Overexpression of Epsin 3 enhances migration and invasion of glioma cells by inducing epithelial-mesenchymal transition

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Abstract. Epsin 3 (EPN3) expression is limited to gastric parietal cells and wounded or pathological tissue rather than normal brain tissue, and although it has been identified as an oncogene in estrogen receptor-positive breast cancer and non-small cell lung cancer, its function in cancer is poorly understood. The present study aimed to investigate the association of EPN3 expression with the clinicopathological features of patients with glioma, as well as the effects of EPN3 on glioblastoma cells and the potential molecular mechanisms for its effects on glioblastoma cell behavior. EPN3 expression was assessed by immunohistochemistry in tissue samples from 167 patients with glioma, as well as by western blotting in 5 glioblastoma cell lines. The U87 and U251 glioblastoma cell lines were used to investigate the effects of EPN3 on glioblastoma cell invasion and migration through gain and loss of EPN3 expression experiments; expression levels were further investigated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses. The results demonstrated that EPN3 expression levels were upregulated in high-grade glioma tissues compared with low-grade tissues, and there were varying expression levels of EPN3 in the five glioblastoma cell lines. No significant differences were observed in EPN3 expression in relation to patient age, sex or tumor size. Overexpression of EPN3 promoted glioblastoma cell migration and invasion, which we hypothesized was through affecting epithelial-mesenchymal transition (EMT). RT-qPCR and western blotting revealed that EPN3 upregulation increased the expression of Notch1 intracellular domain, β-catenin, Slug, Twist and zinc-finger E-box-binding homeobox (ZEB)-1. These results suggested that EPN3 enhances the migration and invasion of glioblastoma cells by activating the transcription factors Slug, Twist and ZEB1, but not Snail 1 or ZEB2, to induce EMT in glioma cells; EPN3 involvement in the Notch and WNT/β-catenin signaling pathways may contribute to this process.

Introduction

Glioblastoma is the most common malignant tumor of the nervous system; it is highly invasive, resistant to treatment and relapses easily (1). Numerous studies have focused on glioblastoma in relation to methylation status of a panel of genes (2), angiogenesis and DNA repair pathways (3,4), epidermal growth factor receptor, p53, isocitrate dehydrogenase 1 and MDM2 mutation status (5), and various therapeutic strategies (6,7); however, the overall survival of patients remains poor, with a median survival time of 12-15 months (8). Therefore, further investigations of the mechanisms and potential therapeutic strategies for glioblastoma are necessary.

Epsin (EPN)-3 is a member of the endocytosis adapter protein gene family, which contains several different interaction motifs, and is involved in clathrin-mediated endocytosis (9,10). The Epsin protein family is conserved between yeast, *Drosophila melanogaster* and mammals. In mammals, the Epsin family includes EPN1, EPN2 and EPN3. EPN1 and EPN2 are ubiquitously expressed, to various degrees, in different tissues, particularly in the brain (11,12). Previous studies have reported that EPN1 and EPN2 may be upregulated in a variety of cancer types and are associated with tumor cell proliferation, migration and invasion (11,12), whereas EPN3 expression is normally limited to gastric parietal cells (13), as well as wounded or pathological tissue, rather than normal brain tissue (14). It has been reported that EPN3 is overexpressed in basal cell carcinoma and ulcerative colitis (13,14). In addition, several studies have suggested that Epsins, as adapter proteins in clathrin-mediated endocytosis, are involved in the regulation of various signal receptors, including receptor tyrosine

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Abbreviations: BMP, bone morphogenetic protein; EMT, epithelial-mesenchymal transition; EPN3, Epsin 3; NICD1, Notch1 intracellular domain

Key words: EPN3, glioma, Notch pathway, WNT/β-catenin, EMT

kinases, Notch ligands and receptors, and WNT receptors; defective regulation of these signaling ligands and receptors is associated with tumorigenesis (15-17).

Notch signaling regulates tissue renewal and development, as well as serving various roles in cell differentiation, proliferation and apoptosis in a tissue context-dependent manner (18). Aberrant loss or gain of Notch signaling components is associated with multiple human diseases (19-21). Activation of Notch signaling requires its receptors to bind their specific ligands, resulting in receptor proteolysis and the release of the active Notch intracellular domain (NICD), which translocates to the nucleus to initiate downstream target gene transcription (22). A previous study has suggested that Epsins are required for Notch signaling pathway activation (9).

WNT/β-catenin signaling is also important in tumor development. WNT signaling is activated through endocytosis (17,23,24), and Epsins are required for the stability of the downstream protein Dishevelled and WNT signaling activation in colon cancer development (17).

Notch and WNT signaling pathways are associated with epithelial-mesenchymal transition (EMT) (25-27). EMT is a vital mechanism in embryonic development and tissue repair, and is an essential mechanism of cancer cell metastasis (28). The characteristics of EMT include the loss of the expression or function of epithelial (E)-cadherin with a concomitant increase in mesenchymal markers, including Vimentin (VIM) and Neural-cadherin (29), potentially leading to the spread and dissemination of cancer cells. EMT may be induced by several transcription factors, including Snail1, Slug and Twist (25).

In the present study, EPN3 was revealed to be highly expressed in high-grade glioma tissues compared with low-grade samples. The effects of EPN3 on glioblastoma cell migration and invasion were observed by inducing the gain and loss of EPN3 expression in U87 and U251 glioblastoma cells; the results revealed that the overexpression of EPN3 promoted glioblastoma cell migration and invasion, and increased the expression of NICD1, β-catenin, VIM, Slug, Twist and zinc-finger E-box-binding homeobox (ZEB)-1, whereas the expression level of E-cadherin was decreased. These results suggested that EPN3 may promote glioblastoma cell migration and invasion by inducing EMT, and that EPN3 may be a potential therapeutic target in glioblastoma.

Materials and methods

Tumor samples and cell culture. Glioma tissues from four different World Health Organization (WHO) grades were collected from 167 patients at the Department of Neurosurgery at Tianjin Huanhu Hospital (Tianjin, China), and included 71 women and 96 men (age 1-73 years; Table I); human colon carcinoma tissue was donated by Professor Li Qi (Tianjin Medical University Cancer Institute and Hospital). Informed consent was obtained from all patients or their guardian, and the use of patient tissue was approved by the ethics committee of Tianjin Huanhu Hospital (2016-3). Glioblastoma cell lines A172, LN229, LN308, U251 and U87 were purchased from the American Type Culture Collection (Genetimes ExCell Technology, Inc., Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), and maintained in a humidified atmosphere with 5% CO_2 at 37˚C.

Plasmid vectors and transfection. All plasmids were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The pGFP-C-shLenti vector encoding short hairpin (sh)RNA against EPN3 (shEPN3; cat. no. TL304750; 5'-CACAACTAC TCCGAGGCAGAAATCAAGGT-3') was used to silence EPN3 expression; Scrambled-shRNA (cat. no. TR30021; 5'-GCACTACCAGAGCTAACTCAGATAGTACT-3') was used as negative control. pCMV6-EPN3 (cat. no. RC212297) was used for the upregulation of EPN3; pCMV6-Entry (cat. no. PS100001) was used as negative control. Cells were seeded at a density of $2x10^5$ cells/well in 6-well culture plates, and plasmid transfections (2,500 ng) were performed using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions, when cell cultures reached 90% confluency; cells were incubated in a humidified atmosphere with 5% CO_2 at 37˚C for 48 h. Gene expression levels were evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis at 48 h post-transfection.

RT-qPCR. Total RNA was extracted from the transfected cells ($5x10^6$) using 1 ml TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA was synthesized using a cDNA Reverse Transcription Kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was performed using Power SYBR Green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and an ABI 7500 series Real-Time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling condition was as follows: Initial denaturation at 95˚C (30 sec), followed by 40 cycles of denaturation at 95˚C (20 sec), annealing at 60˚C (20 sec) and elongation at 72˚C (20 sec). mRNA expression levels were normalized to GAPDH and were calculated using the $2^{-\Delta\Delta Ct}$ method (30). The primers used for PCR are listed in Table II.

Antibodies. Anti-EPN3 was purchased from Sigma-Aldrich (cat. no. SAB1303019; Merck KGaA, Darmstadt, Germany); anti-GAPDH was purchased from ProteinTech Group, Inc. (cat. no. 10494-1-AP; Chicago, IL, USA); anti-E-cadherin (cat. no. sc-8426), anti-Slug (cat. no. sc-166902), anti-Snail 1 (cat. no. sc-393172), anti-ZEB2 (cat. no. sc-271984) and anti-Twist (cat. no. sc-81417) were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); anti-VIM was from BioLegend, Inc. (cat. no. 677801; San Diego, CA, USA); and anti-NICD1 (cat. no. WL03097a), anti-ZEB1 (cat. no. WL01657) and anti-β-catenin (cat. no. WL0962a) were from Wanleibio Co., Ltd. (Shanghai, China). The horseradish peroxidase (HRP)-conjugated goat anti-mouse (cat. no. ZB-2305) and HRP-conjugated goat anti-rabbit (ZB-2301) secondary antibodies were purchased from ZSGB-BIO (OriGene Technologies, Inc.).

Western blot analysis. Total protein was extracted from cells ($1x10^7$) using Radioimmunoprecipitation Assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) on

Table I. Relationship between EPN3 expression and clinicopathological features in patients with glioma.

Clinicopathological feature	n	EPN3 expression		Positive rate (%)	P-value[a]
		Low	High		
WHO grade					
I	49	41	8	17.7	<0.001
II	47	38	9		
III	36	19	17	52.1	
IV	35	15	20		
Sex					0.322
Male	96	62	34	35.4	
Female	71	51	20	28.2	
Age (years)					
<45	90	62	28	31.1	0.715
≥45	77	51	26	33.8	
Tumor size (cm)					
<4.5	121	82	39	32.2	0.963
≥4.5	46	31	15	32.6	

[a]P-values were calculated by χ^2. EPN3, Epsin 3; WHO, World Health Organization.

Table II. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Sequence (5'→3')
EPN3	F: CTTGGCTGACATCTTCGTACCT
	R: TGTGTTCGGCCTAAAACCTG
E-cadherin	F: CAGCACGTACACAGCCCTAA
	R: ACCTGAGGCTTTGGATTCCT
Vimentin	F: AGATGGCCCTTGACATTGAG
	R: TGGAAGAGGCAGAGAAATCC
Notch1	F: ACCAATACAACCCTCTGCGG
	R: GGCCCTGGTAGGTCATCATC
Notch2	F: GGAGGCACCTGTATTGACCT
	R: ATGCCCTGGATGGAAAATGGA
Notch3	F: CTGTGGCCCTCATGGTATCT
	R: ACCGTTCAGGCATGGGTTG
Notch4	F: TTCCCAGAACCCTGTGCCAAT
	R: AACTGGCACGTCTCACCCAG
Snail1	F: CCTCCCTGTCAGATGAGGAC
	R: GTTCCTTATGGAGTCGGACC
Slug	F: GGGGAGAAGCCTTTTTCTTG
	R: AGGACGTGTTTGTACTCCT
Twist	F: GGAGTCCGCAGTCTTACGAG
	R: TCTGGAGGACCTGGTAGAGG
ZEB1	F: GCACAACCAAGTGCAGAAGA
	R: GCCTGGTTCAGGAGAAGATG
ZEB2	F: AAATGCACAGAGTGTGGCAAGG
	R: CTGCTGATGTGCGAACTGTAGGA
GAPDH	F: CAATGACCCCTTCATTGACCT
	R: ATGACAAGCTTCCCGTTCTC

E-cadherin, epithelial cadherin; EPN3, Epsin 3; F, forward; R, reverse; ZEB zinc-finger E-box-binding homeobox.

ice. Protein concentrations were determined by Bicinchoninic Acid assay (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Equal amounts of protein (30 μg) were separated using SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk in TBST (1X TBS + 0.1% Tween-20) buffer for 2 h. Following washing 3 times for 5 min in TBST, membranes were incubated with primary antibodies against EPN3, E-cadherin, Slug, Twist (all at 1:500), VIM (1:5,000), NICD1 (1:500), β-catenin (1:1,000) and GAPDH (1:10,000) overnight at 4˚C. Membranes were washed 3 times (5 min each) in TBST, and subsequently incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:1,000) for 1 h at room temperature. Protein bands were visualized using an Enhanced Chemiluminescence Western Blot Detection kit, and the intensity of protein bands were normalized to GAPDH and quantified by densitometric analysis using Quantity One software (version 4.6.9; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunohistochemistry (IHC). Human colon carcinoma tissues were used as a positive control. Colon carcinoma and glioma tissue samples were fixed using 10% formalin at room temperature for 2 h, embedded in paraffin and sectioned (4 μm), slides were deparaffinized with xylene and rehydrated in a decreasing ethanol series. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide for 15 min. Following heat-induced antigen retrieval at 100˚C for 10 min, sections were incubated with 5% rabbit serum (ZSGB-BIO; OriGene Technologies, Inc.) for 10 min to eliminate non-specific binding. Anti-EPN3 (1:200) was added and the sections were incubated overnight at 4˚C. The sections were washed 3 times (5 min each) using PBS, and the corresponding optimized secondary antibodies (Bond Polymer refine detection; cat. no. DS9800; Leica Microsystems, Ltd., Milton Keynes, UK) were added to the sections and incubated for 1 h at room temperature, followed by incubation with the streptavidin-HRP complex (Santa Cruz Biotechnology, Inc.). Immunoreactivity was visualized by incubating with 3,3'-diaminobenzidine (Sigma-Aldrich; Merck KGaA) for 3-15 min, and the sections were counterstained with hematoxylin. Two pathologists blinded to the clinical data independently scored the percentage of positive-staining cells and the intensity of the EPN3 staining. A three-tiered scale was used to grade the staining intensity (1, negative or weak; 2, medium; 3, strong), whereas the percentage of EPN3-positive tumor cells was scored as follows: 0, ≤10% positive tumor cells; 1, 11-24%; 2, 25-50%; 3, 51-75% and 4, >75%. The product of the intensity and positive percentage score of the cells was used as a staining index (negative, 4; positive, ≥4).

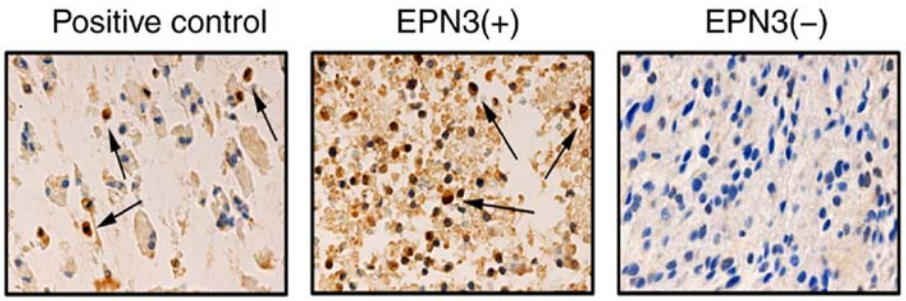

Figure 1. Expression of EPN3 in glioma tissue samples. Representative results for immunohistochemical analysis of EPN3 protein expression in human glioma samples. Left, EPN3 (arrows) is highly expressed in human colon carcinoma tissue, which was used as a positive control. Center, positive expression of EPN3 (arrows) in glioma tissue. Right, negative EPN3 expression in glioma tissue. Magnification, x40. EPN3, Epsin 3.

Transwell migration and invasion assays. Migration and invasion assays were performed using 24-well Transwell chambers either with (invasion) or without (migration) BD BioCoat Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). A total of $5x10^4$ cells were suspended in serum-free DMEM and seeded into the upper chamber; 500 μl DMEM with 10% FBS was added to the lower chamber. Following incubation for 24 h at 37˚C, the non-migratory or non-invasive cells remaining in the upper chambers were gently removed with a cotton swab. The cells that had migrated or invaded to the lower surface of the membrane were washed twice with PBS, fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 15 min. Cells were counted in five different fields of view under a light microscope.

Statistical analysis. All experiments were performed in triplicate. Normally distributed data were presented as the mean ± standard deviation, and were analyzed using Student's t-test in SPSS version 17 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The χ^2 test was used to analyze categorical variables. $P<0.05$ was considered to indicate a statistically significant difference.

Results

EPN3 expression levels are higher in high-grade tumor samples. EPN3 expression was examined by IHC in tissue samples from 167 patients with glioma, including 96 low-grade (WHO grade I/II) and 71 high-grade (WHO grade III/IV) tumor samples (Fig. 1; Table I). The results demonstrated that EPN3 was highly expressed in 17 out of 96 low-grade glioma tissues (17.7%), and 37 out of 71 high-grade glioma tissues (52.1%), and the difference in EPN3 expression levels between low- and high-grade glioma tissue samples was significant ($P<0.001$; Table I). No significant differences were identified in EPN3 expression based on patient age, sex or tumor size ($P>0.05$; Table I).

Overexpression of EPN3 enhances migration and invasion of U87 and U251 glioblastoma cells. Varying EPN3 protein expression levels were detected among the five glioblastoma cell lines examined (A172, LN229, U251, LN308 and U87), with the highest expression levels detected in U251 and U87 cells, and the lowest level in A172 cells (Fig. 2A). U251 and U87 cells were selected for further experiments. Notably, although U87 is different from the original glioblastoma cells, Marie Allen et al have reported that U87 cell line is of CNS origin and is likely to be a bona fide human glioblastoma cell line with unknown patient origin (31). U251 and U87 cells transfected with shEPN3 exhibited reduced EPN3 mRNA and protein expression levels compared with cells transfected with Scra mbled-shRNA (Fig. 2B and C). In addition, cells transfected with EPN3 overexpression vector exhibited increased levels of EPN3 mRNA and protein expression compared with expression levels empty vector-transfected cells (Fig. 2B and C).

The effects of EPN3 on glioblastoma cell migration and invasion were examined using Transwell migration and invasion assays. The results demonstrated that the reduction of EPN3 expression significantly inhibited U87 and U251 glioblastoma cell migration and invasion compared with the control cells ($P<0.001$; Fig. 3A and B, respectively). Conversely, the overexpression of EPN3 significantly promoted U87 and U251 cell migration and invasion compared with control cells ($P<0.01$, $P<0.001$; in Fig. 3). These results indicated that EPN3 overexpression enhanced glioblastoma cell migration and invasion *in vitro*.

Overexpression of EPN3 induces EMT in U87 and U251 glioblastoma cells. EMT serves an important role in the process of tumor cell migration and invasion (32); therefore, expression levels of key molecular markers of EMT, E-cadherin and VIM, were analyzed by RT-qPCR and western blotting in transfected U87 and U251 cells. U87 and U251 cells transfected with shEPN3 exhibited increased levels of E-cadherin mRNA and protein expression (Fig. 4A and B), whereas the expression levels of VIM decreased. Conversely, EPN3-overexpressing U87 and U251 cells exhibited decreased E-cadherin mRNA and protein expression levels (Fig. 4A and B, respectively), whereas the mRNA and protein expression levels of VIM were increased. These results indicated that EPN3 may be associated with EMT, which may contribute to the migratory and invasive ability of glioblastoma cells.

Overexpression of EPN3 increases Notch1 and β-catenin expression in U87 and U251 glioblastoma cells. The Notch pathway is activated in glioblastoma cells and promotes their migration and invasion (33,34); EPN1 and EPN2 were reported to be adaptor proteins in clathrin-mediated endocytosis, which is necessary for the activation of the Notch

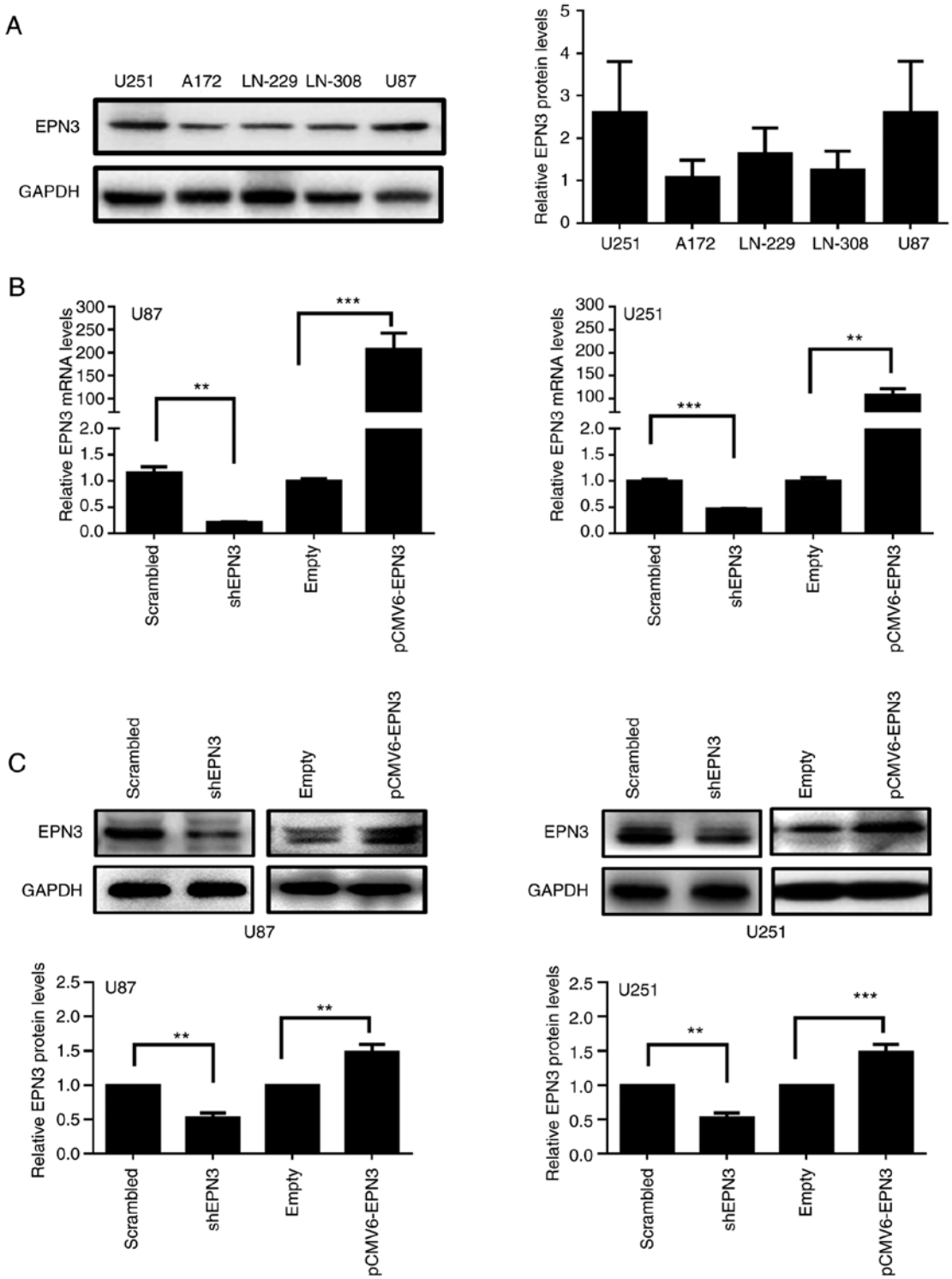

Figure 2. Gain and loss of EPN3 expression levels in glioblastoma cell lines. (A) EPN3 protein expression in five glioblastoma cell lines was determined by western blot analysis. (B and C) U87 and U251 cells were transfected with shEPN3 or pCMV6-EPN3 overexpression vector, or the respective Scramble-shRNA or Empty vector control, and the expression levels of EPN3 (B) mRNA were determined by reverse transcription-quantitative polymerase chain reaction and (C) protein were determined by western blotting; GAPDH was used as a loading control and to normalize the protein expression data. All experiments were repeated at least three times. Data are presented as the mean ± standard deviation; **P<0.01 and ***P<0.001. EPN3, Epsin 3; sh, short hairpin RNA.

pathway (9,35-37). Therefore, whether EPN3 was involved in the activation of the Notch pathway in glioblastoma cells was examined. RT-qPCR analysis was used to identify the effects on Notch receptor expression following the overexpression of EPN3 in U87 cells. The results suggested that Notch1 expression increased when EPN3 was overexpressed, whereas no significant differences were observed in Notch2, Notch3 or Notch4 expression (Fig. 5A). As Notch1 is expressed in primary human gliomas and glioblastoma cell lines (18), the expression of the activated NICD1 was examined by western blotting in transfected U87 and U251 glioblastoma cells. It was demonstrated that the expression levels of NICD1 decreased

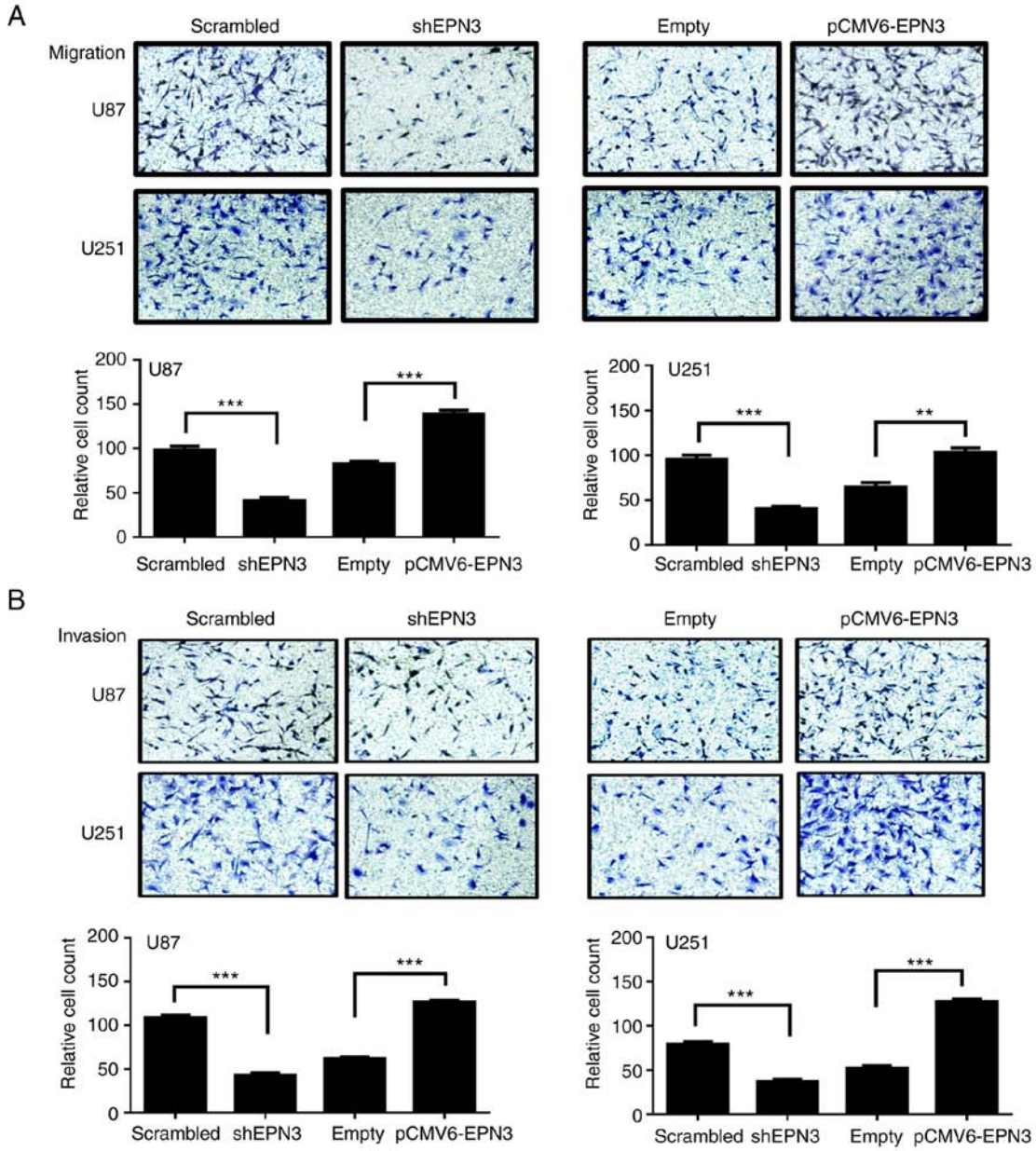

Figure 3. EPN3 expression affects glioblastoma cell migration and invasion. (A and B) U87 and U251 cells were transfected with shEPN3 or pCMV6-EPN3 overexpression vector, or the respective Scramble-shRNA or Empty vector controls, and Transwell assays were used to examine the subsequent effects on (A) migration and (B) invasion. All experiments were repeated at least three times. Data are presented as the mean ± standard deviation; **P<0.01 and ***P<0.001. EPN3, Epsin 3; sh, short hairpin RNA.

when EPN3 was downregulated and increased when EPN3 was overexpressed in U87 and U251 cell lines compared with the respective control-transfected cells (Fig. 5B). WNT/β-catenin signaling is also activated via endocytosis (23,24), and it was previously reported that Epsin is required for WNT signaling activation in colon cancer development (17). In the present study, the expression of β-catenin was decreased when EPN3 was downregulated (Fig. 5C). In conclusion, EPN3 may function to activate Notch1 and WNT signaling pathways to promote tumorigenesis in glioblastoma.

Overexpression of EPN3 increases Slug, Twist and ZEB1 expression levels in U87 and U251 glioblastoma cells. Notch activation induces EMT through the Slug-induced repression of E-cadherin (38) and the upregulation of EMT-inducing transcription factors, including Snail1, Slug, ZEB1, ZEB2 and Twist (25). Whether EPN3 activated these EMT transcription factors was assessed using RT-qPCR and western blotting (Fig. 6). The results demonstrated that Slug, Twist and ZEB1 mRNA expression levels were increased in cells overexpressing EPN3 compared with the respective levels in cells transfected with empty vector (Fig. 6A); Snail1 and ZEB2 mRNA expression levels remained unaffected. Conversely, protein expression levels of Slug, Twist and ZEB1 were decreased in shEPN3-transfected U87 and U251 cells, compared with Scramble shRNA-transfected cells, whereas their expression levels were increased when EPN3 was overexpressed (Fig. 6B-D) Conversely, Snail1 and ZEB2 expression levels were unaltered (Fig. 6E and F, respectively). These results suggested that EPN3 may increase the

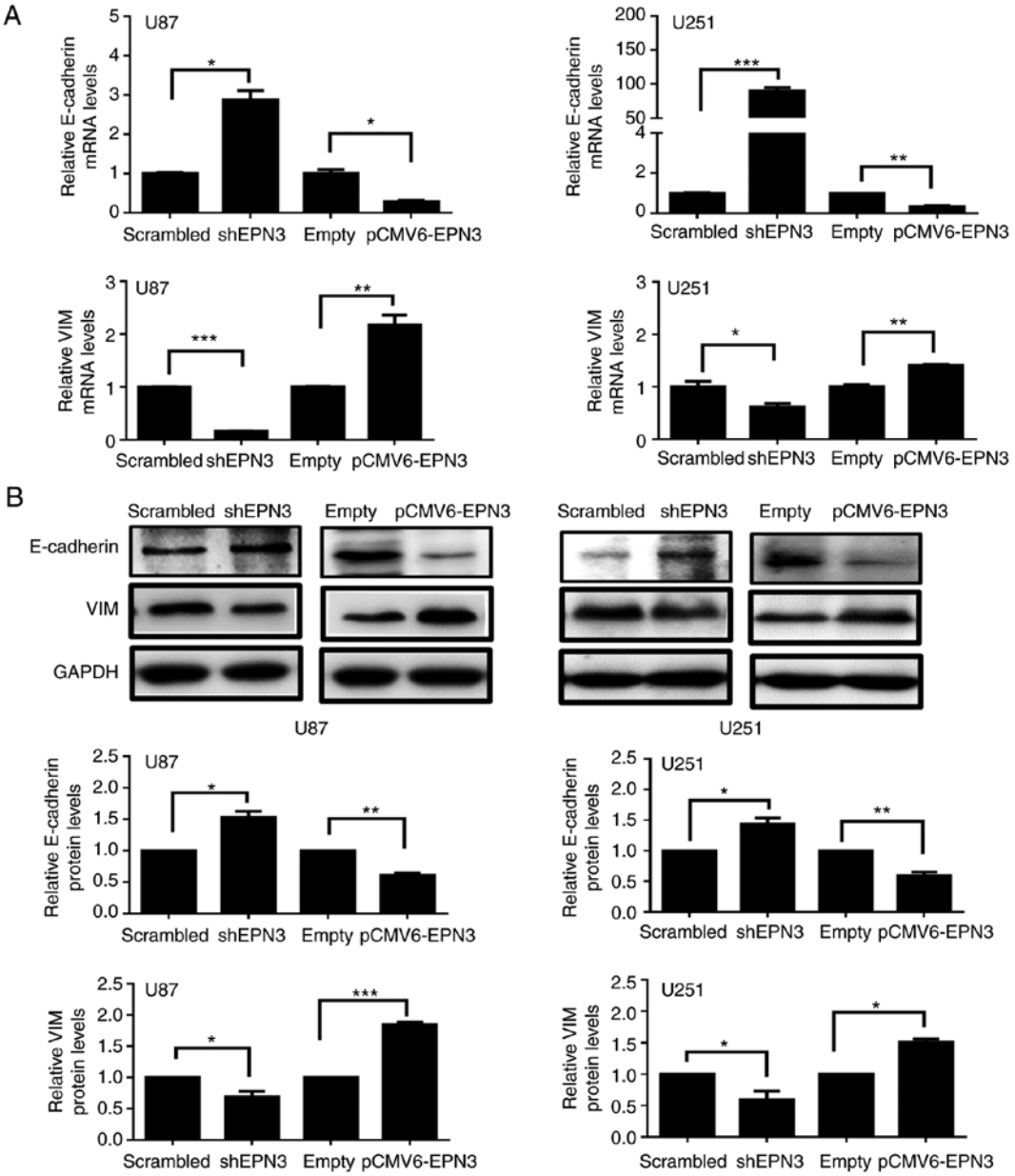

Figure 4. EPN3 overexpression induces epithelial-mesenchymal transition in glioblastoma cells. (A and B) U87 and U251 cells were transfected with shEPN3 or pCMV6-EPN3 overexpression vector, or the respective Scramble-shRNA or Empty vector controls, and the (A) mRNA and (B) protein expression levels E-cadherin and VIM were examined by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively; GAPDH was used as a loading control and to normalize the protein expression data. All experiments were repeated at least three times. Data are presented as the mean ± standard deviation; *P<0.05, **P<0.01 and ***P<0.001. E-cadherin, epithelial cadherin; EPN3, Epsin 3; sh, short hairpin RNA; VIM, vimentin.

transcription of the EMT transcription factors Slug, Twist and ZEB1, rather than Snail-1 and ZEB2, to induce EMT in glioblastoma cells.

Discussion

The Epsin protein family may promote oncogenesis and cancer progression (11); EPN1 and EPN2 have been associated with various types of tumor (39-41), whereas studies regarding EPN3 are limited. A recent study reported that the high expression levels of EPN3 were associated with an increased risk of metastasis in estrogen receptor-positive breast cancer and non-small cell lung cancer (42). Other studies have suggested that EPN3 promotes cellular senescence and is associated with the p53 pathway (43,44). The present study demonstrated that EPN3 was expressed in glioma tissues and glioblastoma cell lines. The results also revealed that the overexpression of EPN3 promoted glioblastoma cell migration and invasion, which indicated that EPN3 may serve as a potential target for glioblastoma therapy.

Relatively little is known about the role of EPN3 in the endosome. Howe and Mobley proposed the signaling endosome hypothesis as a cellular mechanism for distance communication (45); they suggested that activated Tyrosine kinase receptor A and other signaling proteins were internalized into an endocytic organelle, which was then retrograde-transported from the axon tip to the neuron cell body. In addition, Vanlandingham *et al* reported that EPN1

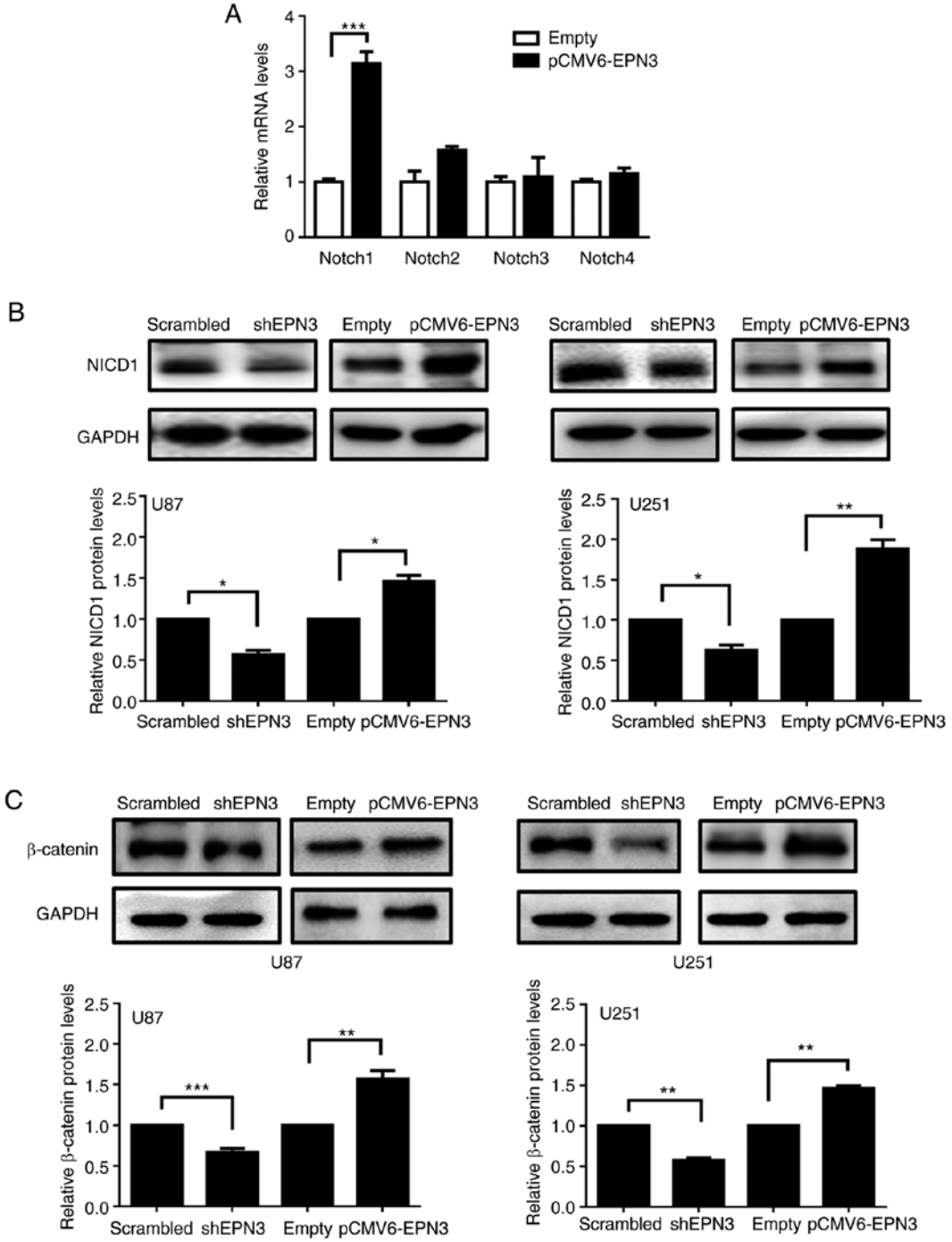

Figure 5. EPN3 overexpression increases the expression levels of Notch1 and β-catenin in glioblastoma cells. (A) Relative mRNA levels of four Notch receptors in U87 cells transfected with pCMV6-EPN3 overexpression vector or Empty vector control were determined by reverse transcription-quantitative polymerase chain reaction. (B and C) U87 and U251 cells were transfected with shEPN3 or pCMV6-EPN3 overexpression vector, or the respective Scramble-shRNA or Empty vector controls, and the protein expression levels of (B) NICD1 and (C) β-catenin were examined by western blotting; GAPDH was used as an internal control and to normalize the protein expression data. Data are presented as the mean ± standard deviation; *P<0.05, **P<0.01 and ***P<0.001. EPN3, Epsin 3; NICD1, Notch1 intracellular domain; sh, short hairpin RNA.

serves an important endosomal role in the efficient retrograde transport of bone morphogenetic protein (BMP) signaling endosomes into motor neuron nuclei, and that EPN1 may negatively regulate BMP signaling at the plasma membrane of the neuromuscular junction, while serving a positive role in nuclear accumulation of phosphorylated Mothers against decapentaplegic (46). These data suggested that EPN3, as an endocytosis adapter protein, may serve different roles in different subcellular localization; however, additional studies to determine these roles are required.

Epsin family proteins are required for Notch pathway activation (9); Notch signaling plays vital roles in glioblastoma cell survival and proliferation (18), migration and invasion (38), radioresistance (21) and EMT (25,26). Similarly, WNT/β-catenin signaling has been reported to be implicated in the proliferation of neural stem cells (47), and the activation

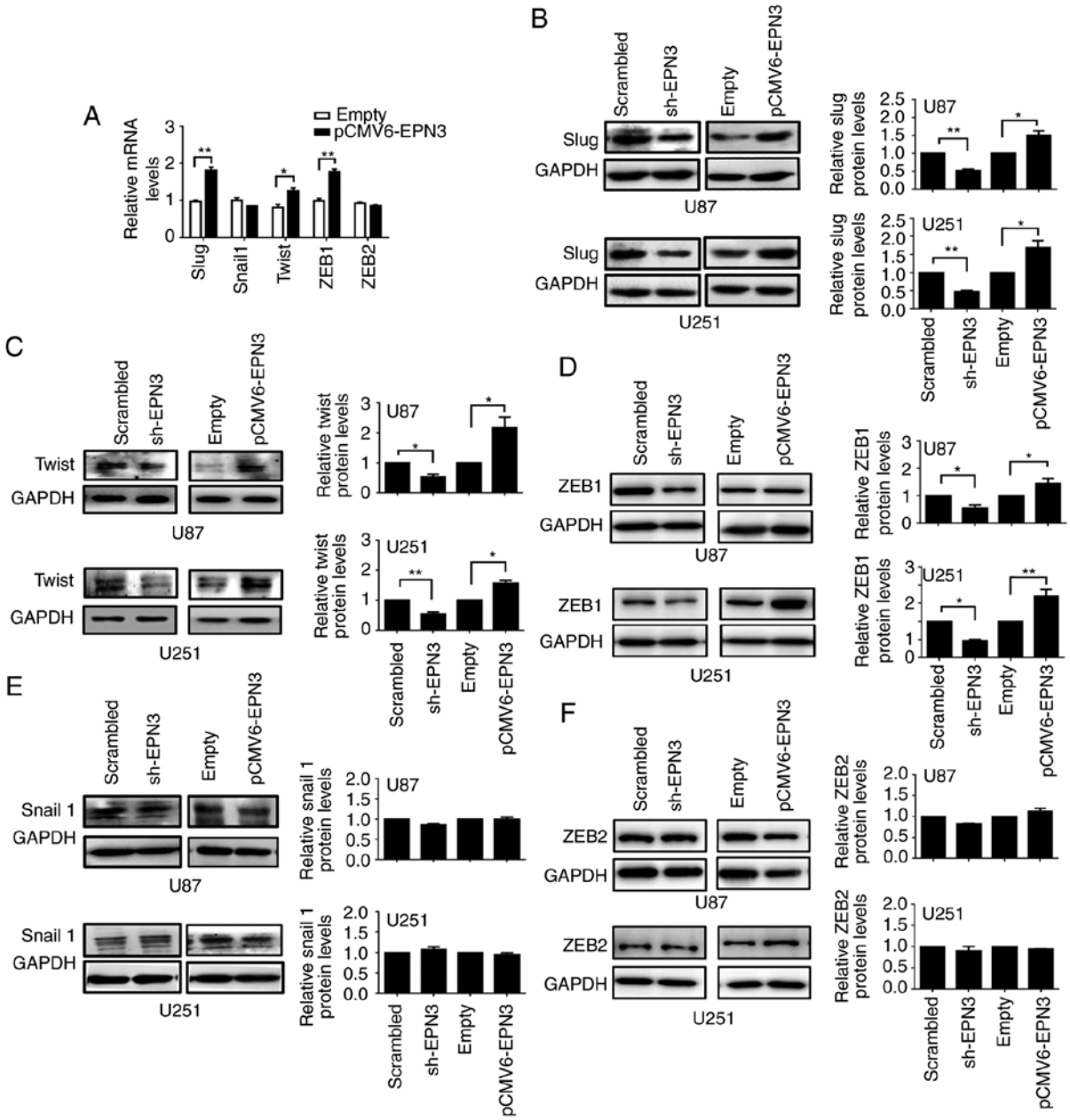

Figure 6. EPN3 overexpression increases the expression of Slug, Twist and ZEB1 in glioblastoma cells. (A) mRNA expression levels of EMT-associated transcription factors were detected by reverse transcription-quantitative polymerase chain reaction in U87 cells transfected with pCMV6-EPN3. (B-F) U87 and U251 cells were transfected with shEPN3 or pCMV6-EPN3 overexpression vector, or the respective Scramble-shRNA or Empty vector controls, and the protein expression levels of (B) Slug, (C) Twist, (D) ZEB1, (E) Snail 1 and (F) ZEB2 were examine by western blotting; GAPDH was used as an internal control and to normalize the protein expression data. Data are presented as the mean ± standard deviation; *P<0.05 and **P<0.01. EPN3, Epsin 3; NICD1, Notch1 intracellular domain; sh, short hairpin RNA; ZEB zinc-finger E-box-binding homeobox.

of canonical WNT/β-catenin signaling enhances the migration and invasion of glioblastoma cells via activating ZEB1 and other activators of EMT (27). Consistently, the results of the present study demonstrated that EPN3 enhanced the migration and invasion of glioblastoma cells, which may have been due to the activation of Notch and WNT/β-catenin signaling. It has been reported that the Notch and WNT pathways interact in breast tumorigenesis (48), and that Notch signaling upregulates the expression of Snail 1 and Slug (49). Slug is important for Notch-mediated EMT as it functions to repress E-cadherin expression, which in turn induces β-catenin activation in human breast cancer (38). However, in colorectal cancer, Notch1 counteracts WNT/β-catenin signaling through chromatin modification (50). Nevertheless, the present study hypothesized that Notch signaling and WNT signaling may act synergistically to promote glioblastoma cell migration and invasion.

The present study results indicated that overexpression of EPN3 may activate EMT in glioblastoma cells and increase the expression of Slug, Twist and ZEB1. In addition, previous studies have reported that E-cadherin may be removed through clathrin-mediated endocytosis, which EPN3 is involved in, and may lead to E-cadherin degradation and the consequent disassembly of adherens junctions (51). Taken together, EPN3

may promote glioblastoma cell EMT, which may be dependent or independent on activating EMT.

In conclusion, the present study results demonstrated the oncogenic role of EPN3 in glioblastoma and its induction of EMT; the activation of Notch and WNT signaling pathways may also have enhanced the migration and invasion of glioblastoma cells induced by EPN3 overexpression. However, there are some limitations in the present study. First, whether the effects of EPN3 on Notch and WNT pathway are related to EPN3-enhanced glioblastoma cells migration and invasion, as well as EMT, remain to be investigated. Second, whether transcription factors are involved in EPN3-induced Notch and WNT pathways also needs further research. However, further investigations are needed to clarify the precise molecular mechanisms of the effects on glioblastoma migration and invasion induced by EPN3 overexpression.

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Availability of data and materials

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

Authors' contributions

YW performed most experiments. WS analyzed the patient data regarding the glioma tissues. PK, CH and ZM drafted the manuscript. XY and QW revised the manuscript critically for important intellectual content; BZ gave final approval of the version to be published. These authors were also involved in the conception of the study. Each author has participated sufficiently in the study to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Informed consent was obtained from all patients, and the use of patient tissue was approved by the Ethics Committee of Tianjin Huanhu Hospital (Tianjin, China; 2016-3).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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