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Original Article

ShRNA-mediated silencing of the PI3K p85α gene suppresses colorectal tumor cell migration and invasion

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Abstract: Phosphatidylinositol 3-kinase (PI3K) plays an important role in cell growth, proliferation and motility. The regulatory subunit, p85α, is the most abundantly expressed regulatory isoform of PI3K and is essential for the activation of PI3K. PI3K p85α is often over-expressed in colorectal cancer (CRC) cells, but its role in the migration and invasion of CRC remains unclear. In this study, lentivirus-mediated PI3K p85α-specific shRNA was used to knock down PI3K p85α expression in CRC cells. Cell wound healing and transwell assays were then performed to examine the effect of PI3K p85α on cell migration and invasion, respectively. Furthermore, the expression of cellular proliferation and migration proteins was assessed in PI3K p85α knockdown CRC cells. Finally, in vivo metastasis assays were performed to identify whether the knockdown of PI3K p85α suppressed spleen and liver metastases in nude mice. The results revealed effective inhibition of the migration and invasion of PI3K p85α knockdown CRC cells, partially by regulating cell adhesion and migration-related proteins such as MMP-2, MMP-9 and COX-2. Moreover, knockdown of PI3K p85α significantly suppressed tumor metastasis in nude mice. Our findings show that PI3K p85α plays an important role in the migration and invasion of CRC. More importantly, further investigations suggest that the PI3K pathway may regulate CRC development by modulating cellular proteins related to extracellular matrix formation and cell adhesion. These results improve our understanding of the detailed mechanism of the PI3K pathway in the regulation of tumor metastasis.

Keywords: Colorectal cancer (CRC), phosphatidylinositol 3-kinase (PI3K), cell migration, cell invasion, matrix metalloproteinase

Introduction

Colorectal cancer (CRC) is the third most common malignant tumor worldwide, accounting for 8% of all cancer-related mortalities [1, 2]. Conventional chemotherapy for CRC provides a promising approach to reduce the incidence and improve the prognosis of CRC; however, its clinical application is limited by the efficacy and toxicity of the chemotherapy treatment [1, 3]. Cancer cell metastasis and invasion are considered the important characteristics of systemic cancer morbidity and mortality [4]. Therefore, a better understanding of the molecular basis related to the interactions between CRC and the host would facilitate the development of new therapeutic targets.

Phosphatidylinositol 3-kinase (PI3K), an intracellular signal transducer, comprises a large and complex family that includes 3 classes with multiple subunits and isoforms [5, 6]. The classI PI3Ks have been extensively studied. They are heterodimers consisting of a number of shared regulatory subunits (p85α, p85β or p55 γ) and a unique catalytic subunit (p110α, p110β or p110δ) [5, 6]. PI3K is known to be involved in a wide range of cellular processes, including cell growth, proliferation, motility and intracellular trafficking [5, 6]. Recent studies have shown that an increase in PI3K activity is associated with the development of colorectal, bladder, and ovarian cancer [7-9], suggesting that PI3K may function as a potential oncogene in these cancers.

The regulatory subunit, p85α, the most abundantly expressed regulatory isoform of PI3K, is essential for the stability of the p110 catalytic subunit and for its recruitment to activated

growth factor receptors [10]. PI3K p85α forms a complex with a protein network associated with oncogenic fusion tyrosine kinases, resulting in constitutive activation of the p110 catalytic subunit and subsequently the PI3K pathway [10]. Therefore, p85α has been identified as one of most important cancer antigens for CRC. However, our understanding of p85α regulation in the development of CRC remains limited.

RNAi is becoming one of the most widely used gene silencing technologies because of its high sequence specificity for target genes [11, 12]. Methods to apply the RNAi effect include siRNA, shRNA, and miRNA. shRNA, an effective means of RNAi that provides effective silencing longevity, a variety of delivery options and low cost, is a promising technique for the precise knockdown of gene expression to achieve a therapeutic effect [13, 14]. The suppression of a particular gene by RNAi to explore its role in CRC oncogenesis has been reported [15, 16], and this technology has opened new avenues in CRC therapeutics. In this study, we constructed PI3K p85α knockdown CRC cells using an RNA interference (RNAi)-based lentivirus, and investigated proteins related to the PI3K pathway. The effect of PI3K knockdown on tumor progression in vivo was also examined. The aim of this study was to elucidate the molecular mechanism responsible for the metastasis of CRC.

Materials and methods

Ethical standards

All animal experiments were conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines and the EU Directive 2010/63/EU for animal experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of The Third Affiliated Hospital of Guangzhou Medical University.

Cell culture

The human CRC cell lines LoVo and SW480 were grown in RPMI-1640 medium (GIBCO, Scotland, UK) supplemented with 10% fetal bovine serum (FBS) (GIBCO) at 37°C with 5% CO_2 in a humidified atmosphere.

Western blot analysis

Cells were harvested and washed with phosphate-buffered saline (PBS) and then resuspended and lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% Triton X-100, and 0.5% deoxycholate, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) for 15 min at 4°C. After quantification and boiling in SDS electrophoresis buffer, the protein samples were electrophoresed in a 10% SDS-PAGE gel and transferred to a polyvinylidine difluoride (PVDF) membrane (Millipore). The membrane was blocked for 1 h in Tris-buffered saline with 0.05% Tween 20 (TBST) containing 5% (W/V) nonfat milk and incubated with the indicated primary antibody overnight at 4°C followed by secondary antibody for 1 h at room temperature. The membrane was then washed 3 times for 10 min each with TBST and incubated with peroxidase-conjugated goat anti-mouse IgG (1:5000; Amersham Pharmacia Biotech, USA) for 1 h. After 3 washes with TBST, the membranes were visualized using an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech, USA). The following primary antibodies were used: antibodies against PI3K p85α (1:1000, Santa Cruz Biotechnology, USA), matrix metalloproteinase-2 (MMP-2) (1:200, Santa Cruz Biotechnology, USA), MMP-9 (1:500, Santa Cruz Biotechnology, USA), cyclooxygenase-2 (COX-2) (1:500, Santa Cruz Biotechnology, USA), and GAPDH (1:1000, Santa Cruz Biotechnology, USA).

Construction of PI3K p85α shRNA lentiviruses

The lentiviral vector LV-008 (Forevergen Biosciences, China) was used to express a short hairpin RNA (shRNA) containing the GFP gene as a reporter. The recombinant lentiviruses were designed to generate a shRNA targeting the PI3K p85α gene (5'-GCUCAGUAUAAUCCCAAAUUG-3') and a negative control (5'-TGGTTTACATGTCGACTAA-3'). Briefly, LV-008-shp85α plasmids and packaging vectors were cotransfected into HEK293 T cells to generate the respective lentivirus. The supernatant containing the lentiviruses was collected at 72 h post-transfection, and then the lentiviruses were concentrated by ultracentrifugation for 1.5 h at 25,000 rpm in a Beckman Instruments (Fullerton, CA, USA) SW28 rotor and resuspended in phosphate-buffered saline (PBS). For lentivirus infection, LoVo or SW480 cells was cultured in 6-well plates and infected with lentivirus in the presence of 5-10 μg/ml of Polybrene. To identify PI3K p85α-knockdown cells, transfected

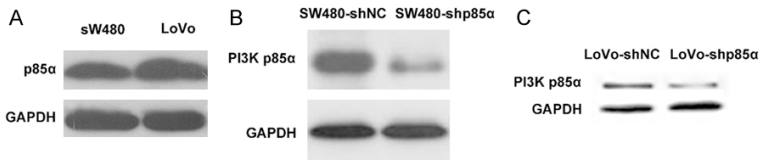

Figure 1. A. PI3K p85α was over-expressed in CRC cells. Western blot analysis was performed to evaluate PI3K p85α protein expression levels in the CRC cell lines. GAPDH was used as a loading control. B, C. Knockdown of PI3K p85α using lentivirus-mediated shRNA in CRC cell lines. The recombinant lentiviruses contained the short shRNA targeting the specific sequence of the PI3K p85α gene or NC shRNA were constructed as described in Material and methods. LoVo or SW480 cells were infected with the lentiviruses, and PI3K p85α knockdown cells were screened using puromycin. Western blot analysis was performed to examine the accumulation of PI3K p85α protein in SW480 and LoVo cells. GAPDH was used as a loading control.

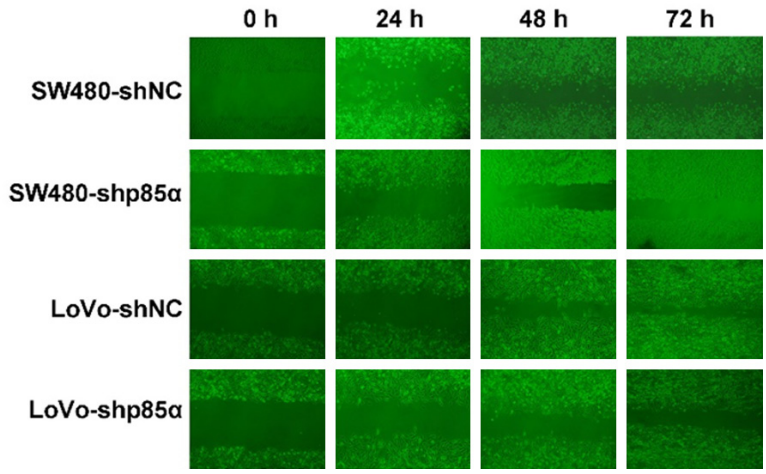

Figure 2. Knockdown of PI3K p85α reduced the wound healing ability of CRC cells. Wound scratch images of PI3K p85α knockdown SW480 and LoVo cells at different time points.

Table 1. Knockdown of PI3K p85α reduced the cell adhesion ability of SW480

Group	N	Mean ± SD	t	P
Control	3	0.271 ± 0.006	5.32	0.006
RNAi	3	0.219 ± 0.015	6	

cells were screened with 2 μg/ml of puromycin for 10-15 days post-infection.

Scratch wound healing assay

The indicated cells were seeded in 12-well plates at a density of 2×10^5 per well. When a monolayer of cells covered the bottom of the well, the surface was scratched vertically with a pipette tip (10 μl), and then photos of the cells were obtained at 0 h, 24 h, 48 h and 72 h to examine the wound healing ability.

Transwell migration and invasion assay

Transwell migration assays were performed using 8-μm pore transwell chambers in 24-well plates (Corning Costar, Cambridge, MA, USA). Cells in the logarithmic growth phase were starved overnight in serum-free RPMI1640 medium. The cell suspension (4×10^5 cells/ml, 100 μl) was then added to the upper chamber, and the lower chamber was filled with 800 μl RPMI1640 medium containing 10% FBS. The cells were then cultured for another 12 h. After swabbing the non-migrated cells from the upper chambers, the cells that had migrated to the lower chambers were fixed with 4% paraformaldehyde in PBS and stained with Giemsa. Finally, the cells that had migrated to the lower chambers were counted under a light microscope (×200). The cell invasion assay procedure was similar to that used for cell migration, except the transwell membranes were precoated with 50 μg/μl of Matrigel® (BD Biosciences, Franklin Lakes, NJ, USA), and the cells were incubated for 48 h. Cells that migrated to the lower chambers were counted in the same manner as described for the cell migration assay.

In vivo metastasis assay

PI3K p85α knockdown SW480 cells (6×10^6 cells) and NC SW480 cells were injected intravenously into the tail vein of 4-6-week-old BALB/c nude mice. After 6 weeks, spleens and livers were excised and examined for tumor growth.

Statistical analyses

SPSS 18.0 statistical software was used for the statistical analysis. Data are presented as the mean ± SD, and all experiments were performed in unique triplicates (n = 3). Data were

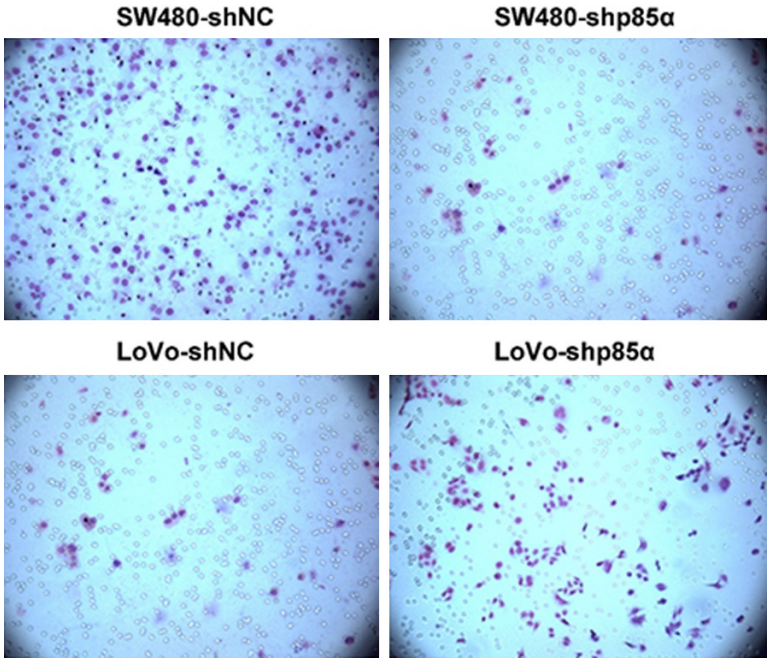

Figure 3. Knockdown of PI3K p85α reduced the migration of CRC cells. Transwell migration assays of PI3K p85α knockdown SW480 and LoVo cells.

evaluated using the Student's t test for comparison between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

PI3K p85α was over-expressed in CRC cells

We first sought to identify PI3K p85α-sensitive cell lines. To achieve this goal, 2 CRC cell lines were used to examine the protein accumulation of PI3K p85α by Western blot analysis. As shown in **Figure 1**, protein accumulation of PI3K p85α in LoVo and SW480 cells was similar to that of GAPDH (**Figure 1A**). These results indicated that PI3K p85α was abundantly over-expressed in LoVo and SW480 cells.

Lentivirus-mediated high-efficiency infection of LoVo and SW480 cells for PI3K p85α knockdown

To examine the role of PI3K p85α in CRC, stable PI3K p85α knockdown LoVo and SW480 cell lines were established using lentivirus-mediated RNAi technology. Briefly, a fragment targeting PI3K p85α was designed and implanted in a lentiviral vector, and a non-targeting fragment was used as a negative control (NC). The lentivirus containing PI3K p85α and the NC fragments was packaged and purified to infect LoVo and SW480 cells, respectively. The efficiency of lentiviral infection was examined by detecting GFP expression (data not shown). Western blot analysis revealed that the protein level of PI3K p85α was dramatically decreased in shp85α compared with NC cells (**Figure 1B**, **1C**).

Knockdown of PI3K p85α reduced the wound healing ability of CRC cells

After establishing p85α knockdown CRC cell lines, we examined the role of p85α in the migration of CRC cells. Next, we determined the cell wound healing activities of SW480 and LoVo cells. As shown in **Figure 2**, the wound healing activity of p85α knockdown SW480 cells was significantly reduced compared to that of NC cells. In NC cells, the "wound" had almost completely healed at 72 h, whereas the "wound" in p85α knockdown cells remained clearly visible at the same time point. Similar results were obtained for LoVo cells. The wound healing activities of LoVo cells from 0 to 72 h were significantly reduced when PI3K p85α was knocked down (**Figure 2**; **Table 1**).

Knockdown of PI3K p85α reduced the migration and invasion of CRC cells

To validate the findings from the wound healing assays and to investigate the role of PI3K p85α in cell migration, transwell migration assays were performed to examine the migration activities of SW480 and LoVo cells. As shown in **Figure 3**, p85α knockdown significantly reduced SW480 and LoVo cell migration through the transwell at 12 h. Moreover, to further investigate cell invasion, transwell invasion assays were performed with pre-coated transwells. As shown in **Figure 4**, p85α knockdown reduced the invasion activity of SW480 and LoVo cell. Taken together, these results indicate that PI3K p85α plays an important role in the migration and invasion of CRC cells.

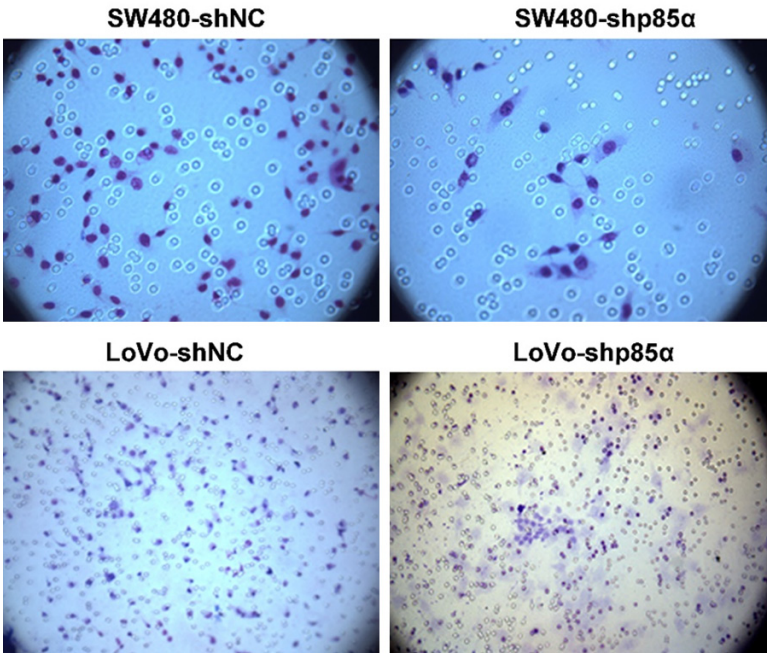

Figure 4. Knockdown of PI3K p85α reduced the invasion of CRC cells. Transwell invasion assays of PI3K p85α knockdown SW480 and LoVo cells.

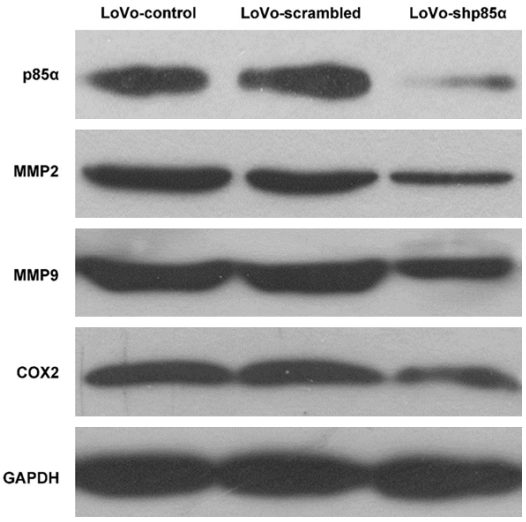

Figure 5. Knockdown of PI3K p85α reduced the accumulation of MMP-2, MMP-9 and COX-2. Western blot analysis was performed to examine the accumulation of MMP-2, MMP-9 and COX-2 in LoVo cells. GAPDH was used as a loading control.

Knockdown of PI3K p85α reduced the accumulation of proteins related to cellular migration and invasion

We examined whether knockdown of PI3K p85α affected proteins related to cellular migration and invasion. To achieve this goal, western blot analysis was performed to measure the levels of MMP-2, MMP-9 and COX-2, which are known to be associated with cancer cell proliferation and invasion [17-19]. As shown in **Figure 5**, the levels of MMP-2, MMP-9 and COX-2 were all significantly reduced when p85α was knocked down.

Knockdown of PI3K p85α suppressed spleen and liver metastases in nude mice

After demonstrating that PI3K p85α plays an important role in the migration and invasion of colorectal cancer cells, we examined tumor metastasis in vivo. To achieve this goal, p85α knockdown LoVo cells and NC cells were injected intravenously into nude mice, and spleens and livers were excised at 6 weeks post-injection. As shown in **Figure 6**, no visible metastatic nodules were observed on the surface of the spleens or livers from nude mice injected with p85α knockdown LoVo cells, whereas injection of NC cells resulted in clear metastases in the spleens and livers. Summarily and consistent with the results obtained from the migration and invasion assays in vitro, PI3K p85α knockdown significantly suppressed tumor metastasis in nude mice.

Discussion

CRC is one of the most common and lethal malignancies worldwide [1]. Consequently, the identification of additional therapeutic targets that contribute to the development and progression of CRC is desirable to combat this deadly disease. The migration and invasion of CRC cells is critical for the process of tumor metastasis [1]. PI3K p85α has been identified as one of most important cancer testis antigens because of its indispensable role in cell growth, proliferation and motility [5, 6, 8, 10]. Thus, in the present study, we aimed to investigate the role of PI3K p85α in the development of CRC.

The PI3K pathway is a key oncogenic signaling pathway in a number of cancers because its

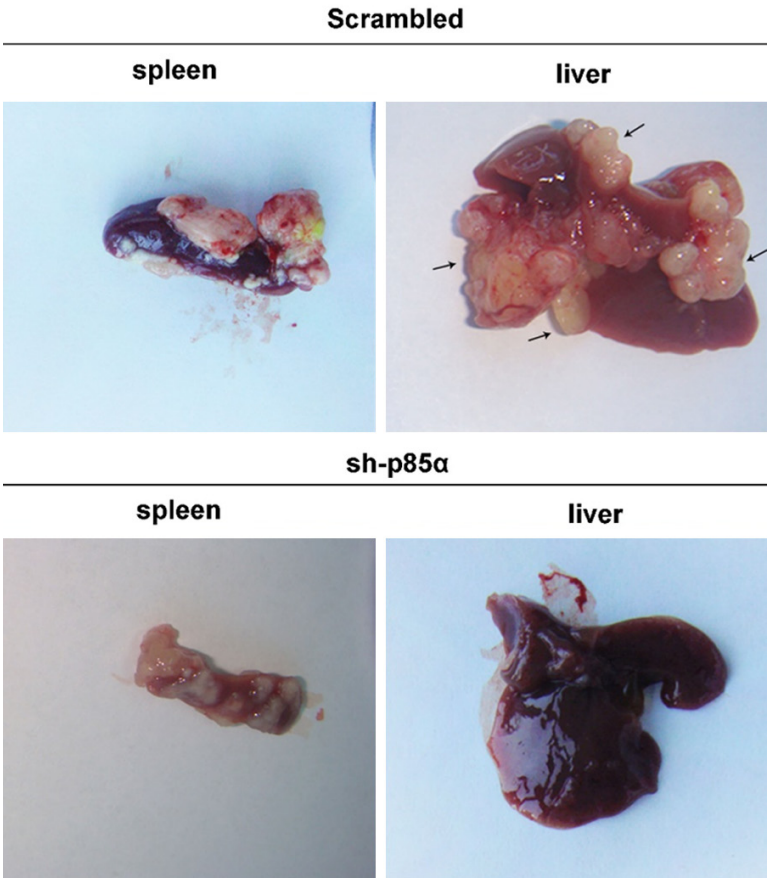

Figure 6. Knockdown of PI3K p85α suppressed tumor metastasis in nude mice. PI3K p85α knockdown SW480 cells were inoculated into nude mice, and the mice were killed after 6 weeks to excise and examine spleens and livers. Nude mice inoculated with NC cells served as a control.

activation is required for tumor cell proliferation and migration. Given that the regulatory p85α submit is essential for the stability of the p110 catalytic submit of the PI3K complex, the regulation of p85α expression levels could affect tumor development [6, 8]. This regulation may depend on a critical molecular balance between the regulatory and catalytic subunits [20]. In our study, knockdown of PI3K p85α significantly reduced the migration and invasion activities of CRC cells. Further investigations have shown that p85α knockdown results in the down-regulation of cellular proteins related to extracellular matrix (ECM) formation and cell adhesion. Our findings reveal detailed mechanisms by which the PI3K pathway regulates cancer cell behavior.

A critical step for tumor cell invasion is the degradation of the ECM, a complex network of extracellular macromolecules that functions as a barrier to the spread of cancer cells to distal sites by restricting tumor growth and invasion [21] Monoclonal. MMPs are initially expressed as inactive pro-enzymes that require proteolytic processing to release the active enzyme [22, 23]. More than 20 different MMPs act on a broad spectrum of substrates, including collagen type I, II, III, and IV and stromyelin [24]. MMP-2 and MMP-9 are thought to a play a key role in the degradation of type IV collagen and gelatin, the 2 main components of the ECM. MMP-2 and MMP-9 are secreted in their latent zymogenic form and are subsequently cleaved by other MMPs or proteases to yield the activated forms [19]. Therefore, MMP-2 and MMP-9 are key proteins involved in the initial breakdown of ECM [19]. Our transwell assays showed that knockdown of PI3K p85α in CRC cells led to remarkable suppression of tumor cell migration and invasion. Moreover, protein expression levels of MMP-2 and MMP-9 were also reduced in these cells. These results suggest that the PI3K signal transduction pathway may control tumor metastasis by regulating the activation of MMP-2 and MMP-9, which in turn affect ECM degradation.

COX is known to have 2 isoforms: COX-1 and COX-2 [17]. Both enzymes can catalyze the conversion of arachidonic acid and other fatty acids to prostaglandins [17]. COX-1 produces metabolites that play a central role in normal physiologic functions [18], whereas COX-2 is an inducible enzyme that is expressed in response to a variety of physiological stimuli such as inflammation, adhesion, and neoplasia [18]. In tumors, the main product of COX-2 is prostaglandin E2, which plays an important role in cancer by promoting cell proliferation, survival and angiogenesis [25]. The cellular effects of PGE2 are mediated through a family of G protein-coupled receptors, including EP1, 2, 3 and

EP4 [26]. The EP4 receptor has been shown to activate extracellular signal-regulated kinase 1 and 2 via the PI3K pathway [26, 27]. Therefore, knockdown of PI3K may down-regulate EP4, decrease the expression of COX-2 and PGE2, and eventually reduce the migration and adhesion of CRC cells.

Conclusions

In summary, this report showed that knockdown of PI3K p85α suppressed the migration and invasion of CRC cells in vitro and suppressed tumor metastasis in vivo. Further investigation showed that the expression levels of proteins such as MMP-2, MMP-9 and COX-2, which are related to extracellular matrix formation and cell adhesion, were reduced in response to p85α knockdown, shedding new light on the potential mechanism by which the PIK3 pathway modulates the progression of CRC. In future studies, we will continue to explore the influence of high or low expression levels of proteins related to the PIK3 pathway on cell migration and invasion, with a greater focus on in vivo models, to reveal the precise molecular mechanisms of PI3K in CRC.

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Disclosure of conflict of interest

None.

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