Dual roles of protein tyrosine phosphatase kappa in coordinating angiogenesis induced by pro-angiogenic factors

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Abstract. A potential role may be played by receptor-type protein tyrosine phosphatase kappa (PTPRK) in angiogenesis due to its critical function in coordinating intracellular signal transduction from various receptors reliant on tyrosine phosphorylation. In the present study, we investigated the involvement of PTPRK in the cellular functions of vascular endothelial cells (HECV) and its role in angiogenesis using *in vitro* assays and a PTPRK knockdown vascular endothelial cell model. PTPRK knockdown in HECV cells ($HECV^{PTPRKkd}$) resulted in a decrease of cell proliferation and cell-matrix adhesion; however, increased cell spreading and motility were seen. Reduced focal adhesion kinase (FAK) and paxillin protein levels were seen in the PTPRK knockdown cells which may contribute to the inhibitory effect on adhesion. $HECV^{PTPRKkd}$ cells were more responsive to the treatment of fibroblast growth factor (FGF) in their migration compared with the untreated control and cells treated with VEGF. Moreover, elevated c-Src and Akt1 were seen in the PTPRK knockdown cells. The FGF-promoted cell migration was remarkably suppressed by an addition of PLCγ inhibitor compared with other small inhibitors. Knockdown of PTPRK suppressed the ability of HECV cells to form tubules and also impaired the tubule formation that was induced by FGF and conditioned medium of cancer cells. Taken together, it suggests that PTPRK plays dual roles in coordinating angiogenesis. It plays a positive role in cell proliferation, adhesion and tubule formation, but suppresses cell migration, in particular, the FGF-promoted migration. PTPRK bears potential to be targeted for the prevention of tumour associated angiogenesis.

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Introduction

Vasculogenesis/angiogenesis is an essential process for embryonic and postnatal development, wound healing and also endometrial angiogenesis during the menstrual cycle in women. Angiogenesis is also vital for the growth and dissemination of solid tumours (1,2). Tumour-associated angiogenesis is pivotal for a solid tumour to grow beyond a certain size (2-3 mm) as it can be restricted by interspatial diffusion of nutrients. The newly formed vasculature also provides a pathway for cancer cells to spread to other parts of the body. Angiogenesis is affected by multiple factors and various cells in the tumour microenvironment (3).

Angiogenesis can be induced or promoted by pro-angiogenic factors, such as VEGF, FGF, TNF-α and HGF which can be produced by cancer cells, stromal cells, inflammatory cells and endothelial cells (4-7). For example, VEGF binds to two tyrosine kinase receptors, VEGFR1 and VEGFR2. VEGFR1 is expressed by hematopoietic and vascular endothelial cells and acts as a negative regulator of angiogenesis, whilst VEGFR2 is mainly expressed by vascular endothelial cells and is crucial for vasculogenesis leading to the formation of primary vascular plexus (8-10). Tie-2 receptor and its ligands, angiopoietins, are also important for angiogenesis. Angiopoietin-2 has been demonstrated as a VEGF negative regulator in several cancers (11,12). In addition to their pro-angiogenic effect, FGF and HGF can also directly promote proliferation, migration and invasion of cancer cells (13,14).

Protein tyrosine phosphatases (PTPs) are involved in regulation of cellular functions by coordinating signal transduction through dephosphorylation of certain signalling molecules. PTPs have also been indicated as important regulators in tumorigenesis and angiogenesis (15-18). Receptor-like protein tyrosine phosphatase beta (PTPRB, also known as vascular endothelial VE-PTP), for example, plays a crucial role in the angiogenesis of breast cancer by regulating several signalling pathways such as the Tie-2 pathway (19). Blocking PTPRB with a small inhibitor, AKB-9778, reduced tumour growth and metastases of breast cancer (20). Furthermore, Src homology 2 domain-containing protein tyrosine phosphatase-1 (SHP-1) suppresses angiogenesis by inhibiting the VEGF signalling in microvascular endothelial cells (21).

To date, aberrant expression of receptor-like protein tyrosine phosphatase kappa (PTPRK) has been observed

Table I. Primer sequences used in the present study.

Gene	Forward primers (5'-3')	Reverse primer (5'-3')
GAPDH	GGCTGCTTTTAACTCTGGTA	GACTGTGGTCATGAGTCCTT
GAPDH (Q-PCR)	CTGAGTACGTCGTGGAGTC	ACTGAACCTGACCGTACACAGAGATGACCCTTTTG
PTPRK	AATTACAATTGATGGGGAGA	CCACTTTTCCACCTGAAGTA
PTPRK (Q-PCR)	AATTACAATTGATGGGGAGA	ACTGAACCTGACCGTACATATTGTGTGACGATGAAAGC

in glioma, lymphoma, prostate and breast cancer (22-26). However, the role played by PTPRK in angiogenesis remains largely unknown. The present study aimed to investigate the role played by this molecule in angiogenesis, in particular VEGF and FGF-promoted angiogenesis.

Materials and methods

Cell lines and cells culture. HECV (human endothelial vascular cell line) cells were purchased from Interlab (Naples, Italy); PANC-1, MSA-MB-231 and MRC-5 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and HT115 cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC; Salisbury, UK). Cells were routinely cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and antibiotics at 37˚C with 5% CO_2. PCR primers were designed using Primer-3 and synthesised by Sigma-Aldrich (Dorset, UK) and the sequences are provided in Table I.

Reverse transcription-PCR. Total RNA extraction from cells was performed using Tri reagent (Sigma-Aldrich). Following reverse transcription, PCR was carried out using GoTaq DNA olymerase (Promega, Southampton, UK). Reactions were carried out with the following process: 94˚C for 5 min, 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec, followed by a final extension of 7 min at 72˚C. PCR products were separated on a 1.5% agarose gel and photographed after staining with SYBR Safe DNA dye (Invitrogen, Paisley, UK).

Real-time quantitative PCR. The level of PTPRK transcripts in HECV cells was determined using a real-time quantitative PCR, based on a previously reported method (27). The reaction was carried out on an iCycler iQ™ (Bio-Rad Laboratories, Hempstead, UK) which is equipped with an optical unit that allows real-time detection of 96 reactions. The reaction conditions were: 94˚C for 12 min, 100 cycles of 94˚C for 15 sec, 55˚C for 35 sec (the data capture step) and 72˚C for 15 sec. The levels of the transcripts were generated from an internal standard that was simultaneously amplified.

Construction of ribozyme transgene targeting human PTPRK and the establishment of corresponding stable transfectants. Anti-human PTPRK hammerhead ribozymes were designed based on the secondary structure of the gene transcript and generated using the Zuker RNA Mfold program (28). The ribozymes were synthesized and then cloned into a pEF6/V5-His TOPO vector (Invitrogen). The verified ribozyme transgenes and empty plasmids were transfected into HECV ($HECV^{PTPRKkd}$ and $HECV^{pEF}$) cells, respectively using an Easyjet Plus electroporator (Equibio, Kent, UK). After a period of selection with 5 μg/ml blasticidin (up to 10 days), the verified transfectants were cultured in maintenance medium containing 0.5 μg/ml blasticidin. Primer sequences of the ribozymes were 5'-CTGCAGTTTGCTCTTTTTTACAATT AATATCTGATGAGTCCGTGAGGA-3' and 5'-ACTAGTTC ATCCTCCTTCTCCTAGTTGTTTCGTCCTCACGGACT-3'.

In vitro cell growth assay. HECV cells (3,000 cells/well) were plated into two identical 96-well plates. Cells were fixed in 4% formalin after 24 and 72 h of culture. The cells were then stained with 0.5% (w/v) crystal violet. Following washing, stained crystal violet was extracted with 10% (v/v) acetic acid (29). Absorbance was then determined at a wavelength of 540 nm using an ELx800 spectrophotometer (BioTek Instruments, Inc., Swindon, UK). Growth rate of day 3 (%) = absorbance of day 3/absorbance of day 1 x 100.

In vitro cell-matrix adhesion. Cells (20,000/well) were seeded to each well of a 96-well plate which was pre-coated with Matrigel (5 μg/well) (BD Biosciences, Oxford, UK). After 40 min of incubation, the non-adherent cells were washed off using balanced salt solution (BSS; comprising 137 mM NaCl, 2.6 mM KCl, 1.7 mM Na_2HPO_4 and 8.0 mM KH_2PO_4 and adjusting the pH to 7.4 with 1 M NaOH). The remaining cells were fixed with formalin and were stained with crystal violet. The number of adherent cells was then counted under a microscope.

In vitro migration/wounding assay. Cells (200,000/well) were seeded into a 24-well plate and allowed to reach confluence. The cell monolayer was scratched using a fine gauge needle to create an artificial wound of ~200 μm in width (30). Images were taken at 0.25, 1, 2, 3 and 4 h after wounding. Migration distances were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell spreading assay. Cells (20,000/well) were seeded into a 96-well plate which was pre-coated with Matrigel (5 μg/well) (BD Biosciences) and the cells were fixed after an incubation of up to 4 h. The fixed cells were stained with fluorescein phalloidin (F432; Life Technologies, Carlsbad, CA, USA) and DAPI (10236276001; Roche Applied Science, Basel, Switzerland). Images were taken using a Leica fluorescence

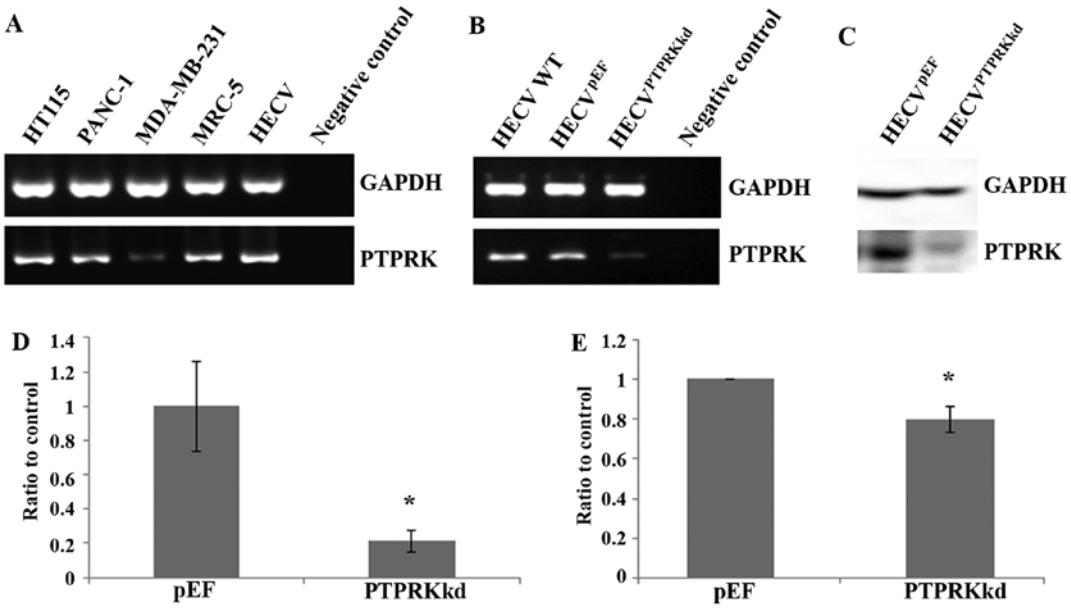

Figure 1. PTPRK gene expression and knockdown of PTPRK in HECV cells. (A) Expression of PTPRK mRNA in different cell lines (HT115, colon cancer cell; PANC-1, pancreatic cancer cell; MDA-MB-231, breast cancer cell; MRC-5, lung fibroblast cell; HECV, endothelial cell). Knockdown of PTPRK was seen in $HECV^{PTPRKkd}$ cells compared with empty plasmid control ($HECV^{pEF}$ cells) using RT-PCR (B), western blot analysis (C) and real-time quantitative PCR (D). (E) PTPRK protein band volume of three repeats which is normalised against corresponding internal control. The intensity shown is integrated band intensity (intensity x area) and was normalised against the corresponding GAPDH signal. *P<0.05.

microscope (Leica DM IL LED). Cell spreading was measured using ImageJ software (National Institutes of Health).

Tubule formation assay. Cells (40,000/well) were seeded into a 96-well plate which was pre-coated with Matrigel (500 μg/well). The cells were fixed with formalin after a 4-h incubation and photographed immediately using a microscope. The sum of tubule perimeter was measured using ImageJ software.

Electric cell-substrate impedance sensing (ECIS). An ECIS 9600 model instrument and 96W1E arrays (Applied Biophysics, Inc., Troy, NY, USA) was also used for migration assays in the study, as previously reported (31). $HECV^{pEF}$ and $HECV^{PTPRKkd}$ cells were seeded at 40,000 cells/well in 200 μl DMEM medium alone or supplemented with 10 ng/ml FGF (F0291; Sigma-Aldrich) or 10 ng/ml VEGF (293-VE; R&D Systems, Abingdon, UK), with small inhibitors; 5 μM Akt inhibitor (Akt124005; Millipore UK, Ltd., Watford, UK), 50 nM c-Src inhibitor Src I1 (Tocris Bioscience, Bristol, UK), 5 nM PI3K inhibitor (wortmannin; Tocris Bioscience) and 5 nM PLCg inhibitor (STK870702; Vitas-M Laboratory, Ltd., Apeldoorn, The Netherlands), respectively. The resistance was measured at 30 kHZ for 5 h after electrical wounding, and data was analysed using an ECIS-9600 software package.

Western blot analysis. Equal amounts of protein were separated using SDS-PAGE and blotted onto nitrocellulose membranes (SC-3724; Santa Cruz Biotechnology, Heidelberg, Germany). Proteins were then probed with the primary antibodies and corresponding peroxidase-conjugated secondary antibodies. Protein bands were visualised using a chemiluminescence detection kit (Luminata; Millipore) and photographed using UVItec imager (Uvitec Printing Ink Co., Inc., Lodi, NJ, USA). Antibodies for GAPDH (sc-32233), PTPRK (sc-28906), c-Src (sc-5266), PLCγ (sc-81) and Akt1 (sc-1618) were purchased from Santa Cruz Biotechnology. Antibodies for FAK and Paxillin were obtained from BD Biosciences.

Statistical analysis. Statistical analysis was performed using SigmaPlot 11 (SPSS, Inc., Chicago, IL, USA). Data were calculated as the mean ± SD, the Student's t-test was used for normally distributed data and one-way ANOVA was used for multiple group comparison. Each assay was performed three times. P<0.05 was considered statistically significant.

Results

Knockdown of PTPRK in vascular endothelial cells using anti-PTPRK ribozyme. The expression of PTPRK in the vascular endothelial cell line HECV, was first determined using conventional PCR with a comparison to its expression in several cancer cell lines including colorectal cancer (HT115), pancreatic cancer (PANC-1) and breast cancer (MDA-MB-231) and also a fibroblast cell line (MRC-5) (Fig. 1A). All these cell lines express PTPRK though levels had subtle variations.

The expression of PTPRK was knocked down using ribozyme transgenes targeting human PTPRK mRNA. Reduced mRNA expression of PTPRK was seen in the cells which were transfected with anti-PTPRK ribozyme transgenes using both conventional PCR (Fig. 1B) and real-time quantitative PCR (Fig. 1D). The knockdown of PTPRK was further confirmed with western blot analysis for its protein expression (Fig. 1C and E).

Effect of PTPRK knockdown on the proliferation and cell-matrix adhesion of endothelial vascular cells. After verification of the knockdown of PTPRK, we also examined its influence

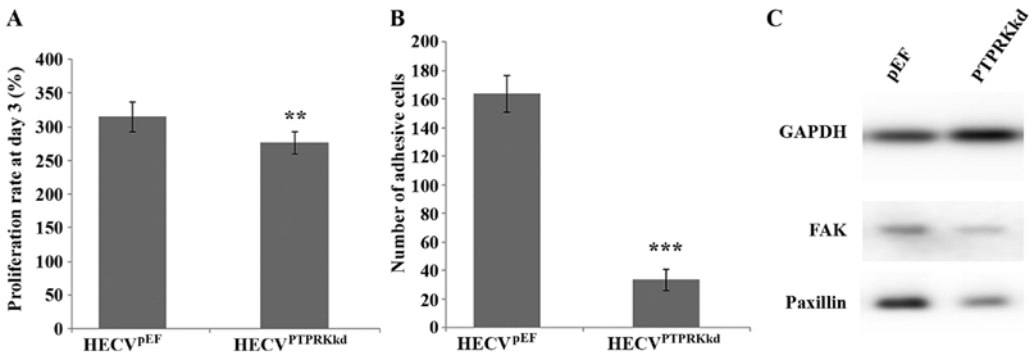

Figure 2. The effects of PTPRK knockdown on cell adhesion of HECV cells. (A) Knockdown of PTPRK reduced cell-matrix adhesion in HECV cells. **P<0.01. (B) Reduced expression of PTPRK resulted a decrease in cell-matrix adhesion in HECV cells. ***P<0.001. (C) FAK and paxillin protein expression levels were decreased in the PTPRK knockdown HECV cells.

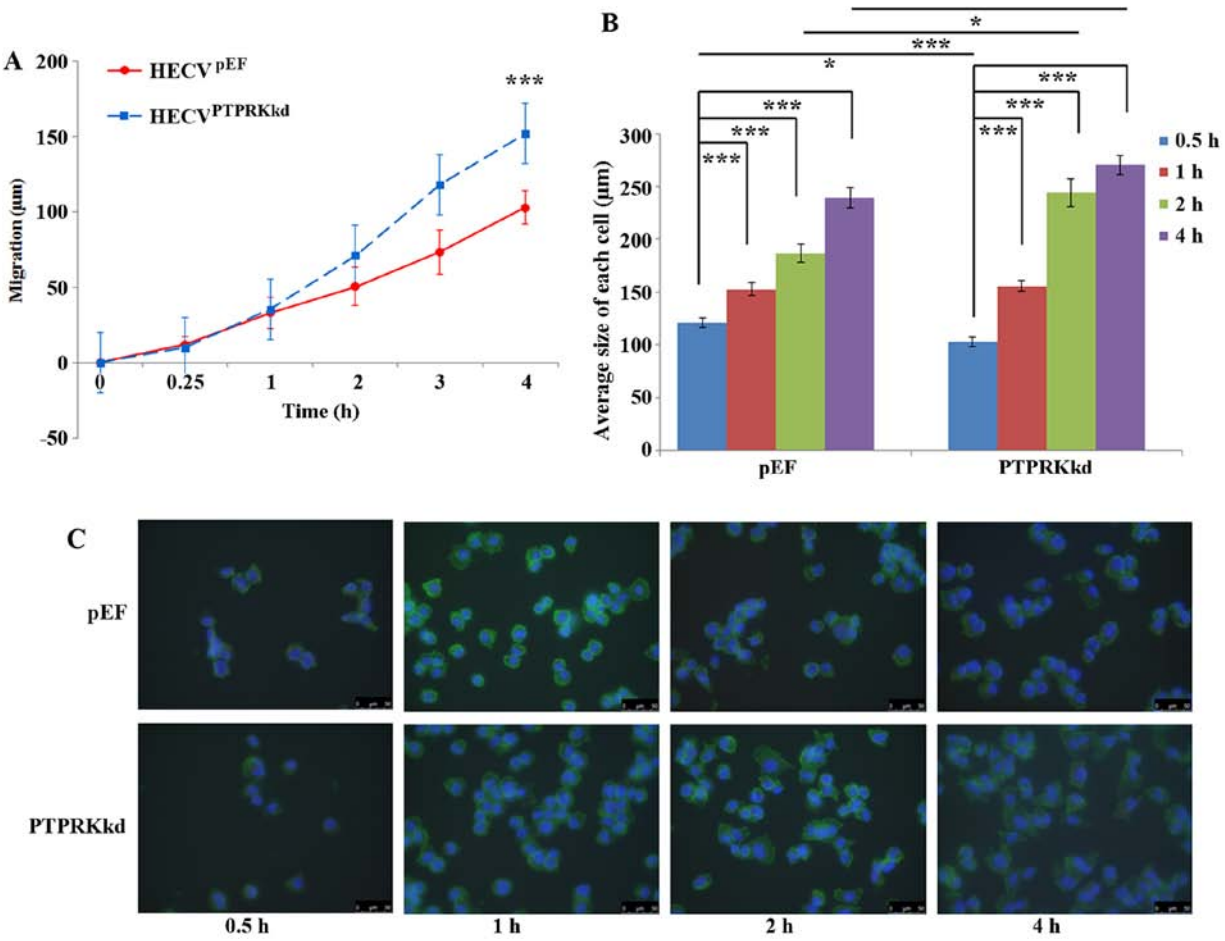

Figure 3. The effects of PTPRK knockdown on cell migration and spreading of HECV cells. (A) Knockdown of PTPRK in HECV cells increased cell motility. (B) Bar graph showed average size of HECV cells at different time points (0.5, 1, 2 and 4 h). (C) Immunofluorescent of phalloidin in HECV cells at different time points. *P<0.05 and ***P<0.001.

on cell proliferation and cell-matrix adhesion. Knockdown of PTPRK significantly reduced proliferation of the vascular endothelial cells compared to the control cells (276.27±16.37 vs. 314.77±21.93%; P<0.01) (Fig. 2A). PTPRK knockdown elicited a significant influence on cell matrix adhesion. The number of adhered HECV^{PTPRKkd} cells (33.33±7.27) was much less than the HECV^{pEF} cells (163.50±12.96), P<0.001 (Fig. 2B). Furthermore, knockdown of PTPRK resulted in a reduced expression of both FAK and paxillin proteins (Fig. 2C).

PTPRK and the migration and spreading of endothelial vascular cells. PTPRK knockdown cells exhibited increased cell motility compared with the control cells. After a 4-h incubation, the distance that HECV^{PTPRKkd} cells migrated was 152.02±18.98 μm compare with that of HECV^{pEF} cells (102.91±11.26 μm), P<0.001 (Fig. 3A). Cell spreading assays showed a very interesting result. After a 30-min incubation, the size of HECV^{pEF} cells were found to be larger than HECV^{PTPRKkd} (120.98±4.66 vs. 102.82±4.25 μm; P<0.05)

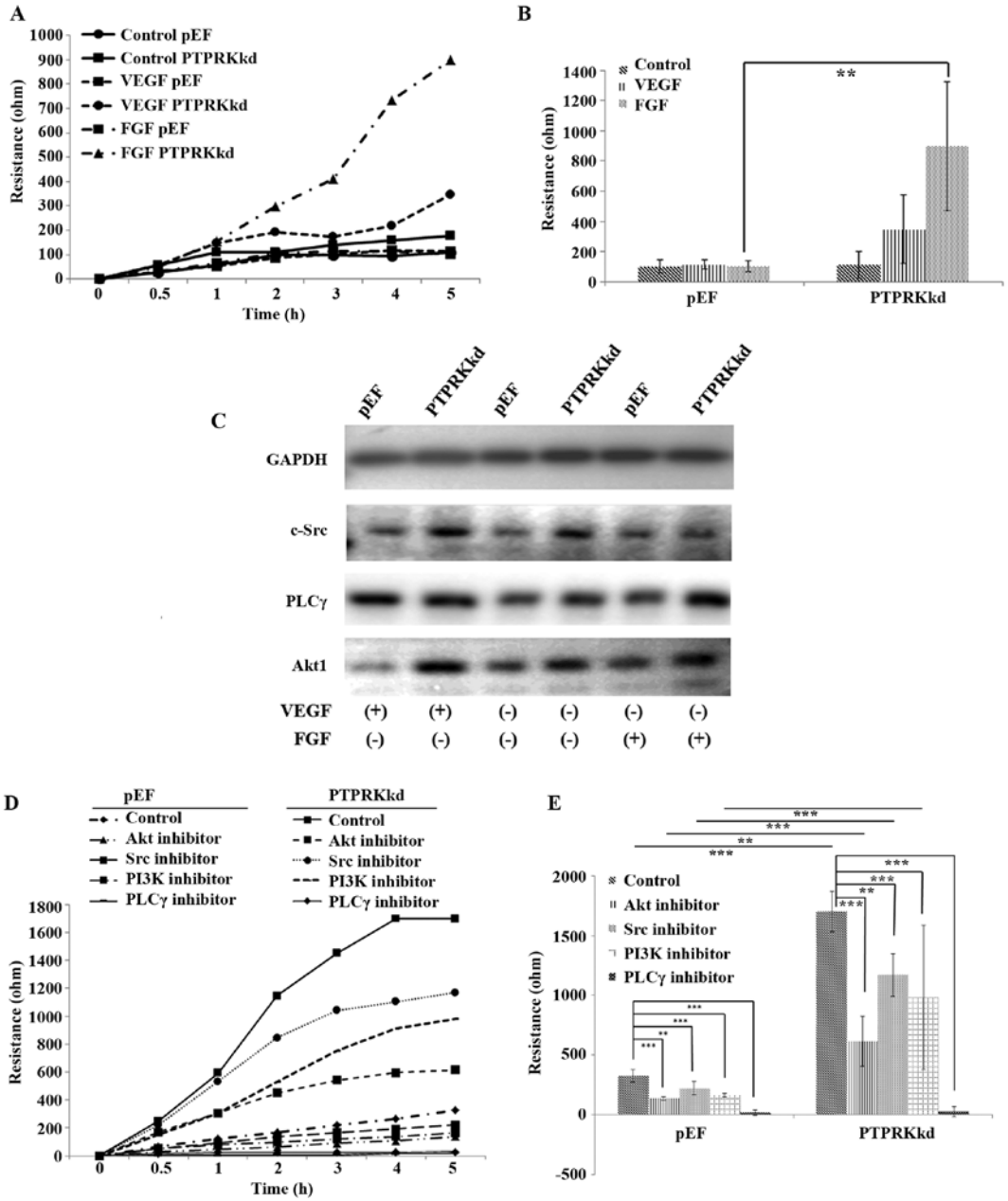

Figure 4. The FGF signalling pathway participates in regulating cell motility of PTPRK knockdown cells. (A) Incubation of HECV^{PTPRKkd} cells with rhFGF promoted cell motility significantly. (B) The overall changes of resistance on the fifth hour with statistical analysis. (C) Alteration of c-Src, PLCγ and Akt1 proteins in PTPRK knockdown HECV cells and their responses to a 2-h treatment of FGF and VEGF, respectively. (D) Involvement of PI3K, Src, Akt1 and PLCγ in the FGF-promoted migration of the HECV cells with PTPRK knockdown. We used the ECIS to determine cell migration of HECV cells with additions of small inhibitors targeting these molecules. The curves are average resistance of each group over a period up to 5 h. (E) The overall changes of resistance on the fifth hour with statistical analysis. Both HECV^{pEF} and HECV^{PTPRKkd} cells were treated with FGF alone or in combination with Akt1 inhibitor (5 μM, Akt124005; Calbiochem), c-Src inhibitor (50 nM, Src I1; Tocris Bioscience) and PI3K inhibitor (5 nM wortmannin; Tocris Bioscience), respectively. **P<0.01 and ***P<0.001.

which appeared to be in line with the inhibitory effect on cell-matrix adhesion. Such an effect was diminished after 1-h incubation. However, an increased spreading was seen in the HECV^{PTPRKkd} cells after 2-h incubation, their size being 244.20±12.93 ImageJ units, ~30% bigger than the size of HECV^{pEF} cells (186.76±8.29 ImageJ units; P<0.001). A similar influence on the spreading was also observed after an incubation of 4 h (Fig. 3B and C).

PTPRK in VEGF and FGF induced migration of vascular endothelial cells. VEGF and FGF pathways play crucial roles for several cell functions including vasculogenesis and angiogenesis (8,32). To explore the involvement of PTPRK in VEGF and FGF induced angiogenesis, we determined the migration of HECV cells with additions of VEGF and FGF, respectively. Little effect on cell migration was seen in the HECV^{pEF} cells exposed to VEGF (10 ng/ml) and FGF (10 ng/ml), respectively. However, the knockdown of PTPRK conferred an increased sensitivity to these two pro-angiogenic factors. HECV^{PTPRKkd} cells were more responsive to the treatment of both VEGF and FGF. A more marked increase was seen in FGF treated HECV^{PTPRKkd} cells (Fig. 4A and B). We then examined the

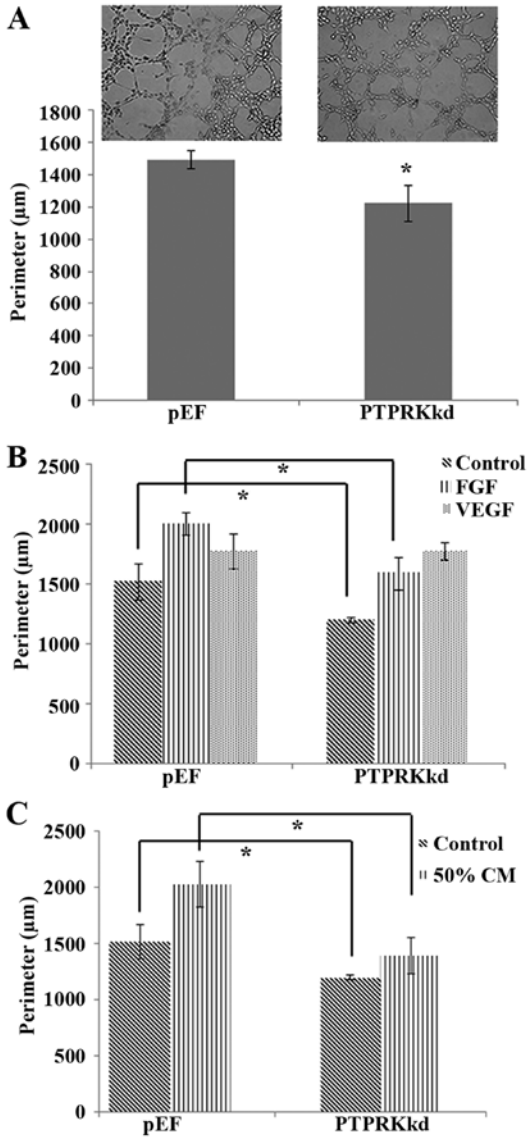

Figure 5. The effects of PTPRK knockdown on cell proliferation and tubule formation of HECV cells. (A) Knockdown of PTPRK decreased the growth rate of HECV cells. (B) Sum of tubule perimeters with different treatments. (C) PTPRK knockdown impaired the tubule formation induced by conditioned medium collected from cancer cells (MDA-MB-231). Tubule formation images were captured with phase-contrast microscopy. *P<0.05.

expression of three key molecules, c-Src, PLCγ and Akt1 which mediate signalling downstream of the respective receptors of VEGF and FGF. To our surprise, an elevated protein level was seen for Akt1 and c-Src in the HECV^{PTPRKkd} cells, which was not observed for the PLCγ. An enhanced expression of Akt1 was seen in the PTPRK knockdown cells when they were exposed to the VEGF, while FGF treated cells exhibited similar levels of Akt1 protein compared with the untreated controls. A similar pattern and expression levels of c-Src were seen in the cells treated with VEGF compared with the untreated controls, while the FGF-treated HECV^{PTPRKkd} cells had a reduced expression of c-Src which was down to a similar level of the HECV^{pEF} cells. VEGF increased the protein level of PLCγ in both HECV^{PTPRKkd} and HECV^{pEF} cells. In contrast to the VEGF-elevated expression of PLCγ, an increased protein expression of PLCγ was only seen in the PTPRK knockdown cells when they were treated with FGF (Fig. 4C). Since an increased response to FGF was seen in the migration of PTPRK knockdown cells, we treated cells with FGF with additions of small inhibitors targeting c-Src, PLCγ, Akt and also PI3K to verify their involvement in the FGF-promoted cell motility. Four inhibitors suppressed the cell migration which were promoted by FGF, however, only the PLCγ inhibitor repressed the migration of HECV^{PTPRKkd} cells to a level similar to the control cells (Fig. 4D and E).

Involvement of PTPRK in pro-angiogenic factor and cancer cell induced tubule formation of vascular endothelial cells. An *in vitro* tubule formation assay was used to assess the influence of PTPRK knockdown on the capability of vascular endothelial cells to form new vasculature. Knockdown of PTPRK resulted in a decrease of proliferation and cell-matrix adhesion, a similar inhibitory effect was also seen in the tubule formation (Fig. 5A), though the motility of endothelial cells was enhanced after the PTPRK knockdown. We then investigated the proangiogenic factor, in particular the VEGF and FGF-induced angiogenesis. The reduced tubule formation in the PTPRK knockdown cells was diminished by an exposure to VEGF (10 ng/ml) and the PTPRK knockdown cells appeared to be more responsive to VEGF compared with the HECV^{pEF} cells but not to a significant level. However, an increased tubule formation was seen in both HECV^{PTPRKkd} and HECV^{pEF} cells which were treated with FGF (10 ng/ml) (1588.92±134.61 vs. 2002.02±96.39 μm; P<0.05). However, the PTPRK knockdown-inhibited tubule formation still existed when the cells were treated with FGF (Fig. 5B).

To mimic the tumour associated angiogenesis, we treated the endothelial cells with medium collected from breast cancer cells (MDA-MB-231). We aimed to clarify the involvement of PTPRK in cancer cell-induced angiogenesis. Cells incubated with 50% medium collected from breast cancer showed similar result with FGF treated cells, it promoted both tubule formation of HECV^{PTPRKkd} and HECV^{pEF} cells but the PTPRK knockdown-inhibited tubule formation was still present (1392.87±157.59 vs. 2029.87±204.46 μm; P<0.01) (Fig. 5C).

Discussion

Our previous studies have shown that the expression of PTPRK is reduced in the breast cancer which is associated with poor prognosis of the disease (26). Knockdown of PTPRK in breast cancer cells promoted their proliferation, adhesion, invasion and migration. In contrast to the reduced expression of PTPRK observed in the breast cancer, an increased PTPRK expression was seen in prostate cancer (25). An inhibitory effect on cellular functions was also seen in prostate cancer cells following PTPRK knockdown. It suggests that PTPRK plays different roles in different cancers which can be cancer specific. It has been indicated that some PTPs participate in the regulation of angiogenesis. For example, SHP-1 (PTPN6) has been identified as an anti-angiogenic regulator via VEGFR2 signalling pathway (21,33). VE-PTP (PTPRB) plays an important role in angiogenesis by targeting the VEGFR2 (34,35) and Tie2 pathways (36-39). However, to date, little is known about the role of PTPRK in tumour-associated angiogenesis.

In the present study, we first confirmed expression of PTPRK in a vascular endothelial cell lines (HECV). PTPRK

was found to be extensively expressed by a variety of different cell lines, including vascular endothelial cells (HECV), colorectal cancer (HT115), pancreatic cancer (PANC-1) and fibroblasts (MRC-5). We then employed the anti-PTPRK ribozyme plasmid constructs to establish a cell model for the present study. Knockdown of PTPRK expression in the HECV cells resulted in a decreased proliferation and cell-matrix adhesion. This is similar to the inhibitory effect observed in the prostate cancer cells with knockdown of PTPRK (25). In contrast, PTPRK knockdown promoted migration of the endothelial cells which was similar to the effect seen in breast cancer cells (26). Such contrasting effects on different cellular functions of vascular endothelial cells suggest that PTPRK elicits more complex functions by interacting with different molecules.

Focal adhesion kinase (FAK)-paxillin signalling pathway regulates many cellular functions such as cell survival and migration; it has also been indicated that PTPs are involved in this mechanism. For example, PTP-PEST interacts with paxillin and Grb2 which are key players in the focal adhesion complex (40). DEP-1 (PTPRJ) inhibits cell proliferation, formation of vinculin and paxillin-containing adhesion plaques and also the activation of FAK (41). DEP-1 can interact with c-Src and promotes cell adhesion through an activation of FAK and paxillin (42). In the present study, knockdown of PTPRK in human endothelia cells reduced cell-matrix adhesion. To examine the involvement of FAK and paxillin in the inhibitory effect on cell adhesion of HECV cells, we investigated the protein expression using western blot analysis. The results showed that protein levels of both FAK and paxillin were decreased in the PTPRK knockdown cells. The reduced FAK and paxillin protein levels are in line with the inhibitory effect on cell-matrix adhesion. It suggests that PTPRK may play a role by either directly mediating the adhesion and/or stabilising focal adhesion complex which includes FAK and paxillin. However, the exact machinery operated by PTPRK in regulation of cell adhesion requires further investigation.

Moreover, knockdown of PTPRK promoted cell motility in endothelial cells and cell spreading assay showed us that the average cell size of PTPRK knockdown cells were smaller than control cell after 30-min incubation. This is consistent with the inhibitory effect of PTPRK knockdown on cell adhesion and also the reduced FAK and paxillin proteins. The cellular spreading of HECV cells following the initial adhesion was enhanced by the PTPRK knockdown due to its effect on cell migration. Furthermore, fibronectin induced endothelial cell migration was regulated by Src-dependent phosphorylation of FGFR1 (43). Fibroblast induced cell-contact-dependent colorectal cancer cell migration and invasion via regulation of the FGF2-FGFRs-Src-αvβ5 pathway (44). In migratory glioma-tropic neural stem cells, promotion of VEGFR2 expression resulted in activation of VEGFR pathway downstream molecules such as PLCγ, FAK and Akt (45). Our results showed that c-Src and Akt were increased at their protein levels after the knockdown of PTPRK. PLCγ appeared to be more involved in the FGF induced effect. Along with c-Src, PLCγ and Akt, we also included PI3K which is a key molecule in mediating signalling for FGF in our experiment with small inhibitors. We treated the HECV cells with FGF and small inhibitors targeting c-Src, PLCγ, Akt and PI3K. The addition of small inhibitors showed that all four molecules play important roles in cell migration and were involved in the PTPRK knockdown promoted cell migration to various levels, in which PLCγ tended to be vital for the migration. However, a more comprehensive method is required to determine PTPRK-regulated protein phosphorylation and signal transduction, for example, the Kinex™ antibody microarray (Kinexus Bioinformatics Corp., Vancouver, Canada).

Studies have shown that certain PTPs contribute to the inhibitory effect on cell proliferation, such as PTPN3 in cholangiocarcinoma, PTPRM and PTPRK in breast cancer (26,46,47). However, studies also showed that same PTPs may confer a favour to the proliferation and inhibition of PTPRM in glioblastoma multiforme cells has been shown to result in decreased cell growth and survival (48). Furthermore, our previous studies have also shown that knockdown of PTPRK in prostate cancer cells reduces cell proliferation due to promotion of apoptosis via JNK pathway (25). In the present study, we tried to investigate which pathway was involved in the regulation of cell growth in human endothelial cells. Little effect was seen in the proliferation of HECV with addition of small inhibitors targeting Src, Akt and also PI3K which is another key player downstream of the respective receptors of VEGF and FGF (data not shown). The mechanism underlying the inhibitor effect of proliferation is yet to be elucidated.

A further *in vitro* tubule formation test showed promotion of tubule formation triggered by the knockdown of PTPRK, which could be the predominant effect of PTRPK knockdown on angiogenesis unless it is further validated by *ex vivo* and *in vivo* evidence. It has been reported that FGF and VEGF pathways participate in the regulation of many cell function such as cell motility and angiogenesis (49,50). Reduction of PTP1B expression increased VEGF-induced migration and proliferation of mouse heart microvascular endothelial cells and FGF-induced proliferation of rat aortic smooth muscle cells (51). SHP-2 was shown to positively regulate endothelial cell motility and angiogenesis *in vitro* and *in vivo* (52). To elucidate the involvement of PTPRK in the pro-angiogenic factors-induced angiogenesis and also the tumour-associated angiogenesis, we treated the HECV cells with VEGF, FGF and also the conditioned medium from breast cancer cell lines. The PTPRK knockdown HECV cells were more responsive to the FGF in their migration suggesting a key role played by PTPRK in suppression of FGF-induced cell migration. In the tubule formation, PTPRK knockdown did not suppress the VEGF-induced tubule formation though it exhibited inhibition on the tubule formation of the untreated cells. In contrast, PTPRK knockdown cells tended to be less responsive to the FGF treatment. Moreover, the PTPRK knockdown cells were less responsive in their tubule formation by an exposure to the conditioned medium from breast cancer cells. It suggests that PTPRK bears inhibitory effect on the tubule formation by suppressing pathways triggered by FGF and cancer cells. Therefore, PTPRK may play a positive role in coordinating cancer cell induced angiogenesis. Further investigation of targeting soluble factors, such as VEGF and FGF released from cancer cells using neutralizing antibodies will help to expand the current understanding of cancer cell-regulated angiogenesis which may help to develop a novel anti-angiogenic strategy.

In conclusion, PTPRK knockdown exhibited diverse effects on different cellular functions of vascular endothelial cells; inhibitory effect on cell proliferation, adhesion and tubule formation, but a positive effect on cell migration. A positive correlation in the expression between PTPRK and focal adhesion complex (FAK and paxillin) contributes to the cell adhesion. Reduced PTPRK expression enhanced FGF-induced migration, but elicited inhibitory effects on the tubule formation that was promoted by FGF and cancer cells. PTPRK tends to be less involved in the VEGF-induced tubule formation. It suggests that PTPRK plays diverse roles in coordinating angiogenesis which can be more specific to certain pro-angiogenic factors.

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