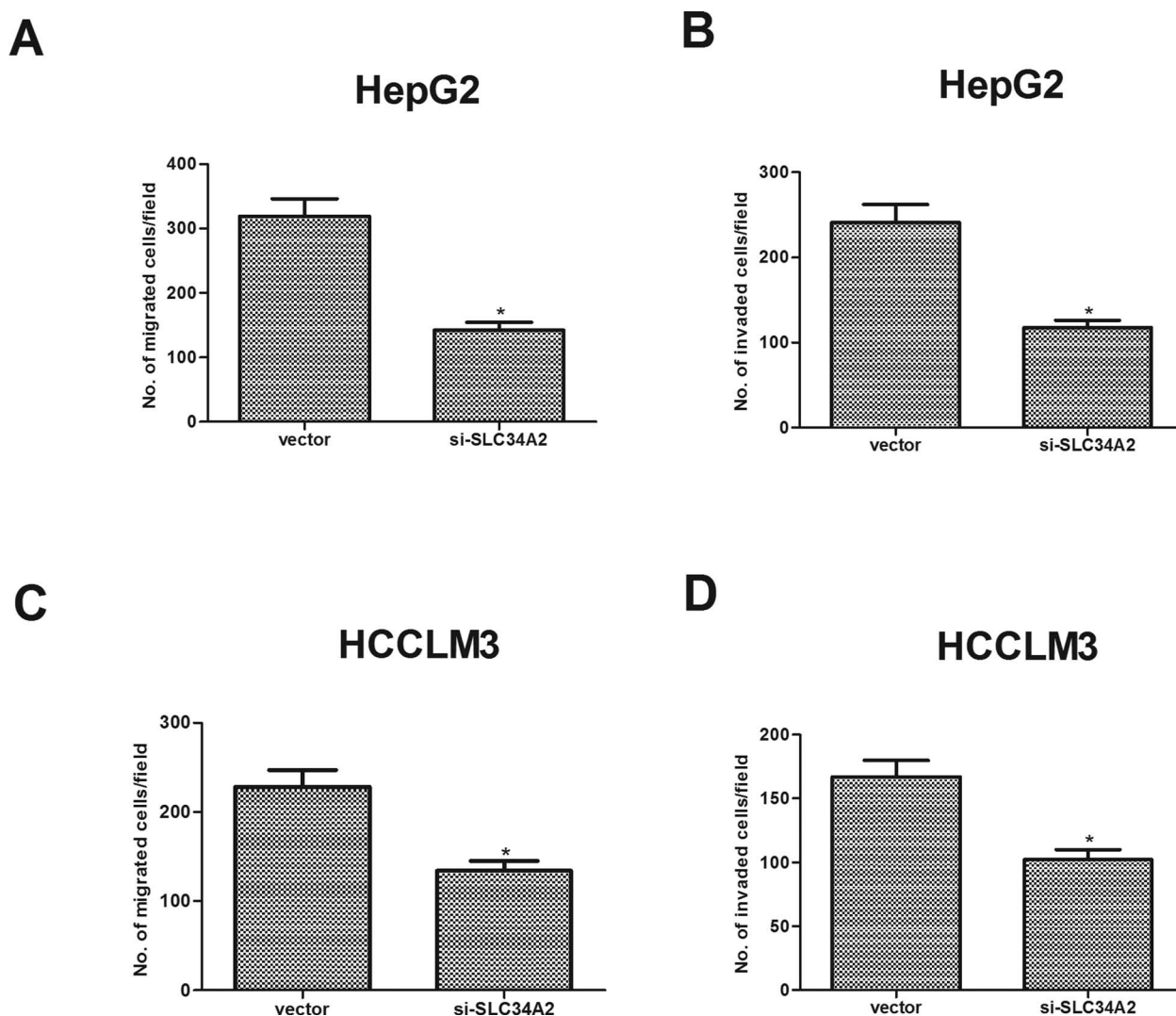


Figure 3. Knockdown of SLC34A2 suppresses migration and invasion in HCC cells. HepG2 and HCCLM3 cells were transfected with si-SLC34A2 or vector for 48 h. (A, C) Cell migration was determined by the Transwell assay. (B, D) Cell invasion was determined using Matrigel-coated Transwell invasion chamber. Results presented are mean \pm SD. *Differences from the vector group at $p < 0.05$.

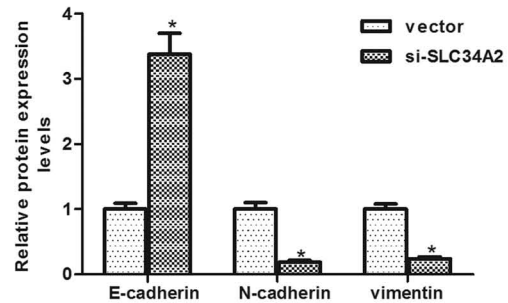
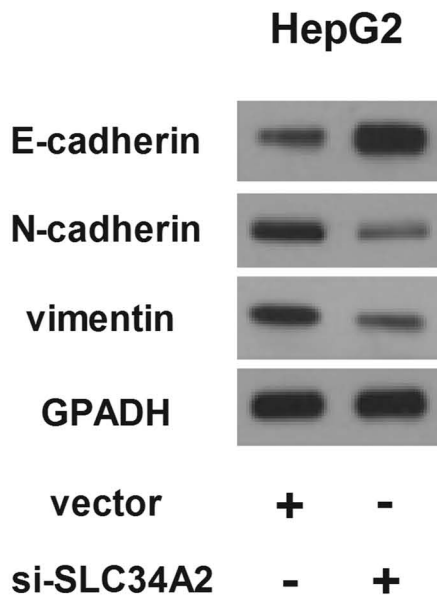
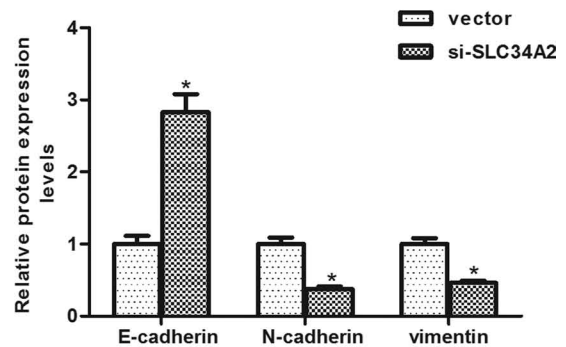
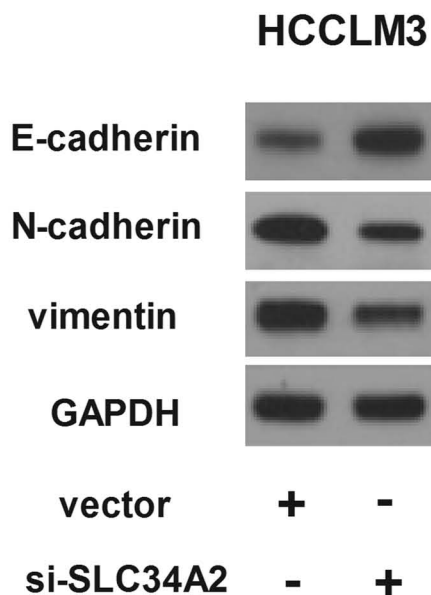
A**B**

Figure 4. Knockdown of SLC34A2 suppresses the EMT phenotype in HCC cells. HepG2 cells were transfected with si-SLC34A2 or vector for 48 h. (A) The expression levels of E-cadherin, N-cadherin, and vimentin were detected in HepG2 cells by Western blotting. (B) The expression levels of E-cadherin, N-cadherin, and vimentin were examined in HCCLM3 cells. Results presented are mean \pm SD. *Differences from the vector group at $p < 0.05$.

of SLC34A2 expression significantly suppressed cell proliferation, migration/invasion, and EMT phenotype in HCC cells. We also provided evidence that knockdown of SLC34A2 efficiently inhibited the expression of p-PI3K and p-AKT in HCC cells.

In recent years, studies have found that SLC34A2 plays an important role in tumor progression, invasion, and metastasis (11–13). A recent study confirmed that SLC34A2 was frequently highly expressed in papillary serous ovarian carcinoma and breast cancer tissues

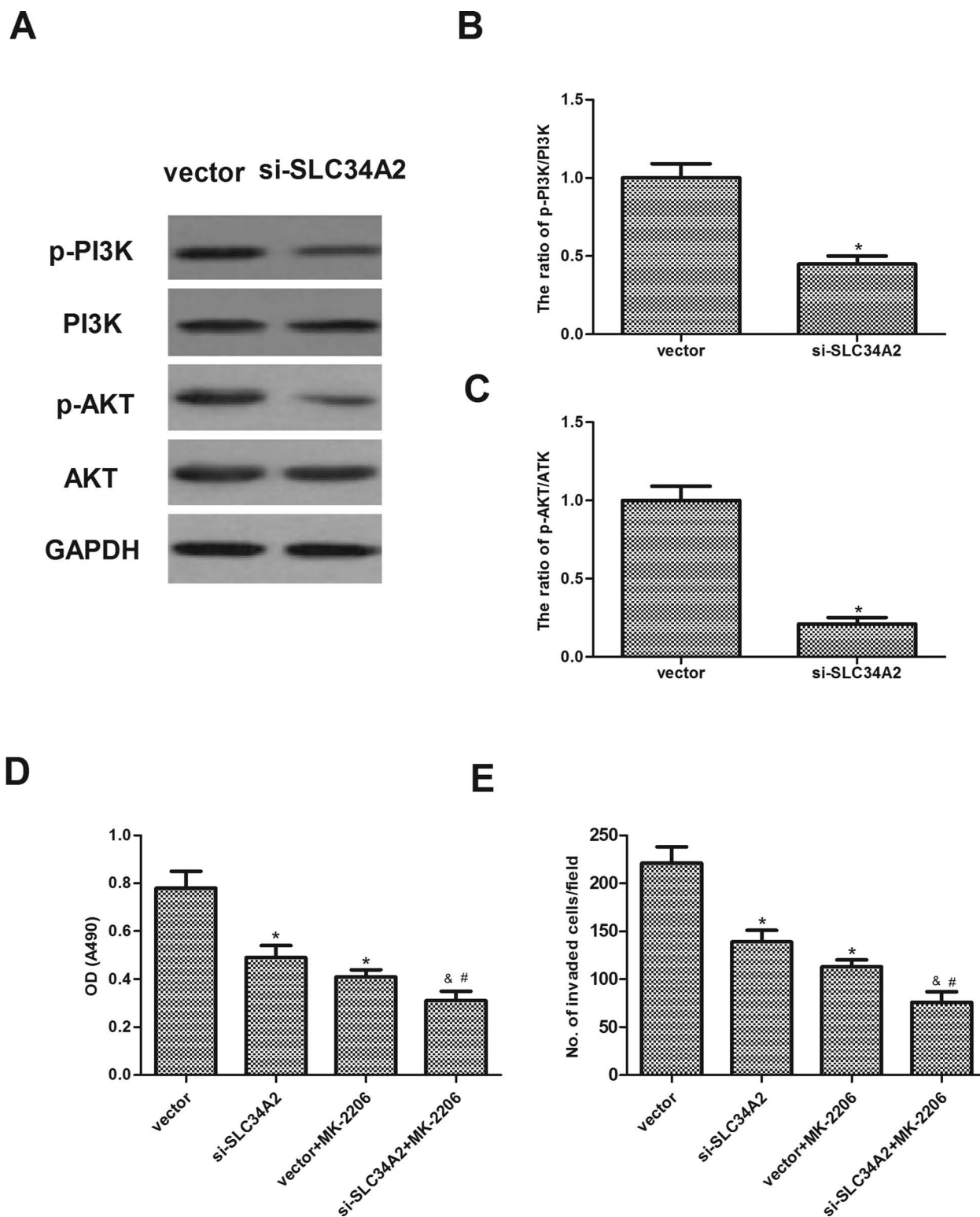


Figure 5. Knockdown of SLC34A2 suppresses the activation of PI3K/AKT signaling pathway in HCC cells. HepG2 cells were transfected with si-SLC34A2 or vector for 48 h. (A) The expression levels of p-PI3K, PI3K, p-AKT, and AKT were detected by Western blotting. (B, C) Protein expression was analyzed using the BandScan 5.0 software and normalized to GAPDH. (D) HepG2 cells were transfected with si-SLC34A2 or vector in the presence or absence of AKT inhibitor (MK-2206; 2 μ M) for 48 h. Cell proliferation was detected by the MTT assay. (E) Cell invasion was evaluated by the Matrigel-coated Transwell invasion chamber. Results presented are mean \pm SD. * p <0.05 versus vector; # p <0.05 versus si-SLC34A2; & p <0.05 versus vector+MK-2206.

(14,15). Besides, SLC34A2 had been reported to be downregulated in human NSCLC cells and patient tissues (16). These findings suggest that SLC34A2 performs an oncogenic or a tumor-suppressing function depending on the cell type or context. However, little is known about the expression and function of SLC34A2 in HCC. To the best of our knowledge, the present study provided the first evidence that SLC34A2 was prominently upregulated in human HCC cell lines. Cancer cell metastasis is related to cell migratory ability and invasiveness (17). Functional analyses demonstrated that knockdown of SLC34A2 expression significantly suppressed cell proliferation and migration/invasion *in vitro*. These data imply that SLC34A2 may behave as an oncogene to promote the development and progression of HCC.

It is well known that EMT plays an important role in cancer cell migration and invasion (18,19). Reduction or loss of E-cadherin expression is one of the well-established hallmarks of EMT (20). In this study, we found that knockdown of SLC34A2 expression greatly increased the expression of epithelial marker E-cadherin and decreased the expression of mesenchymal markers (N-cadherin and vimentin) in HCC cells. These results suggest that knockdown of SLC34A2 blocks the migration and invasion by repressing EMT phenotype in HCC cells.

The PI3K/AKT signaling pathway has been shown to enhance not only cell proliferation but also cell invasion and migration (21,22). PI3K is activated by oncogenes, and activated PI3K catalyzes the formation of phosphatidylinositol 3,4,5 trisphosphate (PIP3), which in turn activates phosphoinositide-dependent kinase-1 (PDK1), leading to activation of AKT (23). AKT is a major regulator in the PI3K/AKT signaling pathway and can be activated upon phosphorylation to subsequently phosphorylate a series of downstream targets, including glycogen synthase kinase 3 β (GSK-3 β), Bad, I κ B kinase (IKK), p27, and mouse double minute 2 (MDM2) (24). Emerging evidence has suggested that inhibiting the PI3K/AKT signaling pathway can inhibit HCC cell proliferation and invasion *in vitro* (25–28). Recently, it has been reported that SLC34A2 might attenuate the tumorigenesis of NSCLC, and the relative mechanism might be related to the changes of PI3K/AKT/mTOR and Ras/Raf/MEK/ERK signal pathways (16). In this study, we found that knockdown of SLC34A2 efficiently inhibited the expression of p-PI3K and p-AKT in HCC cells. These results suggested that the inhibitory effect of siRNA-SLC34A2 on HCC cell proliferation and invasion may be involved in the suppression of the PI3K/AKT signaling pathway.

In conclusion, our findings demonstrated that knockdown of SLC34A2 inhibits HCC cell proliferation and invasion. Therefore, SLC34A2 may play an important role in HCC and may represent a novel therapeutic target for the treatment of HCC.

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