Knockdown of *EPHA1* by CRISPR/CAS9 Promotes Adhesion and Motility of HRT18 Colorectal Carcinoma Cells

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Abstract. *Background: Erythropoietin-producing hepatocellular A1 (EPHA1) is the first member of the EPH superfamily. Its abnormal expression has been reported in various cancer types. However, the contribution of EPHA1 to the regulation of colorectal cancer cell behaviour remains unknown. Materials and Methods: In this study, we investigated the expression profile of EPHA1 in human colorectal cancer and its effect on the adhesion and motility of colorectal cancer cells. We used human colorectal cancer specimens and the colorectal adenocarcinoma cell line HRT18 for this purpose. Results: Our cohort screening data showed that in patients with colorectal cancer, low expression of EPHA1 gene is correlated with a remarkably reduced survival. After EPHA1 is knocked-down in colorectal cancer cells using a clustered regularly interspaced short palindromic repeats–associated nuclease 9 (CRISPR-CAS9) genomic editing system, we observed an increase in the spreading and adhesion of HRT18 cells. Moreover, protein array data indicated that the extracellular-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK) signaling pathways were activated as a consequence. Inhibition of ERK and JNK proteins with specific inhibitors led to suppression of migration of the colorectal cancer cells. Conclusion: EPHA1 suppresses spreading and adhesion of HRT18 colorectal cancer cells through deactivation of ERK and JNK signaling pathways.*

Colorectal cancer (CRC) is the third most common cancer in the UK after breast and lung cancer, accounting for approximately 40,000 new cases each year (1). This tumor type is the second most common cause of cancer-related death in the UK, and after diagnosis, 5-year survival is only about 50% (2). The main reason for this poor survival is the lack of appropriate treatments because the mechanisms that regulate the invasiveness of this tumour type are largely unknown.

The erythropoietin-producing hepatocellular (EPH) receptors represent the largest subfamily of the receptor tyrosine kinases and includes 14 members (3). They are divided into two subfamilies, EPHA and EPHB, according to their extracellular domain sequences and ligand binding (4). EPH receptors are activated in response to binding of their ligands ephrin on adjacent cells and play important roles in multiple biological processes, including axon formation (5), oncogenesis and tumour progression (6).

EPH receptors and ephrin ligands have been reported to be abnormally expressed in many types of human cancer. For example, *EPHA2* is up-regulated in breast, liver, ovarian and colorectal cancer. It has also been shown that high expression of EPHA2 promotes cell motility and it is related to a poor overall survival in patients with colorectal cancer (7). *EPHA3, EPHA4* and *EPHB2* are frequently up-regulated in brain tumors, while *EPHA2* and *EPHB3* are down-regulated in lung cancer (8). Recent data suggest that EPH and ephrin signalling play important roles in tumour development and can be considered as a new potential drug target.

EPH receptor A1 (EPHA1) is the first member of EPH superfamily, originally isolated from an erythropoietin-producing hepatocellular carcinoma cell line (9). Although the abnormal expression of EPHA1 has been described in human cancer, such as colorectal (10), breast (11), prostate (12), and ovarian (13), the function of EPHA1 in tumour development is still uncertain. The up-regulation of *EPHA1* was found to be related to a more aggressive behaviour of cells in ovarian cancer (13). However, its expresion was down-regulated in non-melanoma skin cancer (14) and glioblastoma. Clinical data show that EPHA1 expression varies among patients with colorectal. *EPHA1* has been found to be up-regulated in more than 50% of tumor samples investigated, while its down-regulation was observed in the

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remaining samples (15). Intriguingly, the low expression of EPHA1 was significantly correlated with a poor survival of patients with colorectal cancer (15).

The data above suggest that EPHA1 is involved in carcinogenesis and may play various roles in different types of tumours. However, little is known on the function of EPHA1 in proliferation and cell motility of colorectal cells. In this study, we knocked-down *EPHA1* expression in colorectal adenocarcinoma cell line HRT18 using the clustered regularly interspaced short palindromic repeats–associated nuclease 9 (CRISPR-CAS9) technology to investigate its role in cell proliferation, migration and invasion.

Materials and Methods

Cell culture. Human colorectal adenocarcinoma cell line HRT18 was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (Invitrogen, Paisley, UK) and 1% penicillin/streptomycin solution (Invitrogen). Cells cultures were maintained at 37˚C under a humidified atmosphere with 5% CO_2.

Human colorectal specimens. A total of 165 colorectal samples were collected after surgery and stored at −80˚C for use, with the approval of the Bro Taf Health Authority local Ethics Committee (05/WSE03/92). The samples include 90 colorectal cancer and 75 normal colorectal tissues.

RNA extraction and reverse transcription-polymerase chain reaction (PCR). Total RNA was extracted from cultured cells using TRI reagent (Sigma, Gillingham, UK) according to the manufacturer's instructions, and quantified using an ultraviolet spectrophotometer. Samples were first retro-transcribed using the Super cDNA First-Strand Synthesis Kit and then PCR was carried out using a Ready mix PCR Reaction Mix (Sigma-Aldrich, St Louis, MO, USA) on a 2720 Thermo Cycler (Applied Biosystems, Warrington, UK. The following primers were used: *EPHA1*: forward, 5'-CCTTATGCCA ACTACACCTT-3' and reverse, 5'-GTTCTTCATCCTGGTTCAGC-3'. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): forward, 5'-GGCTGCTTTTAACTCTGGTA-3' and reverse, 5'-ACTGTGG TCATGAGTCCTT-3'. The following cycling conditions were used: 94˚C for 5 min, 30 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s, followed by 72˚C for 10 min. PCR products were separated on a 1% agarose gel and photographed using a VisiDoc-It imaging system (Ultra-Violet Products Ltd., Upland, CA, USA).

CRISPR/CAS9 system targeting human EPHA1 and stable cell line. Human CRISPR/CAS9 guide RNA(gRNA) primers were designed based on the *EPHA1* gene (GenBank accession number: NM_005232). The primer sequences were: *EPHA1* gRNA sense: 5'CACCGCCTGGGGCTGGCCCGCGCGT 3', *EPHA1* gRNA anti-sense: 5'AAACACGCGCGGGCCAGCCCCAG3'. The primer oligonucleotides were annealed using the following parameters: 88˚C for 2 min, 65˚C for 10 min, 37˚C for 10 min and 25˚C for 5 min. The annealing primer was then purified and cloned into the pSpCAS9 (BB)-2A-Puro (PX459) vector (Addgene, Cambridge, MA, USA). The plasmid containing CRISPR/CAS9-EPHA1 was

Table I. *Gene expression of erythropoietin-producing hepatocellular A1 (EPHA1) in a colorectal cancer cohort*.

	Sample size, n	Median copy number of *EPHA1* (IQR)	*p*-Value†
Location			
Left colon	21	0 (0-2789)	
Right colon	27	0 (0-526)	0.01
Transverse colon	2	698*	
Rectum	21	0 (0-225)	0.0408
Tissue			
Normal	75	0.2 (0-8)	
Tumour	90	0 (0-542)	0.002
Differentiation			
Well	2	0.0002*	
Moderate	52	0 (0-1299)	
Poor	14	0 (0-0)	
Dukes' stage			
A	7	0 (0-0)	
B	31	0 (0-1397)	0.2321
C	31	0 (0-222)	0.1935
BC	62	0 (0-681)	0.1914
T stage			
T1	2	0*	
T2	9	0 (0-758)	
T3	38	0 (0-542)	
T4	18	0 (0-1376)	
T2, 3	47	0 (0-526)	
T3, 4	56	0 (0-574)	
T2, 3, 4	65	0 (0-558)	
TNM stage			
1	9	0 (0-758)	
2	28	0 (0-1372)	0.3194
3	25	0 (0-409)	0.2461
4	6	0 (0-0.0556)	0.9397
2, 3	53	0 (0-1126)	0.251
3, 4	31	0 (0-222)	0.3136
2, 3, 4	59	0 (0-590)	0.2866
Node			
0	37	0 (0-1348)	
1	15	0 (0-222)	0.8415
2	15	0 (0-4927)	0.8378
1&2	30	0 (0-223)	0.7964
Invasion			
Non-invasive	47	0 (0-590)	
Invasive	26	0 (0-57)	0.6937
Survival			
Alive	34	0 (0-1040)	
Dead	21	0 (0-1482)	0.7941
Distant metastasis			
Yes	18	0 (0-2613)	
No	48	0 (0-0)	0.1832
Local recurrence			
No recurrence	55	0 (0-227)	
Local recurrence	7	0 (0-19296)	0.4028

IQR: Interquartile range. †Comparison with the first sub-group in each group. *Sub-groups with fewer than three samples were excluded from statistical testing.

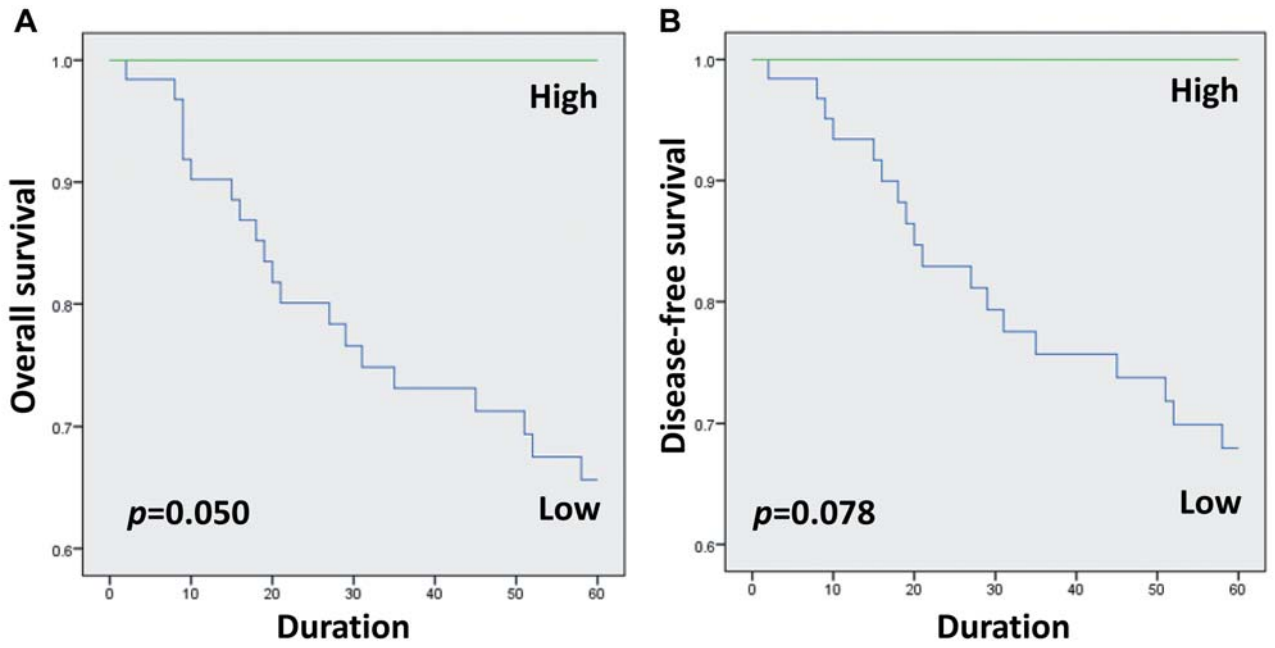

Figure 1. *Kaplan–Meier survival analysis of erythropoietin-producing hepatocellular A1 (EPHA1) expression in colorectal cancer. Using median copy number as a cut-off point, low expression of EPHA1 in primary colorectal tumours appears to correlate with shorter overall survival (A) and disease-free survival (B), although the difference was not significant.*

verified by sequencing analysis. Then CRISPR/CAS9 backbone and CRISPR/CAS9-EPHA1 plasmids were separately transfected into HRT18 cells by electroporation using Gene pulser (Bio-Rad, Hercules, CA, USA). After three weeks of selection with 2 μg/ml puromycin, the verified transfectants were cultured in maintenance medium with 0.5 μg/ml puromycin. Stable cells were named HRT18 CTRL and HRT18 KD, respectively.

Immunofluorescence of EPHA1. Cells were seeded on chamber slides (Nunc™), cultured in an incubator, and fixed in 100% ethanol for 20 min and then washed with balanced salt solution (BSS) buffer. A solution of 0.3% Triton X100 was used to permeabilise cells before staining. Cells were incubated with antibody to EPHA1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. A specific fluorescein isothiocyanate-labelled secondary antibody (Dako, Glostrup, Denmark) was used for 1 h at room temperature. Fluorescent staining was visualized using an Olympus™ BX51 microscope and imaged using a cooled C4742-80 digital camera (Hamamatsu Photonics UK, Welwyn Garden City, UK).

Staining of cells with calcein AM. Cells were stained with calcein AM (Affymetrix eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, cells were seeded on a 24-well plate and incubated with phenol red-free medium with calcein AM (1.2 μl calcein AM/1 ml medium) for 30 min at 37˚C. Cells were observed by immunofluorescence microscopy for their calcein AM-labelled features.

In vitro cell-matrix adhesion. Cells (40,000 cells/ well) were seeded into a 96-well plate pre-coated with Matrigel (50 μl/well; BD Biosciences, Oxford, UK) and incubated at 37˚C for 40 min. Following washing using BSS, cells were fixed with 4% formalin and stained with crystal violet. After capturing images, the adherent cells were dissolved with 10% (v/v) acetic acid, and absorbance was determined.

In vitro invasion and migration assay. A Transwell insert with 8 μm pores was coated with 50 μg of Matrigel for the invasion assay. Non-coated 8 μm pore transwell inserts were used for the migration assay. A total of 40,000 cells were seeded into each insert for migration and invasion assay, respectively. After 72 h incubation, Enzyme-Free Cell Dissociation Solution with calcein AM was added and the transwell inserts were incubated further for 1 h at 37˚C. Fluorescence was determined at a wavelength of 490-510 nm using the GloMax®-Multi Detection System (Promega UK, Southampton, UK).

In vitro wound-healing assay. Cells (40,000) were seeded into a 24-well plate and allowed to reach confluence. The cell monolayer was scraped and photographs were taken at 0.25, 24 and 36 h after wounding. Migration distances were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Electric cell-substrate impedance sensing (ECIS) system-based cell-motility assay. The ECIS system (9600 model; Applied Biophysics Inc., Troy, NY, USA) was used to quantify cell migration. 96WIE arrays were used and cells were seeded at 80,000 cells per well in 200 μl DMEM alone or medium supplemented with 200 nM ERK inhibitor (FR180204) or JNK inhibitor (SP600125) (MERCK, Darmstadt, Germany). After incubation for 20 h, cells were subjected to electric wound, and the multifrequency resistance was recorded for 20 h. The data were analyzed using the ECIS-9600 software package.

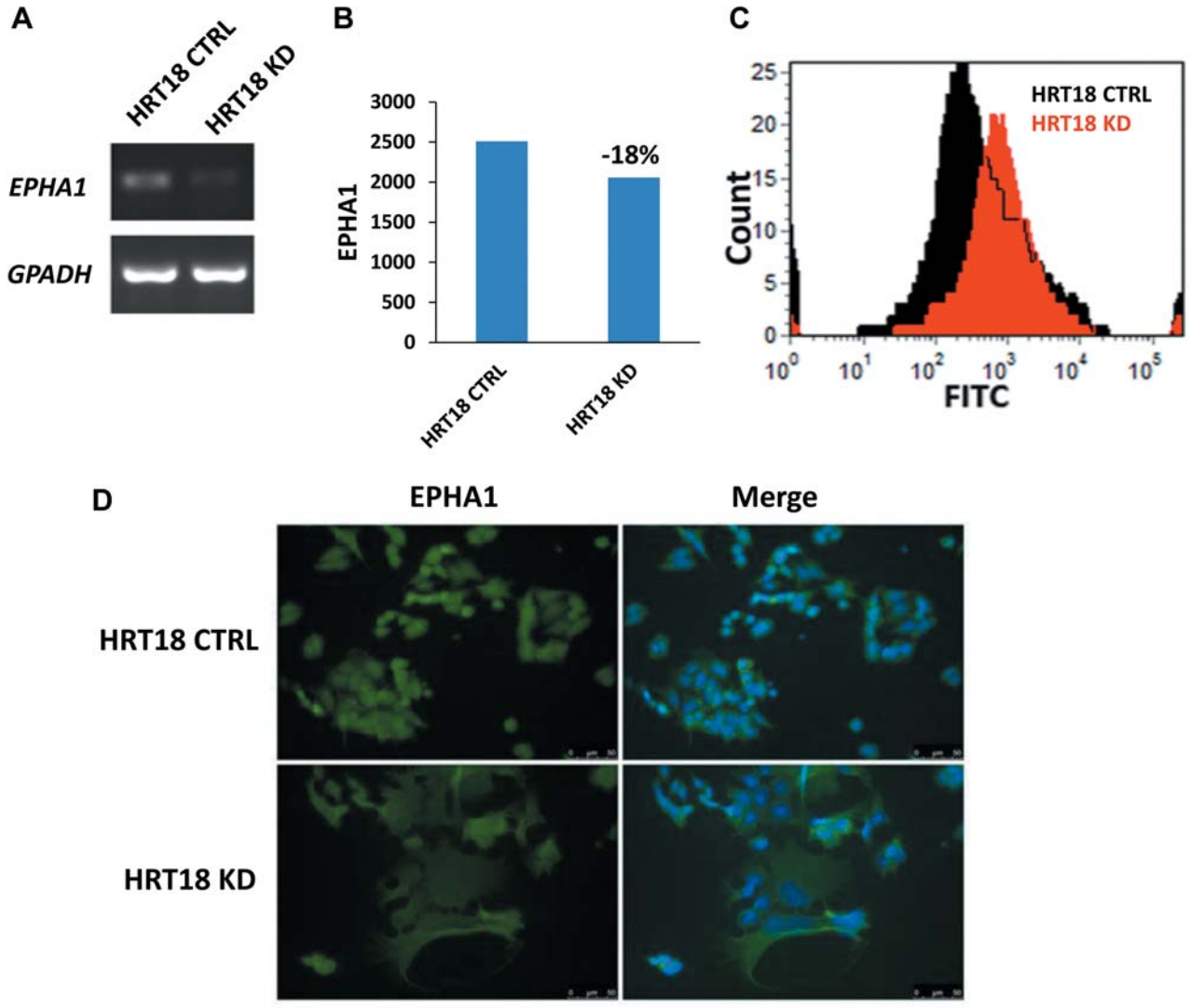

Figure 2. *Knockdown of erythropoietin-producing hepatocellular A1 (EPHA1) in colorectal cancer cells. Knockdown of EPHA1 was seen in HRT18 KD cells using reverse transcriptase polymerase chain reaction compared to empty-plasmid control cells (HRT18 CTRL) (A). Protein expression of EPHA1 was also verified by protein array assay (B), flow cytometric analysis (C) and immunofluorescent staining (D).*

Western blotting analysis. Western blot was performed as previously reported (16). Briefly, whole cell protein was extracted and quantified by NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA, USA) using the BCA protein assay kit. After separation by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were then transferred to a polyvinylidene fluoride membrane (Millipore UK Ltd., Watford, UK). Proteins were probed with primary antibodies anti-ERK or anti-JNK (1:500; Santa Cruz Biotechnology) and incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology). The proteins were detected with Z-ECL chemiluminescence kit (Luminata Forte; Millipore, Hertfordshire, UK) and photographed using G-Box gel documentation system (Syngene, Cambridge, UK). GAPDH was used as an internal control. The intensity values were analysed using Image J software (National Institutes of Health).

Protein array. Protein expression and phosphorylation of selected kinases were assessed using antibody microarray services from Kinexus Bioinformatics Corporation (Vancouver, BC, Canada) which included a panel of approximately 350 pan-specific and 250 phospho-specific antibodies in duplicate.

Statistical analysis. Statistical analysis was performed using SPSS18 (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was used for data which were normally distributed. Overall and disease-free patient survival was analysed using Kaplan–Meier analysis (17). A value of $p<0.05$ was considered statistically significant.

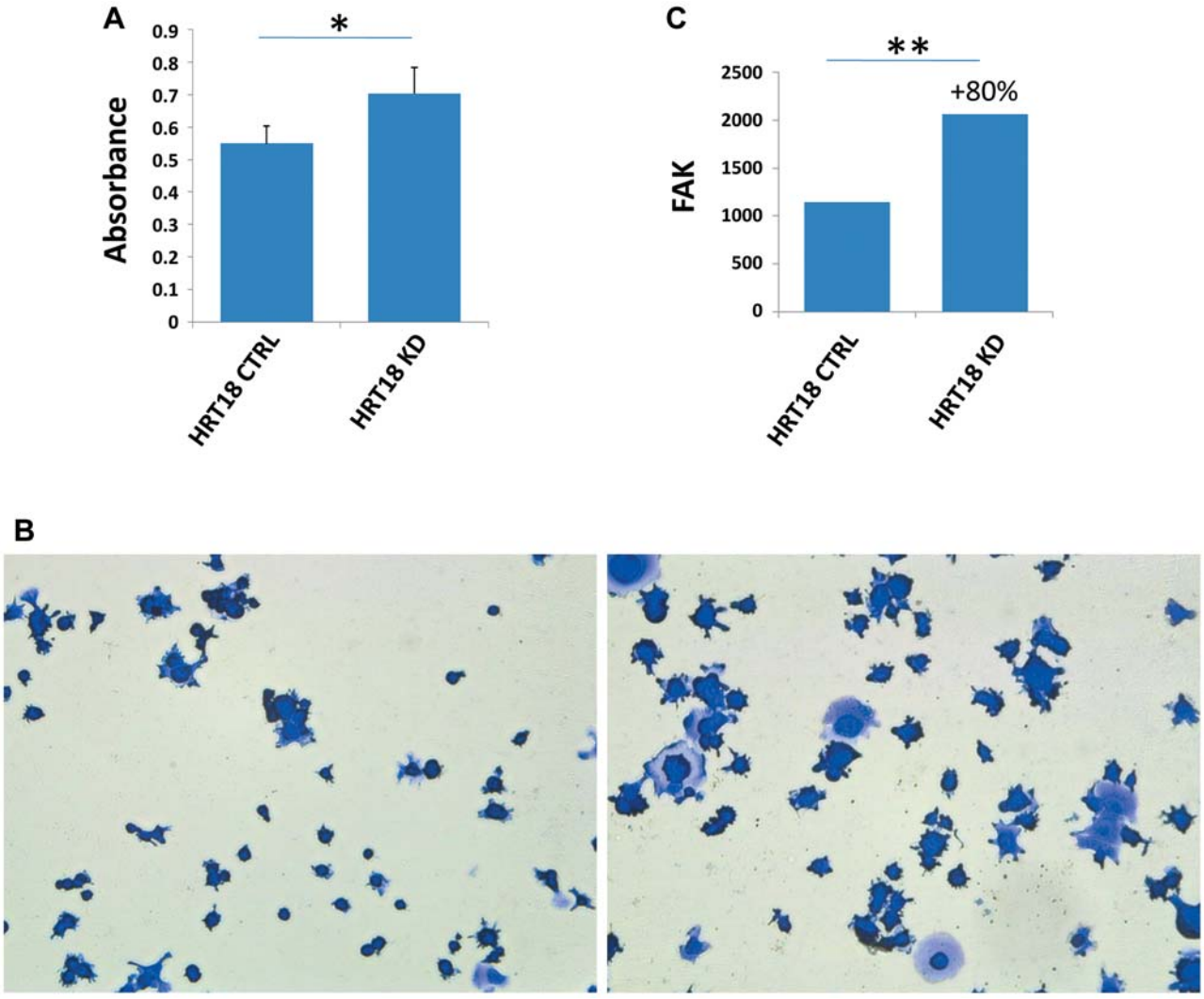

Figure 3. *Effects of erythropoietin-producing hepatocellular A1 (EPHA1) knockdown on cell adhesion of colorectal cells. A: Knockdown of EPHA1 promoted cell-matrix adhesion. B: HRT18 CTRL and EPHA1 knockdown cells (HRT18 KD) stained with crystal violet. C: Focal adhesion kinase (FAK) protein expression was enhanced in HRT18 KD cells compared to control cells by protein array. Data are means±SD. *p<0.05, **p<0.01.*

Results

Expression of EPHA1 in colorectal cancer tissues. The expression of EPHA1 was determined in colorectal cancer cohort tissues using quantitative real-time PCR. The relationship between EPHA1 and clinical pathological data was evaluated (Table I). The number of *EPHA1* transcripts was significantly lower in tumor tissue compared to normal tissue (p=0.02). The Kaplan–Meier survival model was used to analyze the overall and disease-free survival status of patients. It was found that patients with high EPHA1 expression appeared to have longer overall survival and disease-free survival, although the difference was not significant (Figure 1).

Knockdown of EPHA1 in HRT18 colorectal adenocarcinoma cell line. *EPHA1* gene was knocked-down using CRISPR/CAS9 method, targeting human *EPHA1* genomic DNA. The knockdown of *EPHA1* was verified using PCR (Figure 2A) and the result showed that the expression of *EPHA1* significantly decreased in HRT18 KD cells compared to corresponding vector control cells. EPHA1 protein level was reduced as indicated by flow cytometric assay (Figure 2C), protein array (Figure 2B) and immunofluorescence cell staining (Figure 2D). Data from the protein array assay show that EPHA1 protein expression was lower by approximately 18% in HRT18 KD cells compared to HRT18 CTRL cells.

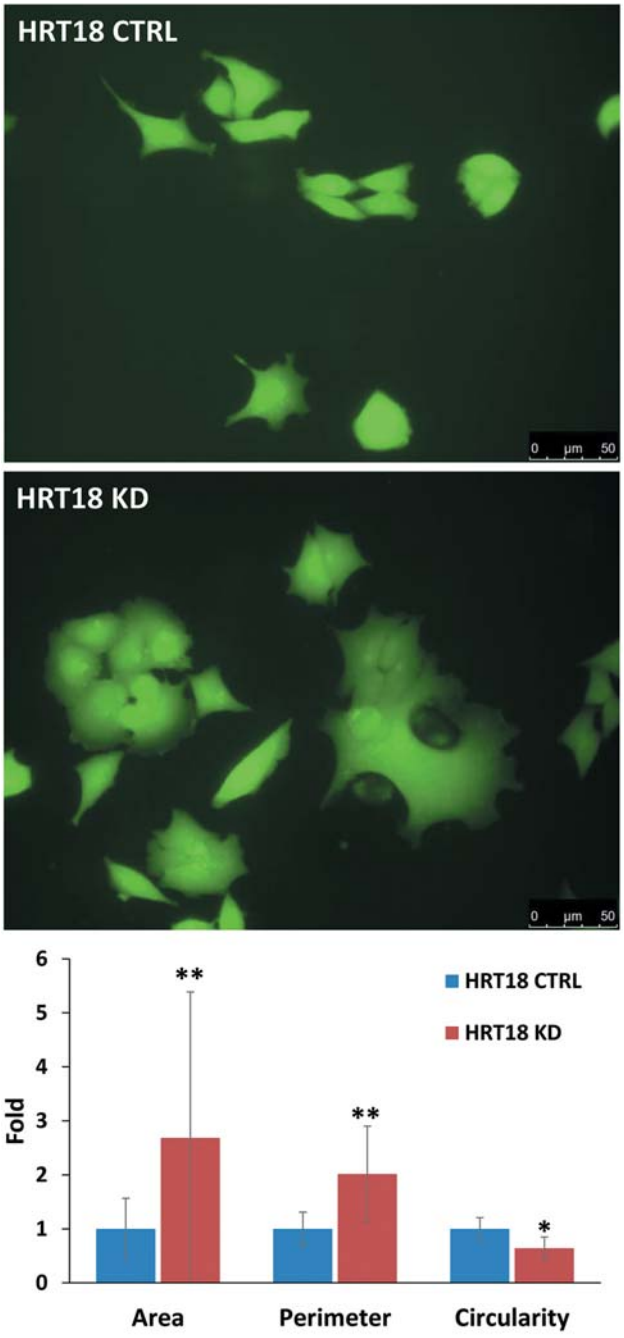

Figure 4. *Effects of erythropoietin-producing hepatocellular A1 (EPHA1) on features of colorectal cells. A: The features of HRT18 cells differed between control (CTRL) and knockdown (KD) cells, as shown by calcein AM staining. B: The area and perimeter of HRT18 KD cells was significantly increased compared to control cells, *p<0.05 and **p<0.01. Data are means±SD.*

Knockdown of EPHA1 by CRISP/CAS9 promotes cell spread, adhesion and motility. The results from immunofluorescence revealed remarkable changes in cell morphology after *EPHA1* knockdown. Most of the HRT18 CTRL cells were round with clear boundaries, while HRT18 KD cells spread well, with more synapse-like structures. To investigate the role of *EPHA1* in the regulation of adhesion and spreading, HRT18 KD and control cells were seeded onto a Matrigel-coated plate. The results showed that the adhesive capability of cells increased when *EPHA1* was down-regulated (Figure 3A). The spread of cells was promoted when EPHA1 was knocked-down in the HRT18 cells compared to control cells (Figure 3B). To evaluate changes of cellular features, cells were stained with calcein AM. The results showed that after EPHA1 knockdown by CRISPR/CAS9, the cells were significantly increased in the perimeter and area and reduced in circle compared with the control cells ($p<0.01$, Figure 4). Focal adhesion kinase (FAK) is a crucial mediator in control of cell adhesion and membrane protrusions. Protein array results showed that expression of FAK protein increased by 80% in HRT18 KD cells compared to HRT18 CTRL cells (Figure 3C).

The effect of EPHA1 on cell invasion and migration was evaluated using transwell and wounding-healing assays. The invasive ability of HRT18 KD cells was increased significantly ($p<0.01$) as was their migration compared to HRT18 CTRL cells.

The role of ERK and JNK in EPHA1-mediated migration of HRT18 cells. The mitogen-activated protein kinase pathway is involved in cell motility. As knockdown of *EPHA1* was shown to affect cell movement, the effect on expression of JNK and ERK in HRT18 cells was analyzed. Western blot results showed a remarkable enhancement in the expression of ERK and JNK in HRT18 KD cells (Figure 6A), that was further confirmed by protein arrays (Figure 6B). These data suggest that JNK and ERK may be involved in the regulation of the movement of HRT18 KD cells. Therefore, HRT18 cells were treated with JNK and ERK inhibitors for 24 h and analyzed for cell migration using ECIS. The HRT18 CTRL cells showed no effect on treatment with JNK and ERK inhibitors, but migration of HRT18 KD cells was dramatically reduced compared with HRT18 CTRL cells ($p<0.01$) (Figure 6C). This suggests that *EPHA1* knockdown may activate some pathways *via* JNK and ERK to promote the motility of colorectal cancer cells.

Discussion

EPH receptors are transmembrane proteins, with the binding site on the extracellular domain. The extracellular domains of two EPH receptors bind two corresponding ligands, forming a complex which mediates communication between two cells (18). It has been reported that EPH receptors play an important roles in cell-substrate adhesion, intercellular junction (19), cell shape and cell differentiation (20).

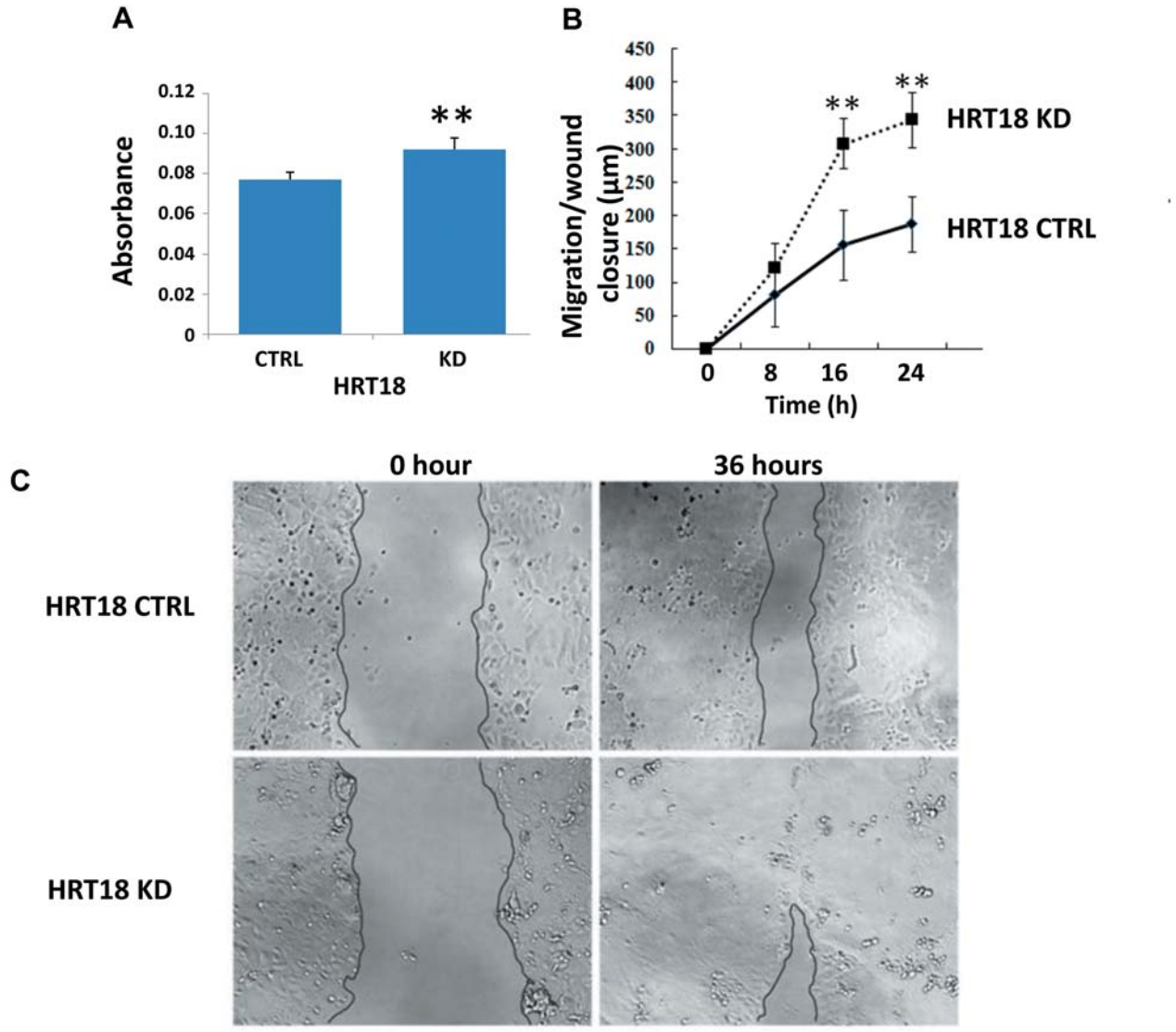

Figure 5. *Effects of erythropoietin-producing hepatocellular A1 (EPHA1) knockdown on invasion and migration of HRT18 colorectal cancer cells. Knockdown of EPHA1 (HRT18 KD) significantly increased cell invasion ability by transwell assay (A) and migration using wounding-healing assay (B, C). **p<0.01. Data are means±SD.*

Previous studies have reported that EPHA1 is involved in cancer development. A high expression of EPHA1 was associated with lymph node metastasis and venous invasion in gastric cancer (21, 22). A low level of EPHA1 was, however, related to a higher invasiveness of breast carcinoma cells (11). Regarding colorectal cancer, EPHA1 expression is very variable. EPHA1 was reported to be down-regulated in 54% but increased in the remaining 46% of colorectal cancer samples in one study (23). Interestingly, EPHA1 expression consistently decreased in all adenoma samples according to the same study. This suggests that EPHA1 may play different roles in the development of different malignancies according to tumor type and stage. However, the function of EPHA1 in cell behaviour of colorectal adenoma is still uncertain.

In this study, the expression of *EPHA1* in a colorectal cancer cohort was analyzed in combination with corresponding clinical and pathological data. Our results showed that a low expression of EPHA1 is related to an advanced stage of the disease and a poor prognosis.

Our study shows that EPHA1 plays a crucial role in colorectal adenocarcinoma cells. Knockdown of *EPHA1* significantly increased the cell-matrix adhesion of HRT18 cells and cell-spreading capability. In particular, there were

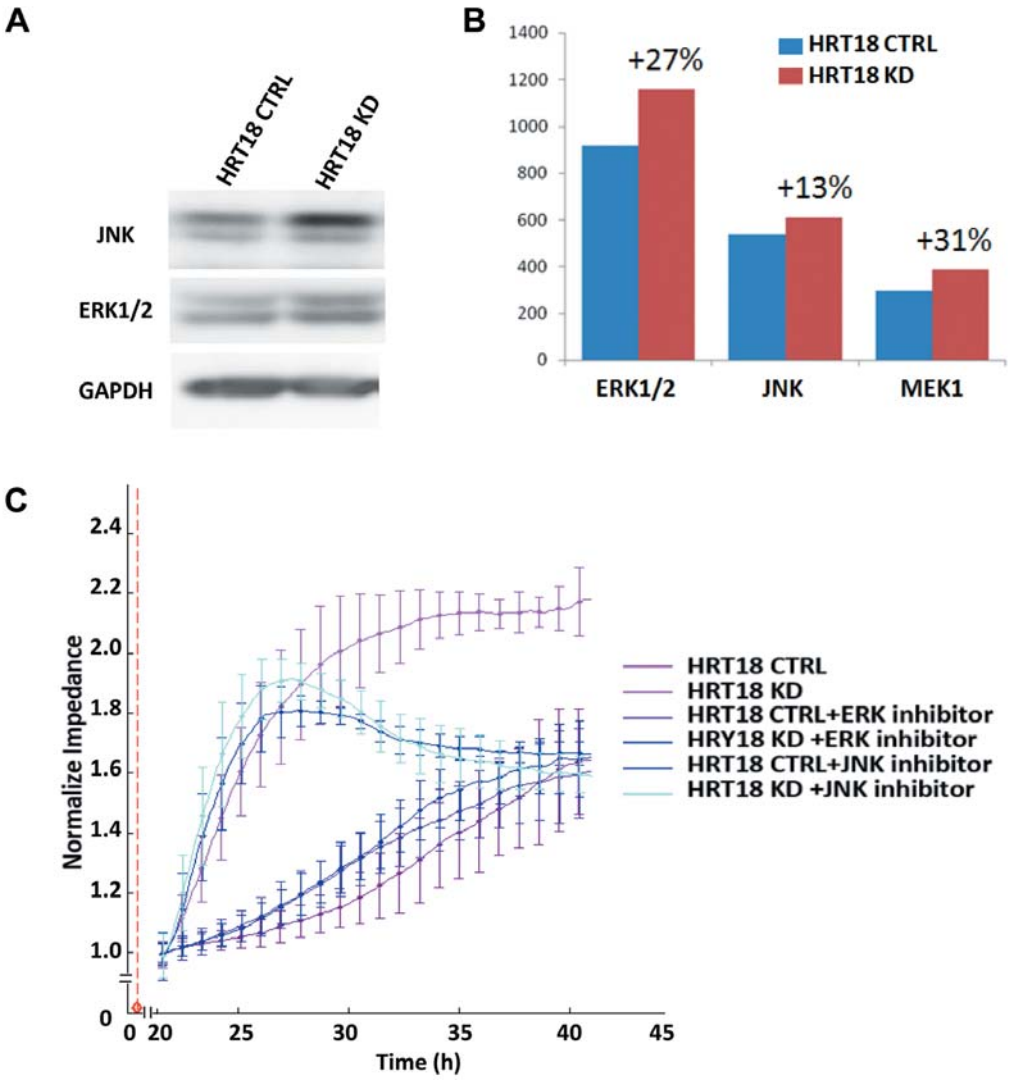

Figure 6. *Knockdown of erythropoietin-producing hepatocellular A1 (EPHA1) in HRT18 cells (HRT18 KD) resulted in increased cell motility via the c-Jun NH2-terminal kinase (JNK) and extracellular-regulated kinase (ERK) pathways. Protein expression of JNK and ERK was enhanced in HRT18 KD cells indicated by western blotting (A) and confirmed by protein arrays (B). Mitogen-activated protein kinase kinase (MEK1) expression was also increased in HRT18 KD cells indicated by protein array. Knockdown of EPHA1 evidently promoted cell migration as indicated by the electric cell–substrate impedance sensing system. Incubation with inhibitors of JNK and ERK significantly diminished such effect (C). **p<0.01. Data are means±SD.*

more spreading cells with extended membrane protrusions when EPHA1 was knocked-down in HRT18 cells compared to the control cells. Moreover, in *EPHA1*-knockdown cells, migration and invasion increased, which were suppressed using JNK and ERK inhibitors. Our data are contrary to a previous study showing that activation of EPHA1 inhibits cell spreading and migration of the HEK2993 colorectal cancer cell line (24). We hypothesize that EPHA1 may have different functions depending on the specific cell type, even for the same cancer type.

Our data and previous reports indicate that EPHA1 receptor may vary according to the tumour sub-type and stage (10). In the early stage of colorectal cancer, EPHA1 expression increases and promotes tumour progression. At later stages however, EPHA1 expression is down-regulated and acts as suppressor of the tumour development (10, 25). Given that colorectal cancer is the most common cancer of the gastrointestinal tract, elucidation of EPHA1 functions in the tumour cell behaviour might be helpful in enhancing our understanding over mechanisms of tumourigenesis.

Conflicts of Interest

The Authors declare no conflicts of interest.

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