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PRECLINICAL STUDY

Pigment epithelium-derived factor (PEDF) inhibits breast cancer metastasis by down-regulating fibronectin

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Abstract Pigment epithelium-derived factor (PEDF) plays an important role in the tumor growth and metastasis inhibition. It has been reported that PEDF expression is significantly reduced in breast cancer, and associated with disease progression and poor patient outcome. However, the exact mechanism of PEDF on breast cancer metastasis including liver and lung metastasis remains unclear. The present study aims to reveal the impact of PEDF on breast cancer. The orthotopic tumor mice model inoculated by MDA-MB-231 cells stably expressing PEDF or control cells was used to assess liver and lung metastasis of breast cancer. In vitro, migration and invasion experiments were used to detect the metastatic abilities of MDA-MB-231 and SKBR3 breast cancer cells with or without overexpression of PEDF. The metastatic-related molecules including EMT makers, fibronectin, and p-AKT and p-ERK were detected by qRT-PCR, Western blot, and Fluorescent immunocytochemistry. PEDF significantly inhibited breast cancer growth and metastasis in vivo and in vitro. Mechanically, PEDF inhibited breast cancer cell migration and invasion by down-regulating fibronectin and subsequent MMP2/MMP9 reduction via p-ERK and p-AKT signaling pathways. However, PEDF had no effect on EMT conversion in the breast cancer cells which was usually involved in cancer metastasis. Furthermore, the study showed that laminin receptor mediated the down-regulation of fibronectin by PEDF. These results reported for the

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first time that PEDF inhibited breast cancer metastasis by down-regulating fibronectin via laminin receptor/AKT/ERK pathway. Our findings demonstrated PEDF as a dual effector in limiting breast cancer growth and metastasis and highlighted a new avenue to block breast cancer progression.

Keywords Pigment epithelium-derived factor · Metastasis · Laminin receptor · Fibronetin · MMP-2 · MMP-9

Abbreviations

PEDF	Pigment epithelium-derived factor
LR	Laminin receptor
FN	Fibronetin
EMT	Epithelial-to-mesenchymal transition

Introduction

The breast cancer is the most common type of tumor and the major cause of cancer mortality in women [1]. The causes of death from breast cancer are usually not due to the primary tumor itself but are the result of metastasis to a distant organ in the body, and the 5-year survival rate is only 23 % for women diagnosed with distant metastasis [2]. Metastasis is a multistep process including local invasion, detachment from the primary tumor, intravasation, survival in the circulation, extravasation, and establishment of a distant lesion [3].

The complex interactions between tumor cells and the extracellular matrix (ECM) play important roles in mediating and regulating many processes during tumor metastasis, including cell migration, cytoskeletal reorganization, and morphological transition [4]. Among the ECM components, Fibronectin (FN) plays an important role in embryonic development, cell adhesion, migration, tumor invasion, and metastasis [5, 6]. The FN expression is inversely associated with the survival and clinical outcome of breast cancer patients [7, 8]. Besides, FN also promotes ovarian cancer and melanoma cancer invasion and metastasis by interacting with its receptor integrins [9–11]. Meanwhile, studies have shown that FN up-regulates MMP-2 and MMP-9 levels resulting in invasion and metastasis of breast cancer [12, 13]. Therefore, the inhibition of FN expression is a potential strategy to suppress breast cancer metastasis.

Pigment epithelium-derived factor (PEDF), a 50-kDa endogenous glycoprotein encoded by SERPINF1, was first discovered in 1991 as a secreted protein from human fetal eye [14] and belonged to the non-inhibitory serine proteinase inhibitor (serpin) superfamily [15]. Pigment epithelium-derived factor was first identified as a neuronal differentiation factor which was produced by human retinal pigment epithelial cells [16]. To date, PEDF is found to be widely expressed in human tissues, including in the brain [17], eyes [16], spinal cord [18], liver [19], plasma [20], and lungs [21]. Furthermore, subsequent studies have shown that PEDF possesses various biological properties in many physiological and pathological processes [22] not only neurotrophic, but also anti-angiogenic, anti-tumorigenic, and anti-metastasis activities [23].

Pigment epithelium-derived factor has been shown to be an anti-tumorigenic agent for many types of tumors, including osteosarcoma, melanoma, glioma, lung, and pancreatic carcinomas [24]. Pigment epithelium-derived factor inhibits tumor growth mostly by angiogenesis inhibition, promoting tumor cell differentiation, and inducing tumor apoptosis [25]. The ability of PEDF to suppress tumor metastasis has been reported in vitro and in several metastatic models [26–30]. In a recent breast cancer study, it has been shown that PEDF is lost in endocrine-resistant MCF-7:5C, MCF-7:2A, and BT474 breast cancer cells, and overexpression of PEDF could restore their sensitivity to tamoxifen [31]. In addition, Cai et al. reported that PEDF expression is decreased in the human breast cancer progression [32], and the intratumoral expression of PEDF is negatively correlated with the microvessel density (MVD) in breast cancer [33]. More recently, by inoculating MDA-MB-231 brain-tropic cell lines into left cardiac ventricle and establishing the hematogenous mouse model of brain metastasis, PEDF has been found to inhibit the proliferation of brain metastasis [34]. All of the above studies indicate that PEDF plays an important role in tumor metastasis. However, the exact mechanisms of PEDF on breast cancer metastasis remain poorly understood.

In our studies, we found that PEDF inhibited breast cancer invasion and metastasis by down-regulating the levels of FN, MMP-2, and MMP-9 via laminin receptor (LR) through ERK and AKT signaling pathways. The findings not only advanced the molecular understanding of PEDF on tumor metastasis, but also provided a therapeutic target for breast cancer treatment.

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Materials and methods

Cell lines, animals, and treatment

The human breast cancer cell lines MDA-MB-231 and SKBR3 breast cancer cells were kindly provided by Prof. Erwei Song (Sun Yat-Sen Memorial Hospital, China), maintained in DMEM (Gibco, Gaithersburg, MD, USA), and supplied with 10 % FBS and 1 % penicillin/streptomycin. The establishment of stable MDA-MB-231 cells expressing Vector/PEDF and animals treated were described in supplemental materials.

Plasmids, small interfering RNAs, and transfected treatment

The plasmids pcDNA3.1 (+) vector, pcDNA3.1-PEDF (+), and shRNA for LR were kept by our own laboratory. The lentivirus encoding hPEDF and control vector lentivirus-GFP were gifts from Prof. Peng Xiang (Sun Yat-sen University, China).The siRNAs for silencing FN were purchased from RIBOBIO (Guangzhou, China). The siRNA for silencing LRP6 was purchased from Genechem (Shanghai, China). The sequences of the FN/LRP6/Laminin receptor-siRNAs and transfection protocols were supplied in supplemental materials.

Western blotting analysis and qRT-PCR

Briefly, protein extracts were resolved through 8–15 % SDS-PAGE, transferred to PVDF membranes, and probed with primary antibodies. The detailed steps of qRT-PCR were descried in supplemental materials.

Migration assay and invasion assay

Migration of breast cancer cells was examined using 24-well Boyden chambers (Corning, Corning, NY) with 8 μm inserts, and Invasion assay of breast cancer cells was performed using 24-well Boyden chambers with 8 μm inserts coated with 50 μl Matrigel. The treatment of breast and cells and detailed steps of migration and invasion assay were descried in supplemental materials.

Alveolar spaces count

Alveolar spaces count assay was established as described by previously [35]. Briefly, alveolar spaces were captured electronically, and the total alveolar area was obtained from the sum of individual alveolar air spaces within the whole lung region of each slide. The area of the lung (*V*p) and the area of the alveoli (*V*a) were definite and calculated. The proportion of alveoli to lung tissue was presented by *V*a/*V*p.

Results

PEDF inhibited breast cancer growth and metastasis in an animal model

Previous investigations showed that PEDF was lost with progression of breast cancer and associated with high MVD in breast cancer [32, 33]. To identify the therapeutic role of PEDF in breast cancer, we inoculated the stable MDA-MB-231 cells expressing PEDF into the mammary pats of nude mouse. The tumor size in lentivirus-hPEDF-treated group was significantly smaller in comparison with control groups from day 20 after cell inoculation gradually (Fig. 1a) and about 40 % reduction on the 31st day after cell inoculation (Fig. 1b). Since PEDF was proved to be antiangiogenic and a potential tumor suppressor, we speculated that the mechanism by which treatment with lenti-PEDF could prevent the tumor growth might lie in the inhibition of tumor angiogenesis. Sections of tumor xenografts were stained for CD31 to measure the MVD. Remarkably, the MVD of tumor tissues was dramatically decreased in lenti-PEDF-treated group compared with that of control group (Fig. 1c).

On the other hand, in order to diminish the effects of the tumor volumes on the metastasis, we sacrificed the control group on day 31 while the PEDF group on day 35 (on which the tumor xenografts both reached to 1.6 cm in diameter), respectively, to evaluate the tumor metastasis to the lungs and livers. As measured by Hematoxylin and eosin (H&E) staining, lentivirus-PEDF led to less massive metastasis, damaged pulmonary areas, and metastasis nodules in the lungs (Fig. 1d, e, S6A, and S6B) of the mice-bearing MDA-MB-231 xenografts as compared with control group. Meanwhile, the number of metastasis nodules and necrosis areas in the livers was also markedly decreased (Fig. 1f and g). Moreover, the penetration of cancer cells into adjacent normal tissues was significantly inhibited in MDA-MB-231 xenografts transfected with lenti-PEDF compared with vector groups (Fig. 1h). Therefore, all this evidence strongly indicated that PEDF could suppress xenografts growth, invasion, and metastasis of breast cancer in vivo.

PEDF suppressed breast cancer cell migration and invasion in vitro

To explore the mechanism by which PEDF impairs the invasion and metastasis of breast cancer, wound-healing assay and migration assay using Boyden chamber were used to evaluate the effect of PEDF on cancer cell motility. During cell migration to close the wound scratches, the migration of MDA-MB-231 cells transiently transfected with PEDF plasmid was much slower

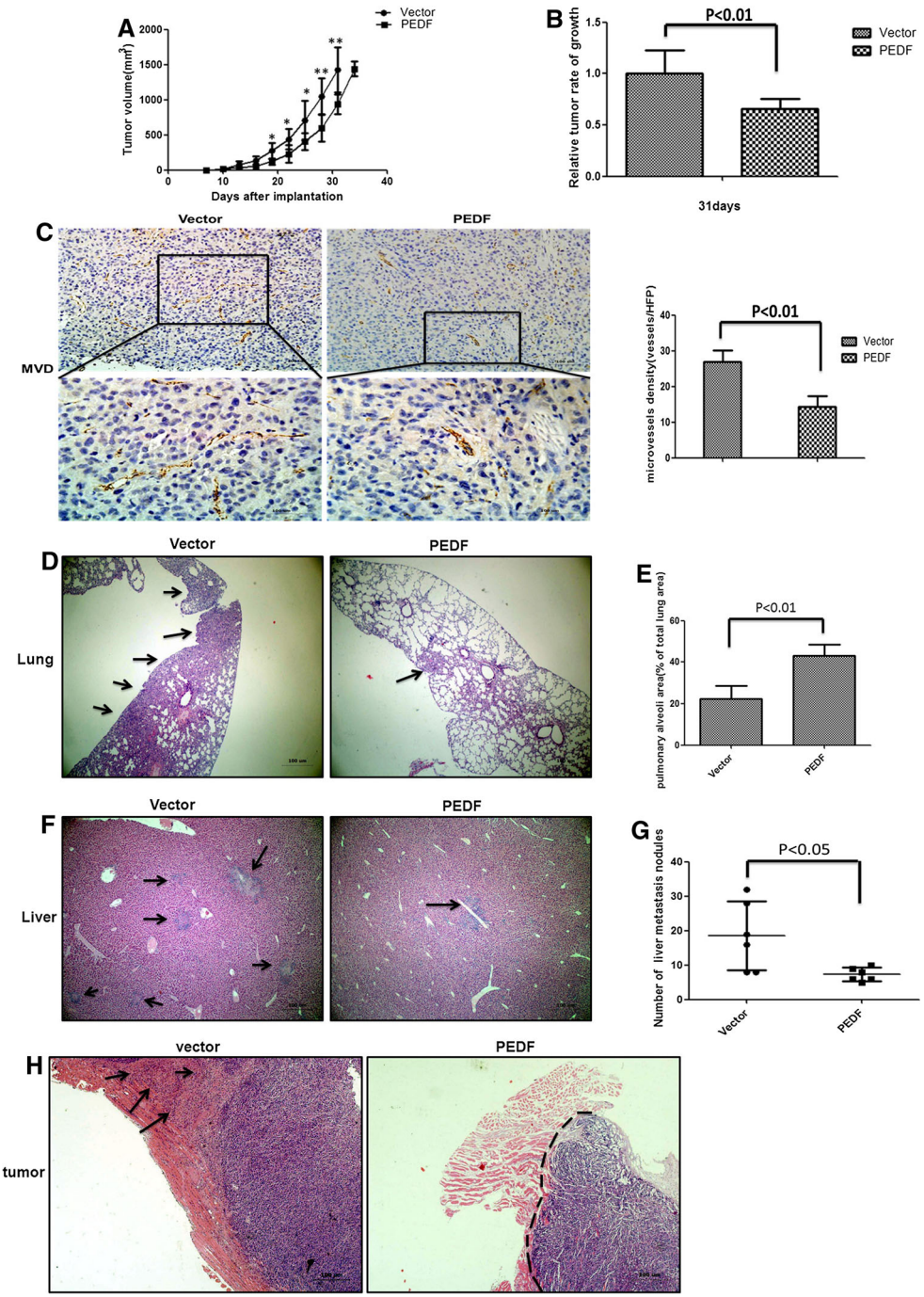

Fig. 1 Inhibition of breast cancer growth and metastasis by PEDF stable expression in MDA-MB-231 xenografts. **a** The growth of mice-bearing MDA-MB-231 breast cancer cells stably expressing Vector/PEDF ($n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with control group). **b** The inhibition rate of mice-bearing MDA-MB-231 cells stably expressing PEDF at the 31st day after implantation. Data are normalized to control group. **c** Microvessel density of mice-bearing MDA-MB-231 xenografts stably expressing Vector/PEDF by immunohistochemistry for staining CD31. *Bars* correspond to mean ± SD. $^{**}P < 0.01$ as compared with control group. *Scar bar* 100 μm. H&E of lungs (**d**) and livers (**f**) of mice-bearing MDA-MB-231 xenografts stably expressing Vector/PEDF when tumor reached to1.6 cm in diameter. *Scar bar* 100 μm. **e** The pulmonary of alveoli area of total lung in each mice-bearing MDA-MB-231 xenografts stably expressing Vector/PEDF. *Bars* correspond to mean ± SD. $^{**}P < 0.01$ as compared with Control group. **g** The number of metastasis nodules under gross inspection in the liver of each mice-bearing MDA-MB-231 xenografts stably expressing Vector/PEDF. *Bars* correspond to mean ± SD. $^{*}P < 0.05$ as compared with Control group. **h** The ability of breast cancer invasion into adjacent tissues in mice-bearing MDA-MB-231 xenografts stably expressing Vector/PEDF. *Scar bar* 100 μm

than that of control cells (Fig. 2a). For the migration assay, the MDA-MB-231 cells transfected with PEDF plasmid for 20 h were suspended in serum-free media and plated on 8 μm cell culture and then inserted in a Boyden chamber. After 8 h of migration, compared with control group, the number of migrating cancer cells was dramatically decreased in PEDF-treated group (Fig. 2b). Furthermore, Boyden chambers were also used to examine the invasiveness of breast cancer cells by plating them on matrix gel-coated inserts in the lower wells. As shown in Fig. 2c, the number of invading cancer cells was reduced to 50 % of control group (Fig. 2c). The migrating and invading numbers of another breast cancer cells line (SKBR-3) which was transfected with PEDF were also significantly decreased compared with those of control group (Fig. 2d). These results demonstrated that PEDF could obviously inhibit the migration and invasion of breast cancer cells.

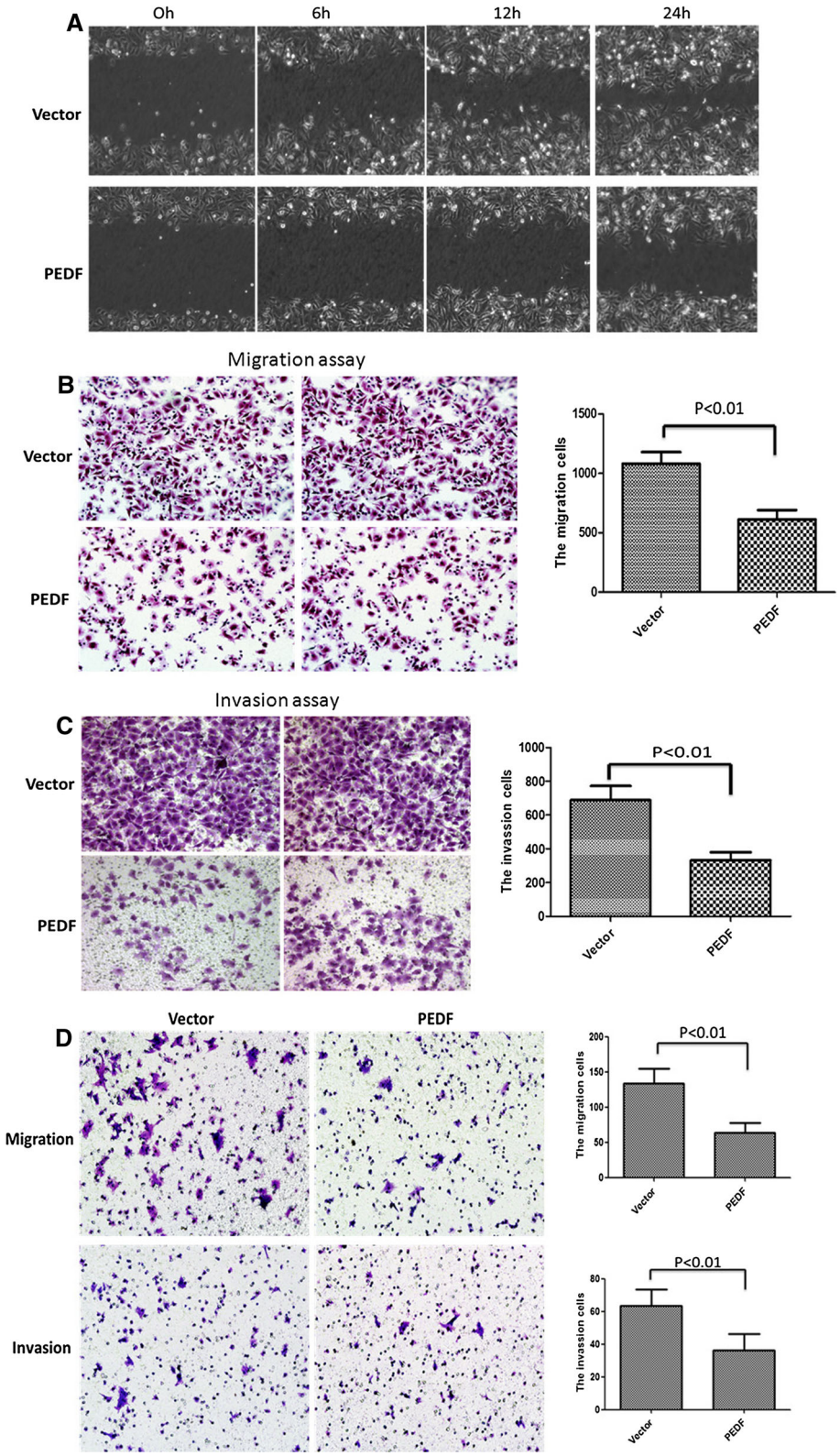

Fig. 2 PEDF suppressed the migration and invasion of breast cancer cells in vitro. **a** Wound-healing assay of MDA-MB-231 breast cancer cells overexpressing PEDF. MDA-MB-231 cells transiently transfected by Vector/PEDF for 20 h were plated for wound-healing assay. **b** Migration assay of MDA-MB-231 breast cancer cells transiently transfected Vector/PEDF for 20 h in a Boyden chamber with an 8-μm inserts for migrating 8 h. The migratory cancer cells are representative as histogram. *Bar* corresponds to mean ± SD. $^{**}P < 0.01$ as compared with Control group. **c** Invasion assay of MDA-MB-231 breast cancer cells transiently transfected Vector/PEDF for 20 h in a Boyden chamber with an 8-μm inserts coated with Matrigel for invading 24 h. The invasive cancer cells are showed as histogram. *Bar* corresponds to mean ± SD. $^{**}P < 0.01$ as compared with Control group. **d** Similar to **b** and **c**, SKBR3 breast cancer cells transiently transfected Vector/PEDF for 20 h and then performed to Migration assay and Invasion assay for 18 h and 30 h, respectively. The migratory and invasive SKBR3 cancer cells are shown as histogram. *Bar* corresponds to mean ± SD. $^{**}P < 0.01$ as compared with Control group

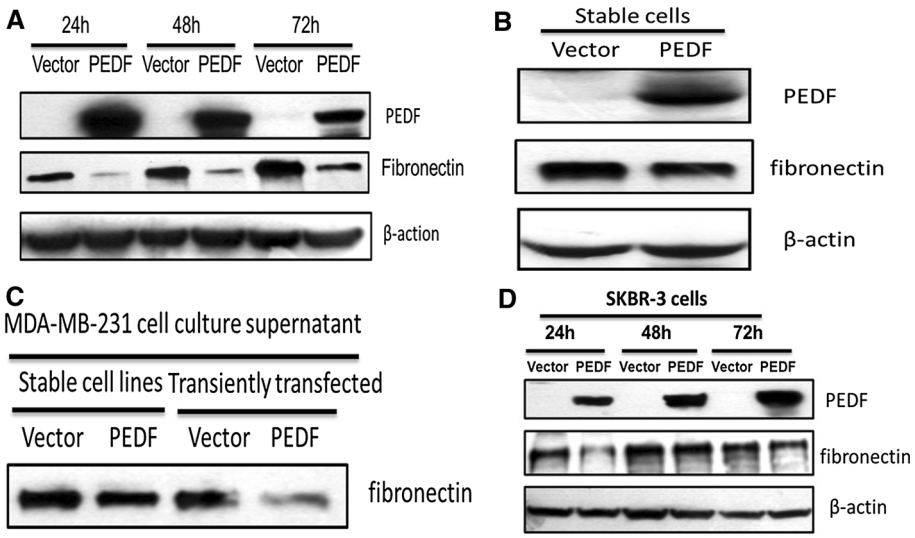

Fig. 3 PEDF significantly reduced fibronectin expression in breast cancer cells. **a** and **b** Western blot analysis for FN in breast cancer cells MDA-MB-231 transiently transfected with Vector/PEDF for 24 h, 48 h, and 72 h (**a**) and in stable MDA-MB-231 cells expressing PEDF. *β*-actin was used as a loading control. **c** Western blot analysis for secreted FN in medium of MDA-MB-231 cells transiently expressing PEDF for 24 h or stable MDA-MB-231 transfected with PEDF. **d** Western blot analysis for FN in breast cancer cells SKBR-3 transiently transfected with Vector/PEDF for 24 h and 48 h

PEDF significantly reduced the expression of fibronectin

Fibronectin has been recognized as the key element in promoting cell adhesion and migration; thus, we deduced that effects of PEDF on cancer cell metastasis might rely on the regulation of FN. Indeed, we noted that PEDF could dramatically inhibit the expression of FN in MDA-MB-231 cells transfected with PEDF for 24, 48, and 72 h or in lenti-PEDF-treated stable cell lines (Fig. 3a and b). Fibronectin can be found in a soluble form or in an insoluble cellular form as an ECM protein. In addition, secreted FN in medium was significantly reduced in MDA-MB-231 cells transfected with PEDF (Fig. 3c). Moreover, similar result of FN expression detected by Western blot assay was obtained from another breast cancer cell line (SKBR-3) (Fig. 3d).

The inhibition of breast cancer invasion by PEDF was partially dependent on FN

FN is an important component of ECM and involved in cells metastasis, growth, and adhesion [5, 6]. To confirm whether FN contributes to the breast cancer cell invasion in our models, we employed FN-siRNA to neutralize FN function. Transfection of cells with FN-siRNA reduced the number of invasive tumor cells by 70 % (Fig. 4a and b). Furthermore, treatment of cells with recombinant FN (20 and 40 μg/ml) enhanced the invasion of cancer cells transfected with PEDF plasmid in a dose-dependent manner (Fig. 4c and d). These findings suggested that the inhibition of breast cancer invasion by PEDF was partially dependent on the reduction of FN.

Previous studies have reported that FN promoted cells invasion and metastasis via up-regulation of MMP-2 and MMP-9, which importantly contributed to the invasion and metastasis of cancer cells [12, 13]. At first, we confirmed the down-regulation of MMP2 and MMP9 by FN using FN-siRNAs. In agreement with previous study, the expression of MMP2 and MMP9 was affected by FN (Figure S1A and 1B). Next, we examined the level of MMP2 and MMP9 to explore the mechanism that PEDF suppressed the invasion through FN. Western Blotting and RT-PCR showed that the protein and mRNA levels of MMP-2 and MMP-9 were remarkably reduced in the cells transfected with PEDF (Fig. 4e and f). Meanwhile, similar results were also obtained by Fluorescent immunocytochemistry (Figure S2).

To further evaluate the ability of PEDF to down-regulate MMP-2 and MMP-9 was through FN reduction, exogenous FN was added to culture medium in MDA-MB-231 cells transfected with PEDF. Western blotting showed that FN abolished the inhibition of MMP2 and MMP9 induced by PEDF (Fig. 4g). Consistent with in vitro data, significant reduction of the expressions of FN, MMP-2, and MMP-9 was observed in breast cancer xenografts in vivo (Fig. 4h). Collectively, these data strongly indicated that the down-regulation of MMP-2/MMP-9 through inhibition of FN

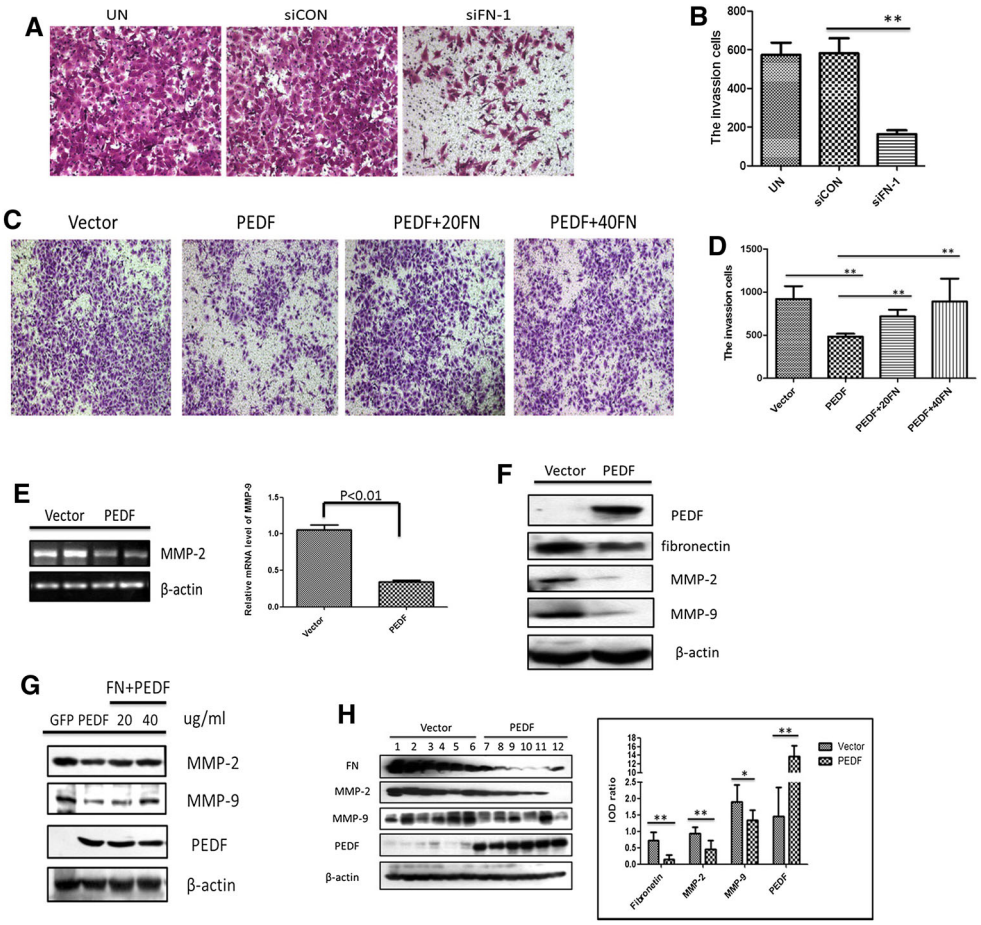

Fig. 4 The inhibitory effect on breast cancer invasion by PEDF was partly dependent on the presence of FN. Invasion assay of MDA-MB-231 breast cancer cells silencing FN by effectively siRNA (**a**). The invasive cancer cells are shown as histogram (**b**). *Bar* corresponds to mean ± SD. UN: untreated; siCON: control interference; $^{**}P < 0.01$ as compared with control group. Invasion assay of MDA-MB-231 cells transiently transfected Vector/PEDF for 24 h and followed by adding 20 and 40 μg/ml FN protein for 12 h (**c**). The invasive cancer cells are shown as histogram (**d**). *Bar* corresponds to mean ± SD. $^{**}P < 0.01$ as compared with control group. Western blot analysis (**f**) and RT-PCR analysis (**e**) for MMP-2 and MMP-9 level in the MDA-MB-231 cells expressing Vector/PEDF for 48 h. *Bar* corresponds to mean ± SD. $^{**}P < 0.01$ as compared with control group. **g** Western blot analysis MMP-2 and MMP-9 in MDA-MB-231 cells expressing Vector/PEDF for 24 h and followed by adding 20 and 4 μg/ml FN protein for 12 h. **h** Western blot analysis FN, MMP-2, and MMP-9 in stably expressing Vector/PEDF MDA-MB-231 xenografts. *Bar* corresponds to mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with control group

contributed to the overall process of inhibitory effect of PEDF on breast cancer invasion and metastasis.

Down-regulation of fibronectin by PEDF via ERK and AKT signaling pathways

Regulation of the processes of cell metastasis and fibrosis by FN may involve MAP Kinase signaling pathway and AKT signaling pathways [36, 37]. We first confirmed that the inactivation of ERK and AKT using inhibitors effectively decreased the FN expression (Fig. 5a and b). Next, to verify whether PEDF reduced FN through the MAP Kinase and AKT signaling, we detected the levels of p-ERK, p-P38, p-JNK, and p-AKT by Western blotting. The results showed that PEDF overexpression could significantly decreased the phosphorylation of ERK and AKT (Fig. 5c) but did not alter the phosphorylation of P38 and JNK (Figure S3). Additionally, treatments of EGF and LPS as agonists of p-ERK could remarkably promote FN expression as well as MMP-2 expression (Fig. 5d). However, it was noted that EGF and LPS could not effectively induce the phosphorylation of ERK in MDA-MB-231 cells transfected with PEDF compared to that of cells with control vector (Fig. 5d), which indicated that PEDF powerfully inhibited ERK phosphorylation. Thus, we concluded that the down-regulation of FN by PEDF might be partial via ERK signaling pathways.

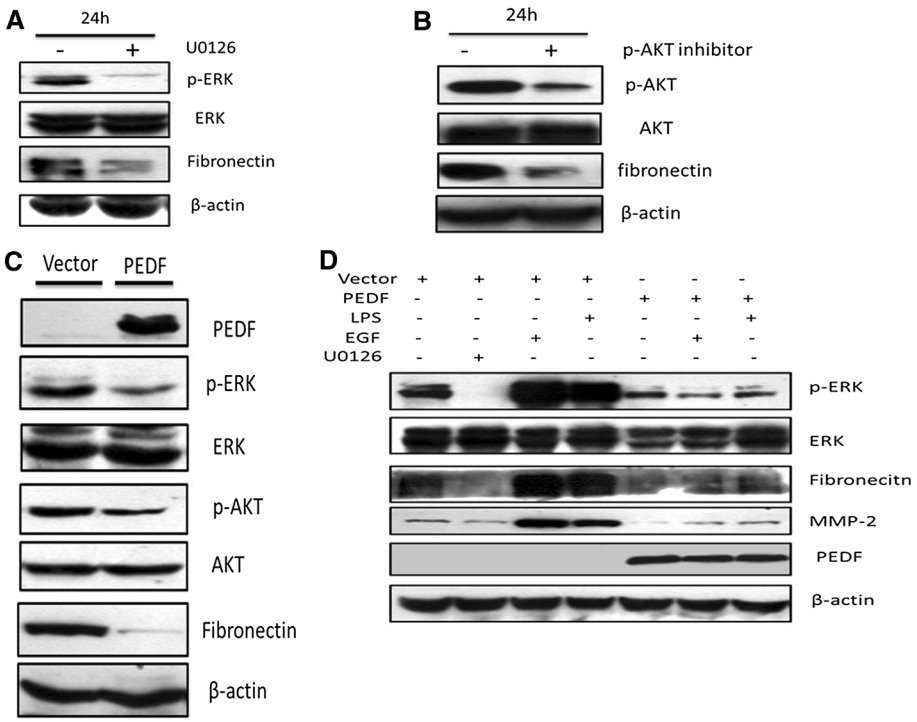

Fig. 5 A schematic diaphragm of PEDF signaling. Western blot analysis of p-ERK (**a**) and p-AKT (**b**) in MDA-MB-231 cells treated with 80 μM U0126 and 1 nM p-AKT inhibitor for 24 h collectively. *β*-actin was used as a loading control. **c** Western blot analysis of p-ERK and p-AKT level in MDA-MB-231 cells expressing Vector/PEDF for 24 h. *β*-actin was used as a loading control. **d** Western blot analysis of p-ERK, FN, and MMP-2 of MDA-MB-231 cells expressing PEDF for 24 h and treated with 80 μM U0126, 100 ng/ml EGF, and 10 μg/ml LPS for 12 h. *β*-actin was used as a loading control

PEDF inhibited the expression of fibronectin via laminin receptor

Previous studies showed that LR mediated anti-inflammatory and anti-thrombogenic effects of PEDF in myeloma [38]. Besides, low-density lipoprotein receptor-related protein6 (LRP6) and ATGL were identified as new PEDF receptor in RPE cell and adipocytes [39, 40]. It was crucial to validate which receptor was responsible for the inhibitory effect of PEDF on FN in MDA-MB-231 breast cancer cells. Firstly, we detected ATGL, LRP6, and LR mRNA levels and found that ATGL was undetectable in MDA-MB-231 cells (data not show). Next, we used shRNA to knockdown LR and LRP6 in MDA-MB-231 cells transfected with PEDF. The shRNA knockdown efficiency was confirmed by RT-PCR (Fig. 6a and b). The results showed that interference with LR, but not LRP6, reversed the down-regulation of p-ERK, p-AKT, FN, MMP2, and MMP9 by PEDF in MDA-MB-231 breast cancer cells (Fig. 6a and b), which suggested that LR was a promising PEDF receptor mediating the regulation of FN in MDA-MB-231 cells.

Discussion

It has been proposed that PEDF may possess both direct and indirect antitumor effects [41]. The direct antitumor effects of PEDF are possibly produced by inducing either anti-proliferative or pro-differentiation activities toward cancer cells, while the indirect antitumor effects are achieved through the anti-angiogenic action of PEDF [42, 43]. To elucidate whether PEDF suppressed breast cancer xenografts growth by the direct inhibition of MDA-MB-231 cells proliferation, we conducted MTT and Colony formation assay. MDA-MB-231 cells transiently transfected with PEDF or stable cells expressing PEDF were measured by MTT assay after incubation, respectively. In comparison to control group, there was no significant difference in cell viability between PEDF-transfected cells and control cells. Likewise, the number and size of colonies between stable MDA-MB-231 cell expressing PEDF and control cells were unchanged (Figure S4A and B), which indicated that PEDF achieved the suppression of breast cancer growth mainly through its anti-angiogenic property.

The epithelial-to-mesenchymal transition (EMT) plays an important role in trans-differentiation of epithelial cells to mesenchymal cells and contributed to metastasis of tumor [44–46]; therefore, we presumed that the inhibitory effect of PEDF on migration and invasion of breast cancer might be ascribed to the reversion of EMT. However, PEDF neither reversed the expression of E-cadherin, a classic epithelial cell marker, nor down-regulated level of vimentin, a mesenchymal cell marker in MDA-MB-231 cells transfected with PEDF for 24, 48, and 72 h (Figure S5A). Meanwhile, the transcriptional levels of vimentin, N-cadherin, E-cadherin, ZO-1 as well as transcription factors Snail and Slug were not obviously changed by PEDF overexpression in MDA-MB-231 cells (Figure S5B–

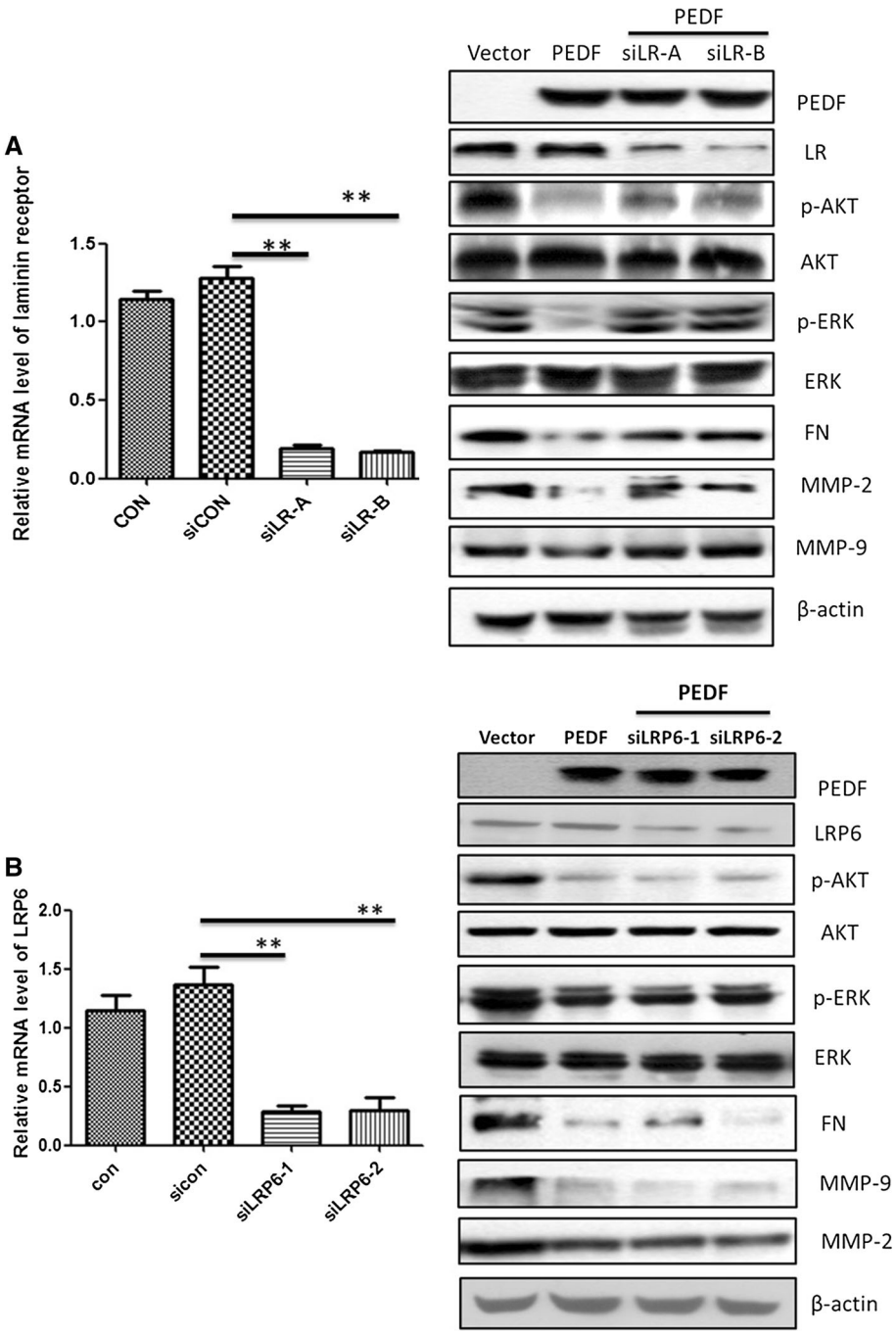

Fig. 6 PEDF inhibited the expression of fibronectin via laminin receptor. **a** Western blot analysis of FN, LR, MMP-2, MMP-9, p-AKT, and p-ERK level in transiently transfected PEDF MDA-MB-231 cells interfered with laminin receptor for 48 h. β-actin was used as a loading control. **b** Western blot analysis of FN, LRP6, MMP-2, MMP-9, p–AKT, and p-ERK level in transiently transfected PEDF MDA-MB-231 cells interfered with LRP6 for 48 h. β-actin was used as a loading control

G). Similarly, the expressions of these markers in stable MDA-MB-231 cells expressing PEDF were unchanged compared to that of control cells (Figure S5H and I). And there was no morphological difference between stable MDA-MB-231 cells expressing PEDF and control cells (Figure S5 J). These results made it more evident that PEDF had no influence on the reversion of EMT in breast cancer cells.

Interactions between the tumor cell and the ECM strongly influence tumor development, affecting cell survival, proliferation, and migration [12]. During cancer progression, the ECM of the tissue in which the tumor growth is extensively remodeled both by degradation of pre-existing ECM molecules and by the neosynthesis of ECM components. Among the ECM components, FN has been recognized as the key element in promoting cell differentiation, growth, adhesion, and migration. In our study, we showed that PEDF overexpression could down-regulate mRNA and protein levels of FN, in line with previous studies which have shown the down-

regulation of FN mRNA level by PEDF in diabetic kidney [47]. In addition, secreted FN in medium was significantly reduced in MDA-MB-231 cells transfected with PEDF (Fig. 3d). These results indicated that although PEDF overexpression was not sufficient to reverse the EMT progress, it did modulate ECM component to affect cell migration and metastasic potential. This was further supported by the data that adding FN to culture medium significantly eliminated the inhibitory effect of PEDF on invasiveness of breast cancer (Fig. 4c and d).

Some studies have shown that FN could promote breast cancer invasion and metastasis by up-regulation of MMP-2 and MMP-9 [12, 13]. We also observed that MMP-2 and MMP-9 levels were down-regulated by PEDF overexpression (Fig. 4e and f, S2) as well as Fn-siRNA in breast cancer cells (Figure S1A and B). In addition, exogenous FN could also abolish the reduction of MMP-2 and MMP-9 expressions by PEDF (Fig. 4g). This implied that MMP-2 and MMP-9 were involved in the modulation of PEDF on breast cancer metastasis. Accordingly, this was strengthened by the similar results found in vivo that PEDF down-regulated the levels of FN, MMP-2, and MMP-9 (Fig. 4h). Besides, PEDF is reported as a substrate for MMP-2 and MMP-9 [48], making it possible that PEDF and MMP-2/9 could form a negative feedback loop in the interaction. Taken together, these evidences supported the idea that the inhibitory effect of PEDF on invasiveness and metastasis of breast cancer was dependent on the down-regulation of FN and subsequently MMP-2/MMP-9 reduction.

The AKT and extracellular signal-regulated kinase (ERK) signaling pathways play an important role in human cancer including breast cancer [49]. MAP Kinase signaling pathway and AKT signaling pathway may be involved in the process of cell metastasis and fibrosis regulated by FN [36, 37]. In our studies, we found that PEDF indeed down-regulated p-AKT and p-ERK levels in breast cancer cells (Fig. 5c) but not the p-P38 and p-JNK levels (Figure S3). Furthermore, AKT and ERK inhibitors significantly reduced FN expression, clarifying that PEDF inhibited breast cancer metastasis might be through AKT and ERK pathways. Recent studies have pointed out that p-AKT and p-ERK signaling pathways exhibit both negative and positive influence on each other at different stages of signal propagation [50]. Other researches have shown that p-AKT inhibition results in a compensatory activation of the ERK signaling pathway in breast cancer, and combined administration of p-AKT inhibitors with p-ERK inhibitors would be more effective [51]. Since PEDF could simultaneously reduce the p-AKT and p-ERK levels, this implied that PEDF would be used as a more effective therapeutic approach in breast cancer.

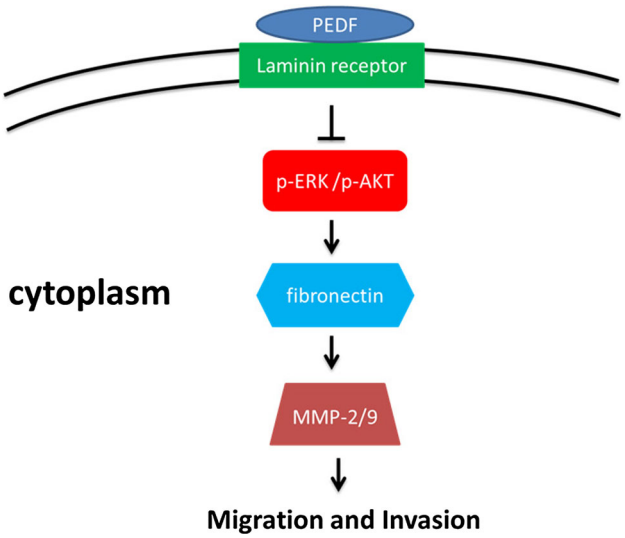

Fig. 7 General view of the paper. The metastatic inhibition of PEDF in breast cancer was related with its effect on decreasing cell migration and invasion by disrupting fibronectin expression via laminin receptor through AKT/ERK signaling pathway

Laminin receptor and ATGL are putative receptors for PEDF [52]. Pigment epithelium-derived factor also serves as an inhibitor of Wnt signaling pathway by binding with LRP6 [39]. However, the mRNA and protein levels of ATGL are hardly detectable in MDA-B-231 breast cancer cells. Laminin receptor localized in the cell membrane to interact with ECM is considered to be a molecular marker of aggressive tumor, such as breast cancer [53]. And Wnt signaling is activated in breast cancer; thus, it is reasonable to assume that LR and LRP6 might be candidates for PEDF receptor. Notably, LR is more abundant than LRP6 detected by RT-PCR in MDA-B-231 cells (data not shown). Differential expression of these receptors on various cells type may provide a partial explanation for the differential effects of PEDF. The ligands modulate the expression of certain receptor in specific cases, while PEDF per se did not regulate the expression of LR. Knockdown of LR, not LRP6, abolished the inhibitory effect of PEDF on p-ERK, p-AKT, FN, MMP2, and MMP9 expression (Fig. 6a and b). Moreover, previous researches have shown that a 34-aa peptide of PEDF (PEDF34) interacts with LR, induces endothelial cell apoptosis, and inhibits EC migration [38]. Thus, we made a plasmid missing the 34 aa region of PEDF, and we found that PEDF without a 34-aa region could not down-regulated the expression of FN (Figure S7), which further support that PEDF suppressed FN expression via the physical interaction with LR at the specific 34 aa region. The interactions of LR with AKT/mTOR and ERK signaling have been reported [54, 55]. However, the exact mechanism by which LR regulates the AKT and ERK pathway remains unclear.

Conclusion

In conclusion, our study has shown that PEDF is a metastasis suppressor in breast cancer. Mechanically, we demonstrated that the metastatic inhibition of PEDF in breast cancer was related to its effect on decreasing cell migration and invasion by disrupting FN expression via AKT/ERK signaling pathway. Furthermore, we identified LR as a potential receptor by which PEDF exhibited its biological function in breast cancer (Fig. 7). Our findings suggested that PEDF would be a promising agent in breast cancer therapy due to its in-direct anti-angiogenesis and direct anti-invasiveness activities.

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Ethical standards Care, use, and treatment of all animals in the present study were in strict agreement with the institutionally approved protocol that followed the guidelines set forth in the Care and Use of Laboratory Animals by the Sun Yat-sen University.

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