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Original Articles

Pigment epithelium-derived factor inhibits lung cancer migration and invasion by upregulating exosomal thrombospondin 1

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ABSTRACT

Exosomes are implicated in cancer cell development, migration and invasion. Pigment epithelium-derived factor (PEDF) is a secreted anticancer protein that can regulate lung cancer progression; however, the role of PEDF in non-small cell lung cancer (NSCLC), including metastasis and cancer cell-derived exosome secretion, is unclear. In this study, we analyzed the effects of PEDF on exosome-mediated migration, invasion, and tumorigenicity of cultured NSCLC cells. The results showed that PEDF overexpression significantly reduced NSCLC invasion and migration, while inducing cell aggregation, whereas PEDF knockdown had the opposite effects. Exosomes from NSCLC cells treated with recombinant PEDF had a significantly reduced ability to promote cancer cell motility, migration, and invasion compared to exosomes from untreated cells. Exosomes from PEDF-treated cells contained thrombospondin 1 (THBS1), which inhibited cytoskeletal remodeling and exosome-induced lung cancer cell motility, migration, and invasion. Furthermore, PEDF-overexpressing NSCLC cells formed smaller xenograft tumors with higher THBS1 expression compared to control tumors. Our findings indicate that PEDF decreases the metastatic potential of NSCLC cells through regulation of THBS1 release in cancer cell-derived exosomes, thus uncovering a new mechanism of lung cancer progression.

1. Introduction

Lung cancers, including non-small cell lung cancer (NSCLC), adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, are the main cause of cancer-related death worldwide [1]. NSCLC is the most frequent type of lung cancer and the leading cause of cancer mortality in Taiwan because in the majority of cases, the disease is diagnosed at the advanced stage when complete surgical resection is impossible, which accounts for a very low 5-year survival rate of less than 5% [2]. Therefore, new approaches are urgently needed for the prevention, early detection, and treatment of NSCLC.

Extracellular exosomes are nanovesicles originated from the cell membrane and secreted by all types of cells; they contain numerous signaling molecules, including nucleic acids, proteins, and lipids and play an important role in intercellular communication. In cancer, tumor and stromal cells may exchange biological material through exosomes, which have been shown to promote angiogenesis and tumor progression as well as cancer cell migration and distant metastasis [3,4]. In

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particular, it was found that lung cancer-derived exosomes act as paracrine signaling mediators that regulate cancer epithelial-to-mesenchymal transition [5]; for example, lung cancer-derived exosomal miR-23a appeared to modulate tumor vasculature and stimulate tumor angiogenesis under hypoxic conditions [6]. Therefore, it was suggested that circulating exosomes isolated from liquid biopsy or blood samples of NSCLC patients could be useful diagnostic and prognostic tools for screening, prediction of treatment response, and real-time monitoring of the disease [7]. Furthermore, exosome-mediated cancer cell communication may represent a potential therapeutic target for future treatment approaches in cancer, including NSCLC. However, the mechanisms underlying the effects of cancer-derived exosomes on tumor cell migration, invasion, and motility are still unclear.

Pigment epithelium-derived factor (PEDF) is a 50-kDa secreted glycoprotein encoded by the *SERPINF1* gene, which belongs to the serpin superfamily of serine protease inhibitors regulating proteolytic cascades related to key biological processes such as blood coagulation, inflammation, angiogenesis, protein folding and transport [8]. PEDF is a multifunctional molecule known to exert cardioprotective, differentiating, neurotrophic, anti-angiogenic, anti-apoptotic, and anti-tumorigenic effects [9,10], and previous findings indicate that PEDF could prevent cancer progression by increasing tumor cell differentiation, inhibiting neovascularization, and suppressing cancer cell invasion and metastasis [11,12].

Accumulating evidence suggests that some circulating tumor cells release PEDF-containing exosomes into the pre-metastatic niche, which results in immunosuppression and cancer spread [13]. Thus, it has been demonstrated that osteosarcoma-derived exosomes are rich in mRNA molecules encoding annexin 2, Smad2, phosphorylase, Cdc42-interacting protein 4, and PEDF and can mediate cancer metastasis [14]. However, the association between PEDF and cancer cell-derived exosomes and the role of PEDF in lung cancer require further investigation.

Thrombospondin 1 (THBS1) is a protein encoded by the *THBS1* gene in human and is a member of the thrombospondin family of matrix glycoproteins with potent anti-angiogenic activity [15], which can also exert anti-cancer effects [16]. Recent studies indicated that THBS1 expression inversely correlated with lung cancer. Thus, THBS1 is downregulated in NSCLC, in contrast to upregulated THBS2, and is strongly associated with 5-year survival [17]; furthermore, its abundance is decreased in exosomes of nasopharyngeal cancer cell lines CNP460 and NP69 [18]. Falero-Perez et al. [16] discovered that PEDF knockout in endothelial cells downregulated the expression of THBS1, but not THBS2, which corresponded to increased angiogenesis, suggesting a link between PEDF and THBS1 activity in cancer [19]. However, the correlation between PEDF and THBS1 in lung cancer remains unknown.

The objective of this study was to investigate the regulatory effect of PEDF on the tumorigenic activity of cancer-derived exosomes in NSCLC. We used a human NSCLC cell line and a xenograft tumor animal model to explore the involvement of exosomes derived from PEDF-treated tumor cells on lung cancer cell migration, invasion, and motility. Our findings indicate that PEDF suppresses cancer-derived exosome-mediated metastatic activity by promoting THBS1 expression and increasing its content in exosomes. As THBS1 inhibited cytoskeletal remodeling and suppressed migration and invasion of tumor cells, the presence of THBS1-containing exosomes in the pre-metastatic niche should prevent cancer progression.

2. Materials and methods

2.1. Cell culture

The human cancer cell line A549 was obtained from BCRC (Bioresource Collection and Research Center, Taiwan), derived from ATCC (American Type Culture Collection/Bioresource Collection and Research Center, ATCC number CCL-185). These cells have performed STR-PCR profile at BCRC (D7S820: 8, 11 CSF1PO:10, 12 TH01: 8, 9.3 D13S317: 11 D16S539: 11, 12 vWA: 14 TPOX: 8, 11 Amelogenin: X, Y D5S818:11). A549 were cultured in F12K growth medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% FBS (Invitrogen, Carlsbad, CA, USA), 100 units/mL of penicillin, and 100 pg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO, USA), at 37 °C in a humidified atmosphere of 95% air-5% CO_2. Culture medium was replaced every 4–5 days, and cells subcultured 3–12 times were used for experiments.

2.2. Cell migration and motility assays

IBIDI™ Culture Inserts (IBIDI, Martinsried, Germany) were placed into 6-well culture dishes and 1×10^4 cells/mL were seeded into the two reservoirs of the same insert [20]. After 24 h, the insert was removed with caution to create a 0.5-mm gap, and cell migration was assessed by bright-field microscopy at 0 h and 24 h after insert removal. The migrated cells were photographed and cell-covered areas measured using the WimScratch software program (Wimasis, Munich, Germany). Cell motility, tracks, displacement, and speed were assessed by time-lapse confocal microscopy (SP2; Leica, Exton, PA, USA) at various time points over a 20 h period.

2.3. Cell invasion assay

Cell invasion was evaluated by a modified Matrigel Boyden chamber assay using Bio-Coat Matrigel invasion chambers (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions [20]. Cells (1×10^5/mL) in serum-free medium were seeded on Matrigel-coated filters, and 5% FBS was added to the lower chambers as a chemoattractant. After incubation for 24 h, membranes were washed with PBS, the upper side wiped with a cotton ball, and cells invaded the lower side were removed using 0.1 mM EDTA in 10 mM Tris-HCl (pH 8.0) and counted.

2.4. Quantitative real-time PCR

Total RNA (2 μg) was reverse-transcribed using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and miRNA was isolated with a miRNA extraction kit (Invitrogen) [20]. The qPCR analysis was performed in concordance to the MIQE-guidelines in the revised manuscript [21]. Quantitative (q)RT-PCR was performed using a TaqMan assay (LightCycler FastStart DNA Master SYBR Green I; Roche, basel, Switzerland) and the following primers (Integrated DNA Technologies, MDBio Inc.): human *PEDF* forward, 5′-ATT CCC GAT GAG ATC AGC A-3′ and reverse, 5′-CTT AGG GTC CGA CAT CAT GG-3′; mouse *PEDF* forward, 5′-TCG AAA GCA GCC CTG TGT T-3′ and reverse: 5′-AAT CAC CCG ACT TCA GCA AGA-3′; human *THBS1* forward, 5′-CAC CAA CCG CAT TCC AGA G-3′ and reverse, 5′-TCA GGG ATG CCA GAA GGA G-3′; mouse *THBS1* forward, 5′-GCA GCA CAC ACA GAA GCA TT-3′ and reverse: 5′-CAA TCA GCT CTC ACC AGC AG-3′; human *GAPDH* forward, 5′-AGC CAC ATC GCT CAG ACA C-3′ and reverse, 5′-GCC CAA TAC GAC CAA ATC C-3′, mouse *GAPDH* forward, 5′-AAC GAC CCC TTC ATT GAC CT-3′ and reverse, 5′-CAC AGT AGA CTC CAC GAC A-3′.

2.5. Western blotting

Western blotting was performed as described previously [22]. Protein concentrations of cell and tissue lysates were measured using the Lowry assay, and 30 μg of total protein was separated by SDS-PAGE in 7.5, 10, or 12.5% gels depending on the expected molecular weight of the target. Proteins were transferred onto nitrocellulose membranes, which were blocked with 5% non-fat dry milk, and incubated with with primary antibodies against PEDF (1:1000; ab115489, Abcam, Cambridge, MA, USA), CD81 (1:500; GTX101766, Gene Tex, Irvine, CA, USA), CD63 (1:500, NBP2—42225SS, Novus Biologicals, Littleton, CO,

USA), THBS1 (1:500; Rev 031506 K, Neomarkers, Fremont, CA, USA), HSP70 (1:1000, MA-006, ABR Affinity BioReagents, Golden, CO, USA), Calnexin (1:500, GTX109669, GeneTex), β-actin (1:2000; GTX109639. Gene Tex), α-tubulin (1:2000; T5168, Sigma, Louis, MO,USA) and GAPDH (1:2000, sc-137179, Santa Cruz Biotechnology, Santa Cruz, CA, USA). And then incubation with horseradish peroxidase-conjugated secondary antibodies. The signals were visualized using an enhanced chemiluminescence detection kit (TOOLS Extreme ECL-HRP Substrate, Taiwan).

2.6. Construction of a human PEDF lentiviral vector and cell transduction

For cloning of human PEDF (GenBank accession number AF400442.1), total RNA was isolated from A549 cells, and cDNA was synthesized using MMLV reverse transcriptase according to the manufacturer's manual (Invitrogen). The hPEDF lentiviral vector was constructed and cell transduction performed as described previously [22] and recombinant clones stably expressing hPEDF were selected with 1 mg/mL puromycine.

2.7. PEDF silencing

A549 cells were seeded in 6-well plates for 24 h, transfected with 20 nM of human PEDF small interfering (si) RNA (SC-40947; Santa Cruz Biotechnology; Santa Cruz, CA, USA) using Lipofectamine 300 transfection reagent (Invitrogen) [22] and analyzed after 48 h.

2.8. Protein identification by nano ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

Total protein (10 μg) in solution was precipitated with acetone (1:10 v/v) and centrifuged for 10 min at 20,000 × *g*. The dried protein residue was mixed with 16 μL of 25 mM NH_4HCO_3 and 2 μL dithiothreitol and incubated at 25 °C for 30 min; then, 2 μL iodoacetamide was added for another 30 min at 25 °C Finally, Trypsin/Lys-C Mix (0.1 μg/μL, Mass Spec Grade) was added and proteins were digested for 16 h at 37 °C. The digested peptide solution (2 μL) was injected into the nanoACQUITY UPLC system (Waters) and detected using a LTQ Orbitrap Discovery hybrid Fourier Transform Mass Spectrometer (Thermo Fisher Scientific, MS, USA) in the positive ion mode at a resolution of 30,000. Protein elution was performed with 0.1% formic acid as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. After elution from a desalting column (Waters Symmetry C18, 5 μm, 180 μm × 20 mm) using mobile phase A at a flow rate of 5 μL/min for 3 min, peptides were separated in an analytical column (Waters BEH C18, 1.7 μm, 75 μm × 150 mm) at a flow rate of 300 nL/min at the following conditions: 1% B for 0–1 min; 1–45% B for 1–20 min; 45–85% B for 20–30 min; 85% B for 30–35 min; 85–1% B for 35–45 min; and 1% B for 45–60 min. MS/MS data were acquired under the data-dependent mode, and raw data files were processed and proteins identified using the Mascot Distiller software and Mascot server (Matrix Science Inc., Boston, MA), respectively.

2.9. Field-emission transmission electron microscopy (FE-TEM)

FE-TEM was performed as described previously [23]. In brief, cells were fixed with 2.5% glutaraldehyde for 2 h at 4 °C, washed, post-fixed in 1% osmium tetroxide for 2 h, dehydrated in graded acetone, infiltrated, and embedded in Epoxy resin. Ultrathin 70-nm sections were cut using a Leica RM2165 microtome (Leica RM2165, Japan) and examined under an FE-TE microscope (HITACHI HT-7700, Japan) at an accelerating voltage of 80 kV.

2.10. Field-emission scanning electron microscopy (FE-SEM)

FE-SEM was performed as described previously [22]. Cultured cells were seeded on 0.17 mm-thick cover slips and fixed in 2.5% glutaraldehyde overnight at 4 °C, post-fixed in 2% osmium tetroxide for 1.5 h at 4 °C, and dehydrated in ascending grades of alcohol (50%, 75%, 85%, 95%, and 100%). Samples were dried using a critical point drier (CPD 030, Bal-TEC) for 1 h, coated in gold, and examined under a FE-SE microscope (Hitachi-8010, Japan) at accelerating voltage of 10–25 KV.

2.11. Tumorigenic potential of PEDF-overexpressing A549 lung cancer cells

Male 6–8 week-old SCID mice purchased from BioLasco Company (Taipei, Taiwan) were housed in a special pathogen-free room with a 12-h light/12-h dark cycle and 40–70% humidity at 19–25 °C and had free access to standard rodent diet and water *ad libitum*. Mice (n = 8 per group) were subcutaneously inoculated in the flanks with 0.1 mL PBS containing 1×10^7 of control or PEDF-expressing A549 cells and monitored for tumor growth twice a week for 40 days using a digital caliper. Tumor volume was calculated as V (mm^3) = length (L, mm) × width (W, mm^2) × 0.5. The protocol for the animal study was approved by DCB Institutional Animal Care and Use Committee (Approval No:102050).

2.12. Immunostaining

To assess PEDF and THBS1 protein expression, 5 μm-thick paraffin tissue sections or A549 cultured cells were incubated in blocking buffer (0.5% bovine serum albumin and 0.05% Tween 20 in PBS) for 1 h, at room temperature and then with specific primary antibodies against PEDF (1:1000; ab115489, Abcam), CD81 (1:500; GTX101766, Gene Tex), α-SMA (GTX100034, Gene Tex) and THBS1 (1:500; Rev 031506 K, Neomarkers) for 1 h and staining was developed using a fluorescence detection system (Ventana Medical Systems, Invitrogen). Samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) to visualize cell nuclei. After washing, sections were mounted in VECTASHIELD® mounting medium (Invitrogen) and examined under a confocal laser microscope (Leica, FL, USA).

2.13. Flow cytometry

Cancer-derived exosomes were labeled with a fluorescent dye Dil (Thermo Fisher Scientific) for 1 h and then added to A549 cultures for various times (0 h, 4 h, 8 h, and 24 h). Cells was collected and a minimum of 1×10^5 cells was analyzed in an LSR II Flow Cytometer (BD Biosciences, CA, USA).

2.14. Statistical analysis

The data were presented as the mean ± SEM and statistically analyzed by ANOVA and then by Dennett's test using SigmaStat version 3.5 (Systat Software Inc.). A p value less than 0.05 was considered statistically significant.

3. Results

3.1. PEDF regulates lung cancer cells aggregation, invasion, and migration

To investigate the role of PEDF in lung cancer, PEDF was overexpressed or silenced in cultured A549 cells by lentiviral transduction or RNA interference. The transduction with the *PEDF*-containing virus significantly upregulated whereas transfection with PEDF siRNA significantly downregulated PEDF expression both at the mRNA and protein levels (Fig. 1A, B), which corresponded to an increase and decrease, respectively, of PEDF release into culture medium (Fig. 1B). As metastatic cells have a reduced propensity for aggregation and cell-cell interaction [24], we examined whether PEDF affected cell aggregation ability. For this, we tested F-actin distribution in A549 cells with upregulated or downregulated PEDF expression or in cells treated with a

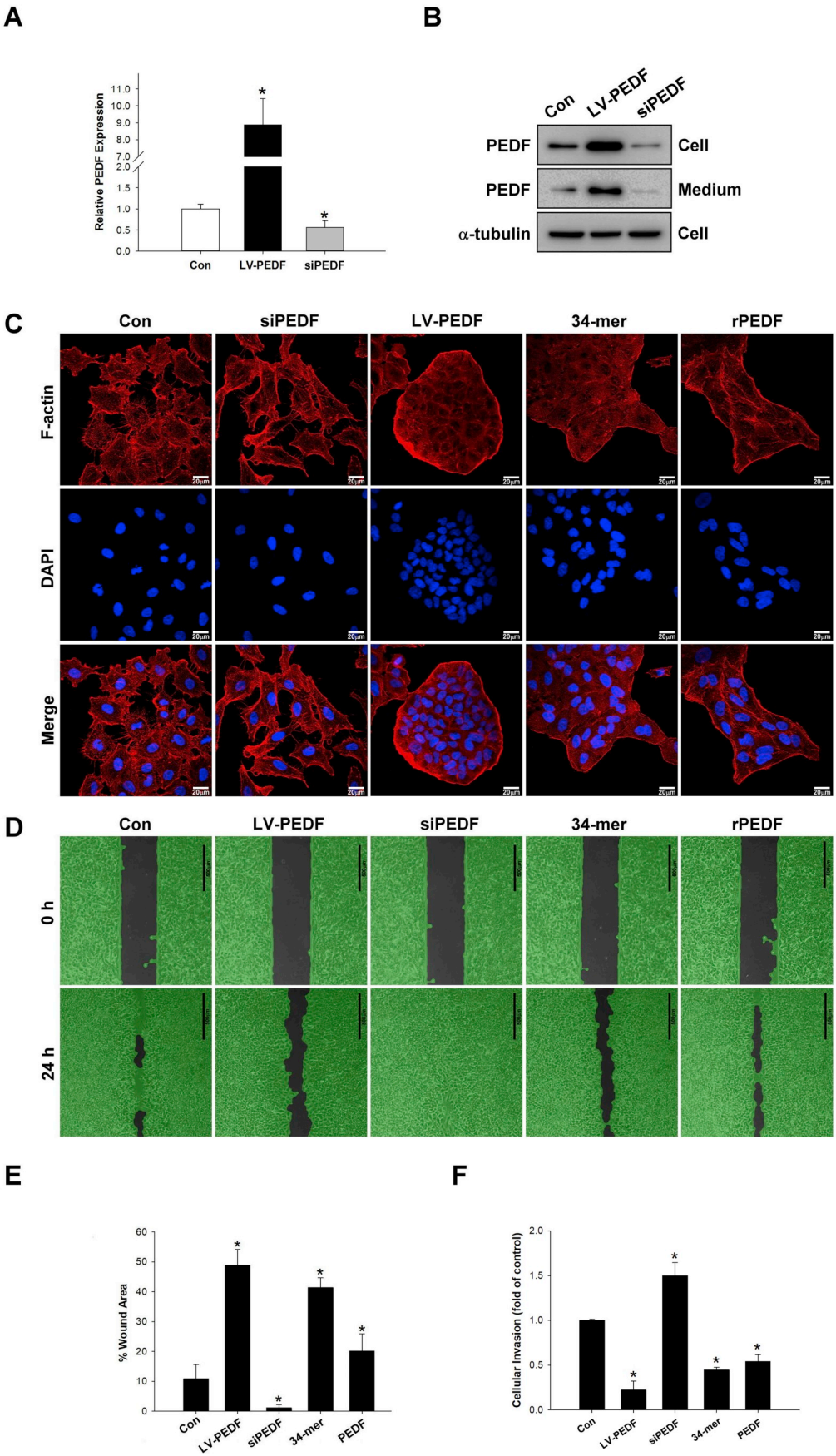

Fig. 1. PEDF regulates lung cancer cells aggregation, invasion, and migration. Lung cancer A549 cells were transfected with LV-PEDF or siPEDF or treated with recombinant 34-mer or full-length PEDF for 24 h (A, B) Overexpression or silencing of PEDF in A549 cells was assessed at the mRNA and protein levels by qRT-PCR (A) and western blotting (B). C, A549 cells were analyzed for cell-cell adhesion by immunocytochemistry (red, F-actin; blue, nuclei); scale bar, 20 μm. (D, E) A549 cell migration was analyzed by the wound healing assay; scale bar, 500 μm. (F) A549 cell invasion ability was examined by the Boyden chamber assay. *$P < 0.05$ compared with control (con). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

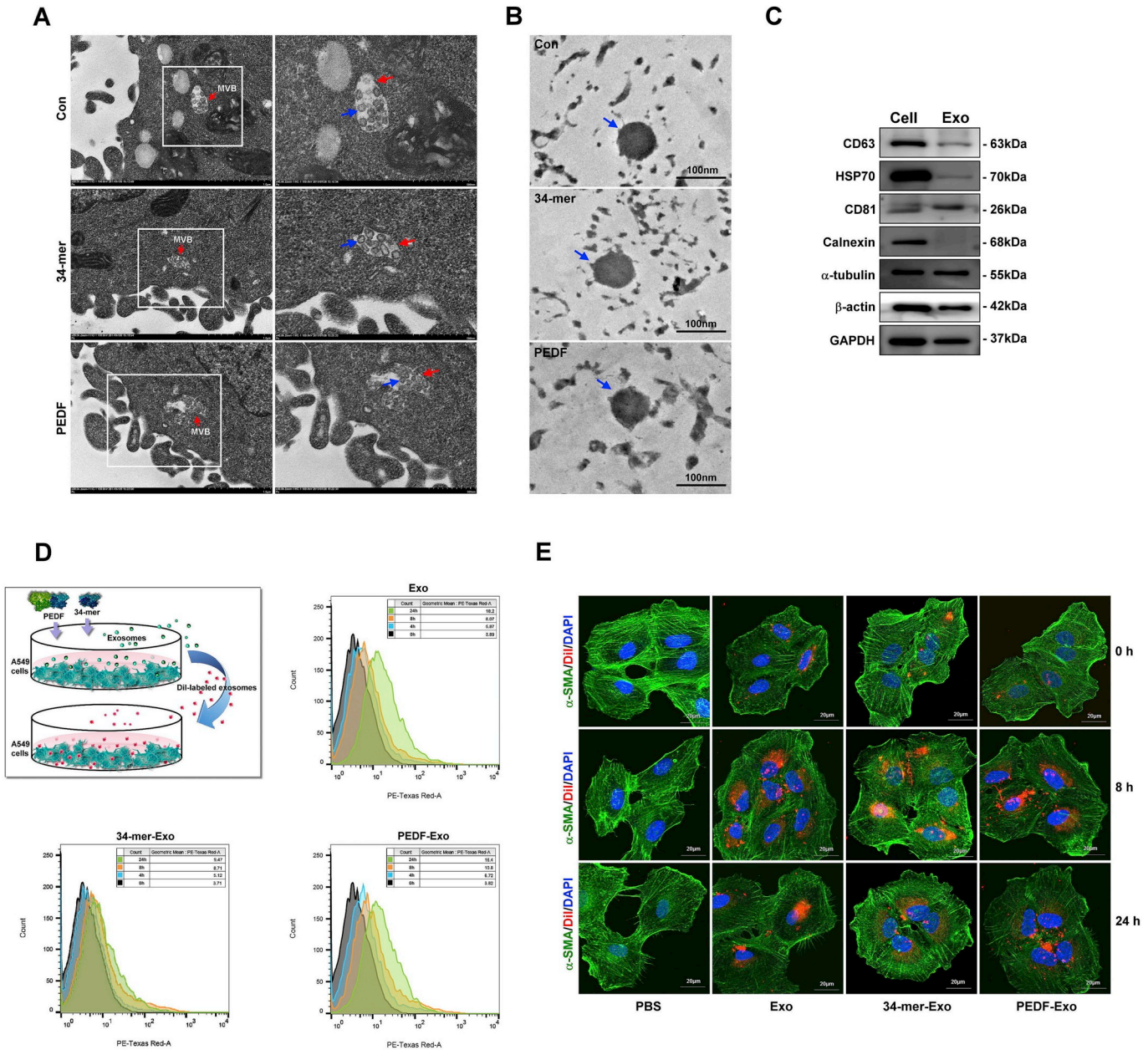

Fig. 2. Exosomes/microvesicles provide cancer cell-to-cell communication. A549 cells were treated with PBS or recombinant PEDF (34-mer or full-length) for 24 h (A, B) The presence of MVBs and exosomes was analyzed by FE-TEM (red arrow, MVB; blue arrow, exosome; scale bars, 1 μm and 500 nm) (A) and isolated exosome from A549 ells (scale bars, 100 μm) (B). (C). Exosomal markers CD63, CD70, and CD81, cytoskeleton proteins α-tubulin and β-actin, and endoplasmic reticulum marker calnexin were analyzed by western blotting. (D, E) Exosomes were labeled with Dil for 1 h and added to A549 cell cultures for incubated for the indicated times; cells were then analyzed for exosome uptake by flow cytometry (D) and confocal laser scanning microscopy (green, α-SMA; red, exosomes; blue, nuclei); scale bar, 20 μm (E). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

recombinant full-length PEDF or a 34-mer PEDF peptide for 48 h. Confocal microscopy analysis suggested that PEDF overexpression or treatment with recombinant PEDF (34-mer or full-length) significantly increased cancer cell aggregation, whereas PEDF silencing markedly reduced cell-cell adhesion compared with the other groups (Fig. 1C). Furthermore, wound healing and Boyden chamber assays indicated that lung cancer cell migration and invasion were inhibited by PEDF overexpression or treatment with external PEDF; in contrast, PEDF silencing significantly promoted cancer cell migration and invasion compared with control (Fig. 1D–F).

3.2. Exosomes/microvesicles are uptaken by lung cancer cells

As tumor-secreted extracellular vesicles, including microvesicles and exosomes, play a significant role in cancer development [25,26], we examined exosomes located in multivesicular bodies (MVBs) of lung cancer cells by FE-TEM (Fig. 2A). The results indicated that in both control and PEDF-treated cells, the isolated exosomes showed size distribution between 50 and 250 nm (Fig. 2B) and expressed exosomal markers such as CD63, HSP70, and CD81, cytoskeletal proteins α-tubulin and β-actin, and GAPDH, whereas the endoplasmic reticulum marker calnexin was not detected (Fig. 2C). To study the effects of PEDF on exosome uptake and internalization by lung cancer cells, exosomes isolated from A549 cells treated or not with the 34-mer or full-length

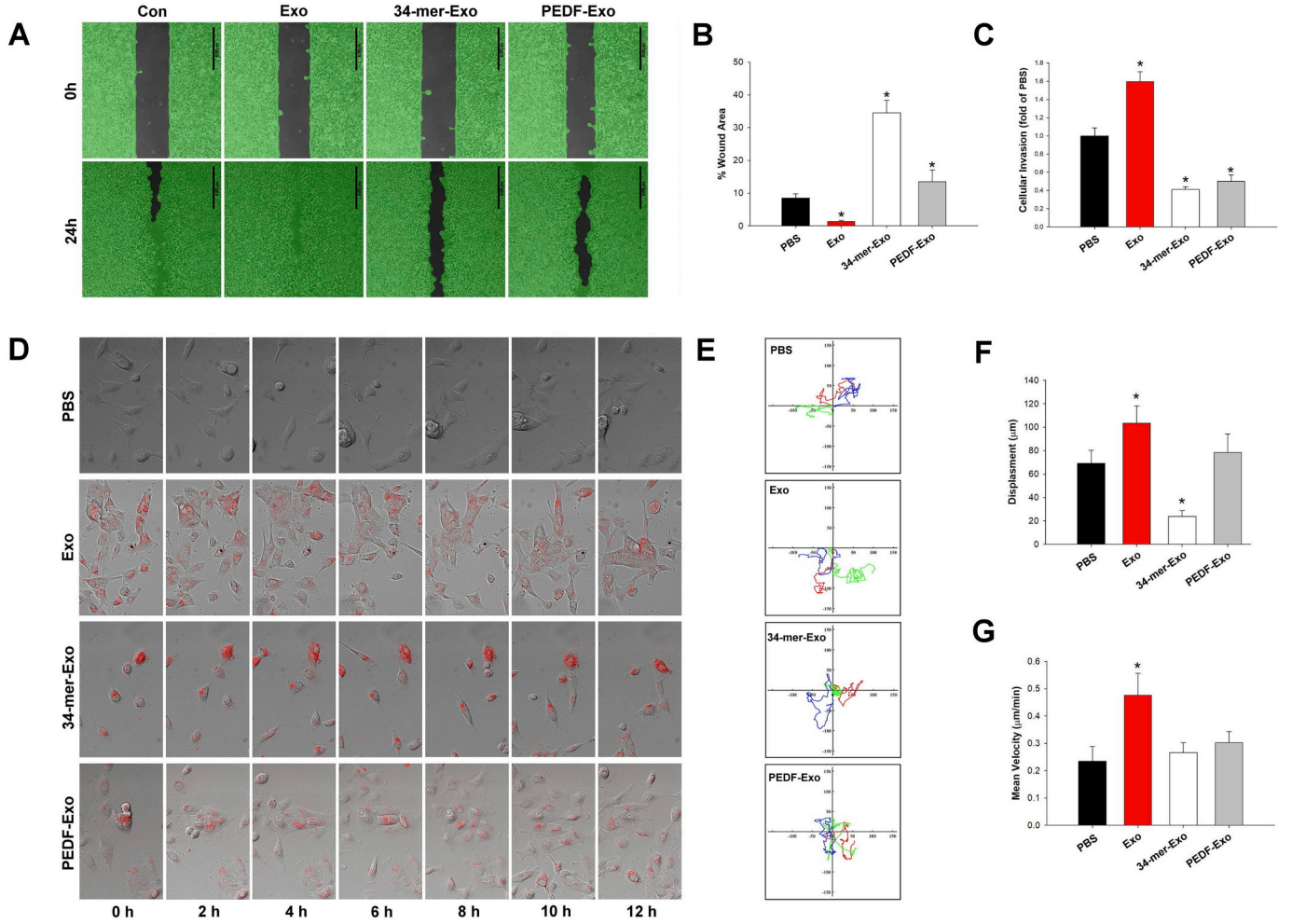

Fig. 3. PEDF attenuates exosome-mediated lung cancer cell migration, invasion, and motility. A549 cells were incubated for 24 h with PBS or Dil-labeled exosomes isolated from cells treated with PBS (Exo), 34-mer peptide (34-mer-Exo), or full-length PEDF (PEDF-Exo). (A, B) Cell migration was analyzed by the wound healing assay (scale bar, 500 μm). (C) Cell invasion was analyzed by the Boyden chamber assay. (D–G) Cells were analyzed by time-lapse microscopy for migration distance and speed (magnification, × 20) (E), displacement (F), and mean velocity (G). *$P < 0.05$ compared with PBS.

PEDF for 24 h were labeled with fluorescent dye Dil and added to fresh A549 monolayers for various times. Cells were analyzed for the presence of Dil granules by flow cytometry (Fig. 2D) and confocal laser microscopy (Fig. 2E); staining for alpha-smooth muscle actin (α-SMA), a cell skeleton protein involved in cell motility, structure, and integrity was used to observe Dil intracellular distribution. The results indicated that exosomes from lung cancer cells treated or not with recombinant PEDF were taken by other lungs cancer cells and localized around the nucleus.

3.3. PEDF attenuates lung cancer cell migration, invasion, and motility induced by cancer cell-derived exosomes

To determine whether PEDF could regulate cancer-derived exosome-mediated lung cancer migration and invasion, exosomes were isolated from the medium of A549 cells pretreated or not with 34-mer or full-length PEDF for 24 h and added to new A549 cells, which were then analyzed for migration (Fig. 3A and B) and invasion (Fig. 3C). The results indicated that PEDF treatment significantly reduced lung cancer cell migration stimulated by cancer cell-derived exosomes. To confirm the inhibitory effect of PEDF on exosome-induced cancer cell migration, labeled exosomes were added to A549 cells for 12 h and the mean cell migration speed and trajectories were analyzed by time-lapse microscopy (Fig. 3D and E). The data showed that cancer cell-derived exosomes markedly increased cell motility (migration distance and speed); however, exosomes from A549 cells pretreated with 34-mer or full-length PEDF significantly inhibited cell motility compared with the other groups. Analysis of cell displacement (Fig. 3F) and mean velocity (Fig. 3G) also indicated that exosomes derived from PBS-treated cancer cells promoted cell movement, whereas those from 34-mer- or full-length PEDF-treated cells had no effect. It is particularly noteworthy that exosomes obtained from 34-mer PEDF-pretreated cells significantly inhibited cell displacement compared with the other groups, which may be related to the inhibitory effect of the 34-mer on Wnt signaling [27] involved in tumor progression, metastasis, and epithelial-to-mesenchymal transition [28].

3.4. PEDF regulates protein content of lung cancer cell-derived exosomes

To compare protein composition of cancer cell-derived exosomes, they were isolated from A549 cells treated or not with 34-mer or full-length PEDF and subjected to proteomic analysis using UPLC-MS/MS (Fig. 4A). A549 cell-derived exosomes contained a number of proteins implicated in cancer invasion, including osteopontin-α (OPN-α), TGF-β1, α2-macroglobulin (ACTBL2), gelsolin (GSN), α2-Heremans-Schmid glycoprotein (AHSG), apolipoprotein (APO), type XII collagen (COL12A1), fibulin-1 (FBLN1), CD83, E2-mediated cathepsin D (CTSD), collagen alpha-3(V) (COL5A3), and epidermal growth factor receptor (EGFR). However, treatment of A549 cells with 34-mer or full-length PEDF resulted in the appearance of THBS1 in exosomes, which was

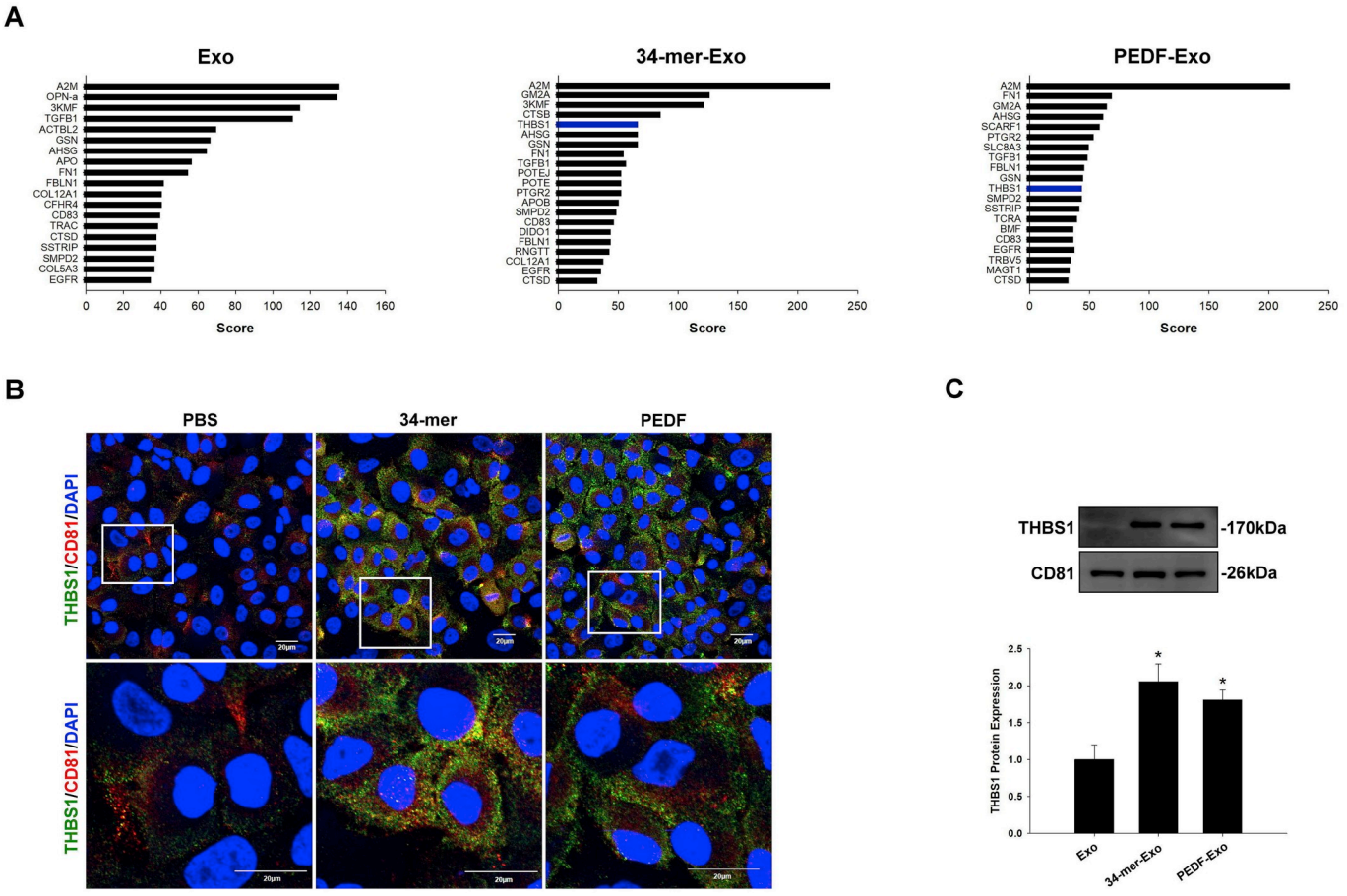

Fig. 4. PEDF regulates lung cancer-derived exosome-mediated secretion *in vitro*. Exosomes were isolated from A549 cells treated with PBS (Exo), 34-mer peptide (34-mer-Exo), or full-length PEDF (PEDF-Exo) for 24 h. (A) Proteomic analysis of exosomes performed by UPLC-MS/MS. (B, C) THBS1 expression in exosomes was verified by immunocytochemistry (B) and western blotting (C). Green, THBS1; red, CD81 used as exosomal marker; blue, nuclei. Scale bars, 20 μm $^*P < 0.05$ compared with Exo. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

further confirmed by immunocytochemistry (Fig. 4B) and western blotting (Fig. 4C). These findings indicate that PEDF increased THBS1 presence in lung cancer cell-derived exosomes.

3.5. THBS1 inhibits lung cancer cell migration and invasion stimulated by cancer cell exosomes

To clarify the effects of THBS1, A549 cells were incubated with recombinant THBS1 (1 μg/mL) or exosomes derived from cells treated with THBS1 alone or together with its peptide inhibitor LSKL (Leu-Ser-Lys-Leu, 20 μM), and analyzed for cell migration and invasion (Fig. 5A–C). The results showed that THBS1 and exosomes from THBS1-treated cells significantly downregulated cancer cell migration and invasion, but LSKL reversed the effect. These findings were supported by time-lapse microscopy analysis of cell migration distance, speed, and trajectories (Fig. 5D–G and Supplemental Videos 1–4), which confirmed that THBS1 strongly inhibited exosome-induced cancer cell migration. Collectively, these data indicate that PEDF may inhibit lung cancer cell metastasis through increase of THBS1 content in cancer cell exosomes.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.canlet.2018.10.031.

3.6. THBS1 increases the surface area and decreases the thickness of cancer cells by regulating the formation of lamellipodia and filopodia

To examine whether THBS1 has anti-metastatic effects in lung cancer, A549 cells were incubated with THBS1 or exosomes derived from cells treated or not with THBS1 or THBS1 + LSKL, and analyzed for cell morphology, surface area, and formation of lamellipodia and filopodia using FE-SEM. The results showed that THBS1 increased cancer cell surface area (Fig. 6A) and decreased cell thickness, while significantly inhibiting the formation of filopodia (Fig. 6B–F). Cytoskeletal rearrangements could promote the formation of filopodia, which was shown to be associated with β1 integrin-dependent adhesion to intact THBS1 [29]. In contrast, exosomes stimulated the appearance of large lamellipodia and increased the number of filopodia and their length. Inhibition of THBS1 by LSKL did not effectively inhibit the formation of filopodia by cancer cells.

3.7. PEDF suppresses tumorigenicity of NSCLC cells in vivo

To verify the contribution of PEDF to tumorigenic potential of lung cancer cells, PEDF-expressing and control A549 cells were subcutaneously transplanted into the flanks of SCID mice and monitored for tumor growth (Fig. 7A). Mice were euthanized 40 days after implantation and analyzed for tumor volume; the expression of PEDF and THBS1 in tumor tissue was evaluated by RT-PCR, western blotting, and immunohistochemistry. The results showed that PEDF-overexpressing A549 cells formed smaller tumors (Fig. 7B and C), which had higher THBS1 expression both at the mRNA (Fig. 7D) and protein (Fig. 7E–G) levels compared to control, suggesting that PEDF inhibited the growth of lung cancer cells through downregulation of THBS1.

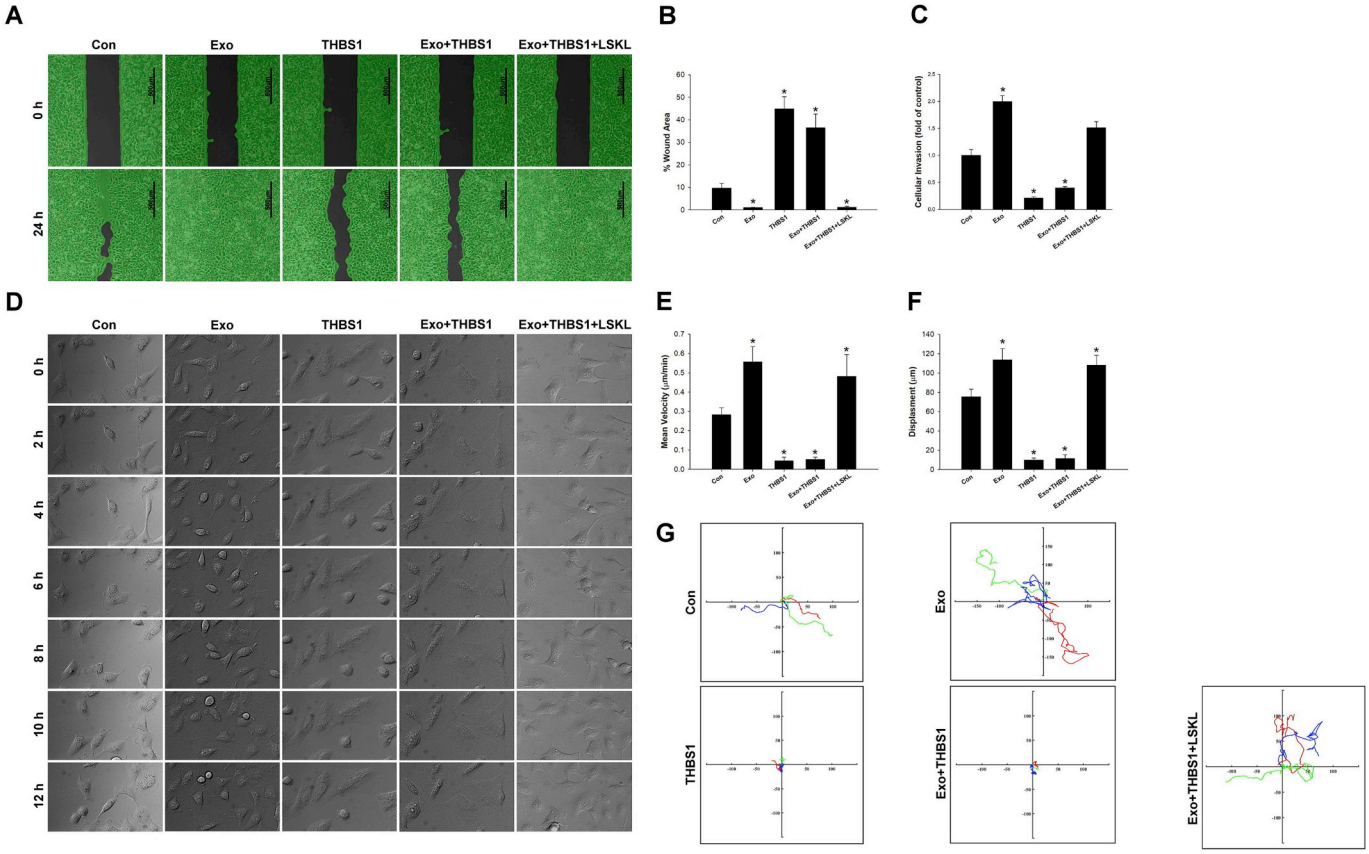

Fig. 5. THBS1 inhibits exosome-mediated migration and invasion of A549 cells. A549 cells were incubated for 24 h with PBS or recombinant THBS1 or exosomes isolated from cells treated with PBS (Exo), THBS1, or THBS1 with LSKL. (A, B) Cell migration was analyzed by the wound healing assay (scale bar, 500 μm). (C) Cell invasion was examined by the Boyden chamber assay. (D–G) Cells were analyzed by time-lapse microscopy for migration distance and speed (magnification, × 20) (D, E), displacement (F), and mean velocity (G). *$P < 0.05$ compared with control.

4. Discussion

In this study, we showed that PEDF attenuated lung cancer cell migration, invasion, and motility by regulating the protein content of cancer cell-derived exosomes. In lung cancer cells, PEDF significantly enhanced intracellular expression and exosomal levels of THBS1, which resulted in the increase of cell adhesion and inhibition of cancer cell migration, invasion, and motility.

PEDF is a secreted glycoprotein with anti-angiogenic, immunomodulation, and neuroprotective activities, which also showed anti-tumorigenic effects in many types of cancers, including breast cancer [30], prostatic cancer [12], renal cell carcinoma [31], pancreatic carcinoma [32], and lung cancer [33]. Our data revealed that both full-length and 34-mer recombinant PEDF effectively stimulated aggregation and inhibited migration and invasion of NSCLC cells, indicating decrease of their metastatic potential.

In lung cancer, PEDF is detected in plasma and pleural effusion where it is phosphorylated at Ser24 and Ser114 by casein kinase 2 (CK2) and at Ser227 by protein kinase A (PKA), which can be associated with PEDF anti-cancer effects [34]. It was also reported that exosomes present in pleural fluid of lung cancer patients contained PEDF and THBS1 [35], suggesting a role of PEDF/THBS1-positive exosomes in lung cancer. Therefore, we tested a hypothesis that PEDF could inhibit NSCLC progression through an exosome-mediated mechanism. Our results indicate that NSCLC cell-derived exosomes contain multiple proteins implicated in cancer development (such as OPN-α, TGF-β1, α2-ACTBL2, GSN, AHSG, APO, COL12A1, FBLN1, CD83, CTSD, COL5A3, and EGFR) and execute paracrine crosstalk among lung cancer cells, regulating their migration, invasion, and motility. However, exosomes from PEDF-treated A549 cells demonstrated diminished cancer-stimulating activity which correlated with dramatic increase in exosomal THBS1, suggesting that PEDF inhibited NSCLC aggressive behavior by increasing THBS1 content in exosomes.

THBS1 is an extracellular matrix protein playing an important role in the tumor microenvironment. The interaction between THBS1 and cell surface molecules modulate a number of carcinogenesis-related processes, including angiogenesis [36], tumor cell-cell adhesion [37], proliferation [38], apoptosis, chemoresistance, invasion, migration [39], and motility [40]. Serum THBS1 level was shown to reflect tumor aggressiveness and has been suggested as a possible prognostic marker in patients with primary resected NSCLC [41]. However, there is no information regarding the association of lung cancer-derived exosomes and THBS1 functional activity in the tumor microenvironment. Our finding showed that recombinant PEDF significantly upregulated THBS1 expression in the cytosol and its presence in cancer-derived exosomes, indicating that PEDF promotes the secretion of THBS1 by exosomes, which may be a mechanism underlying PEDF inhibitory activity in the pre-metastatic niche. THBS1 decreased cell migration characteristics, including distance and mean velocity, which are increased by cancer cell-derived exosomes, suggesting that PEDF inhibits lung cancer metastatic activity through regulation of THBS1 expression.

It was shown that cancer metastasis correlates with cytoskeletal rearrangement manifested by the formation of cellular protrusions such as lamellipodia and filopodia [41]. Our results indicated that exosomes induced the formation of lamellipodia and filopodia by NSCLC cells, whereas THBS1 and exosomes from THBS1-treated cells reduced cellular protrusions and induced cancer cell morphological changes manifested by significant increase in cell surface area and decrease in thickness. These results are consistent with a recent report that a THBS1-specific monoclonal antibody inhibited the formation of

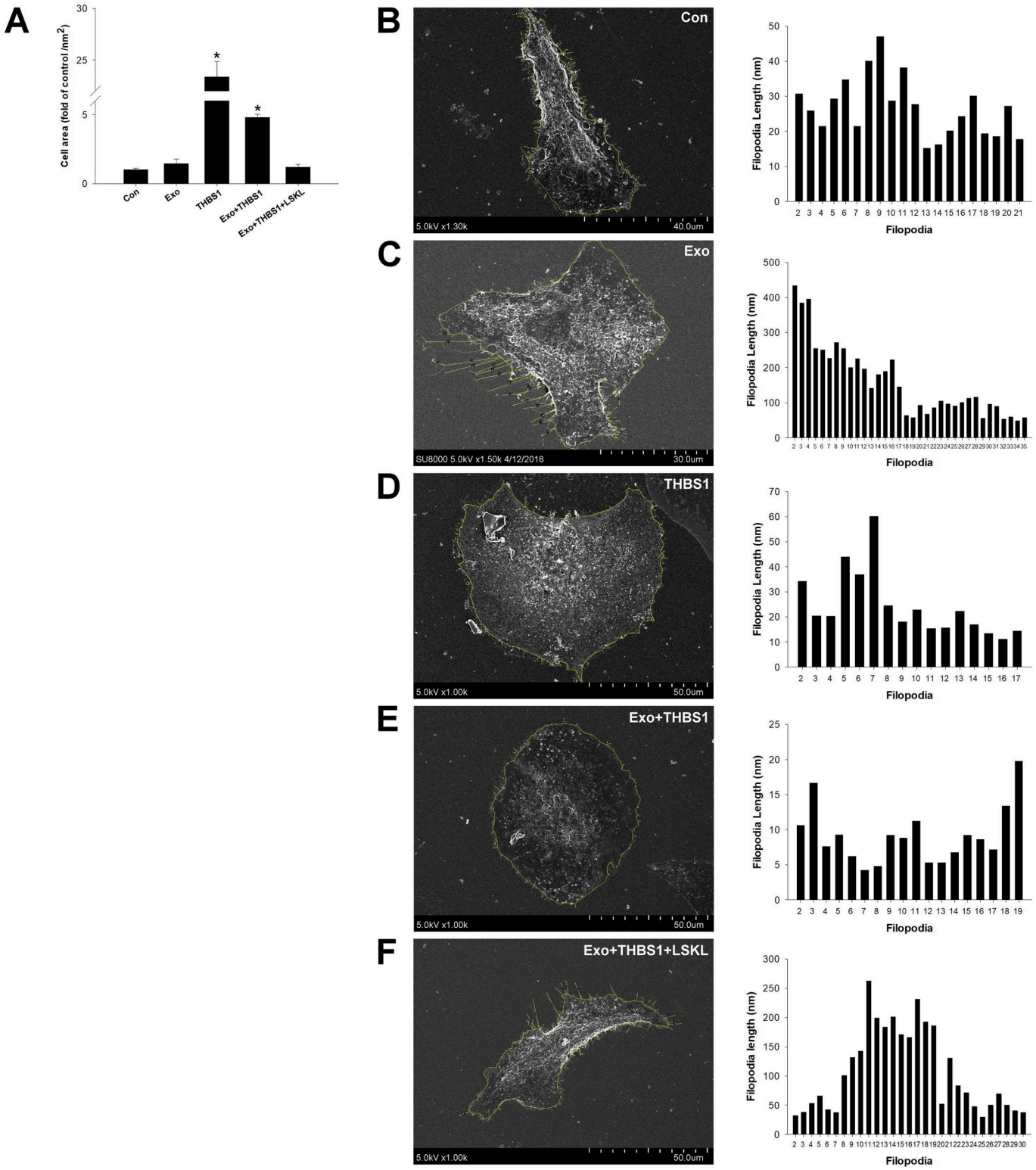

Fig. 6. THBS1 regulates cancer cells surface area and formation of lamellipodia and filopodia. A549 cells were incubated for 24 h with PBS or recombinant THBS1 or exosomes isolated from cells treated with PBS (Exo), THBS1, or THBS1 with LSKL. (A) Cell surface area measured by FE-SEM; *$p < 0.05$ compared with control. (B–F) The formation of filopodia was analyzed by FE-TEM.

pseudopodia by adherent T cells, suggesting that THBS1 participates in an adhesion-dependent mechanism controlling T lymphocyte spreading and migration [42]. Currently, the mechanistic understanding of THBS1 effects on cytoskeletal remodeling is limited. There is a report that *THBS1* gene knockout induces the expression of a small GTPase Cdc42, which regulates actin organization, and promotes filopodium formation in retinal endothelial cells, leading to neoangiogenesis [43].

Our study has some limitations as most of the experiments were performed *in vitro*. First, exosomes were isolated only from cultured lung cancer cells and not from animals. Second, the inhibitory effects of PEDF or THBS1 were studied in an NSCLC cell line, and should be further validated using PEDF and/or THBS1 knockout animal models.

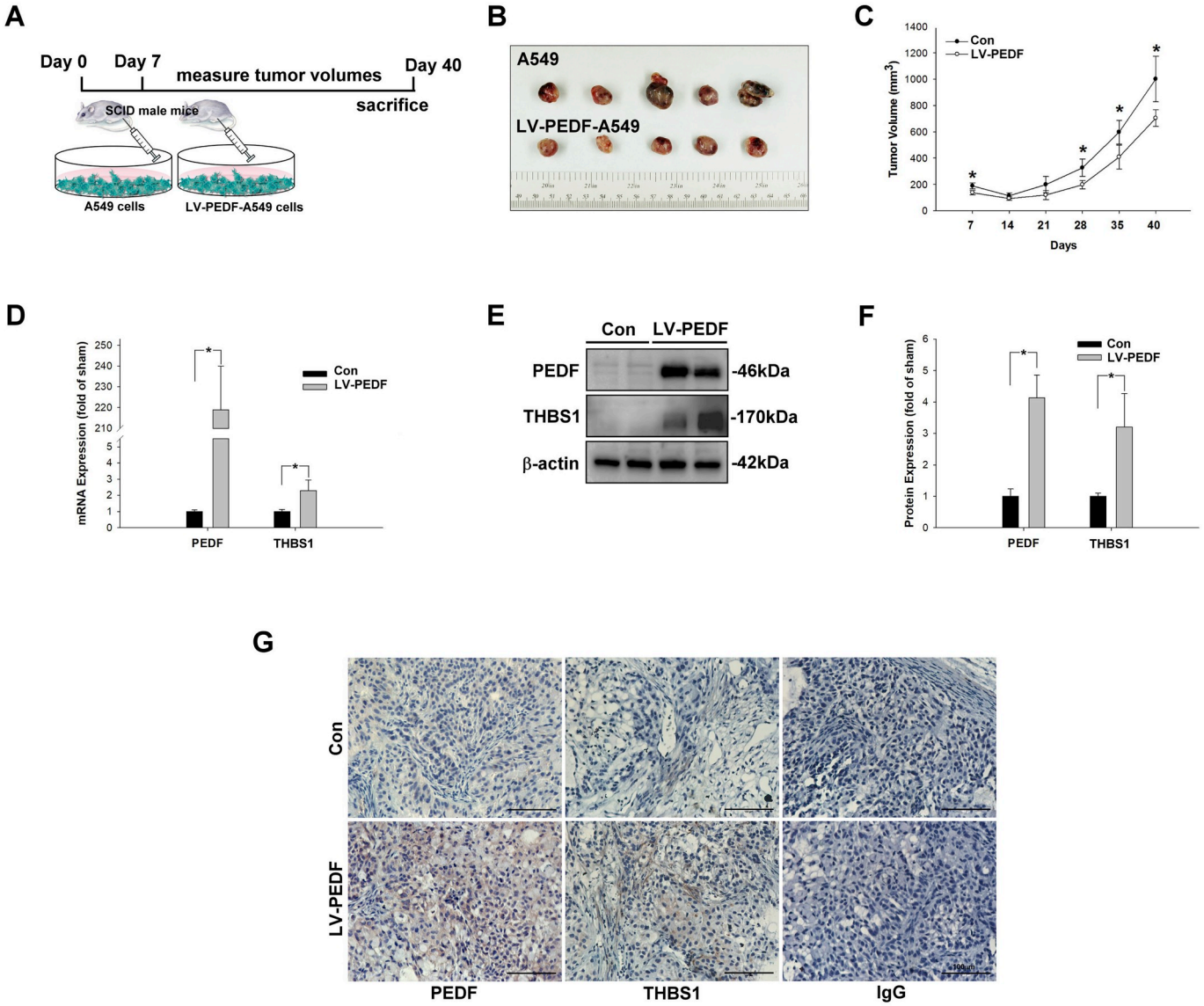

Fig. 7. PEDF inhibits tumorigenicity of lung cancer cells *in vivo*. (A) Schematic diagram of the experimental process. A xenograft model was established in SCID mice subcutaneously inoculated with control or PEDF-overexpressing (LV-PEDF) A549 cells (n = 8 mice/group). (B, C) Size of tumors was measured at day 40. (D–G) Expression of PEDF and THBS1 mRNA and protein in tumor tissues was assessed by real-time PCR (D), western blotting (E, F), and immunohistochemistry (G). *$P < 0.05$. compared with control.

In conclusion, our results suggest that PEDF exerts anti-lung cancer effects through regulation of THBS1 expression and release in cancer cell-derived exosomes. PEDF and THBS1 significantly reduced the metastatic potential of NSCLC cells by suppressing their migration, invasion, and motility. THBS1 prevented cancer spread-related cytoskeletal rearrangements such as the formation of lamellipodia and filopodia and increased cell surface area (Fig. 8). Further studies of PEDF-regulated signaling pathways implicated in exosome secretion and cancer progression are required to validate the therapeutic potential of PEDF in lung cancer.

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Conflicts of interest

There are no potential conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.10.031.

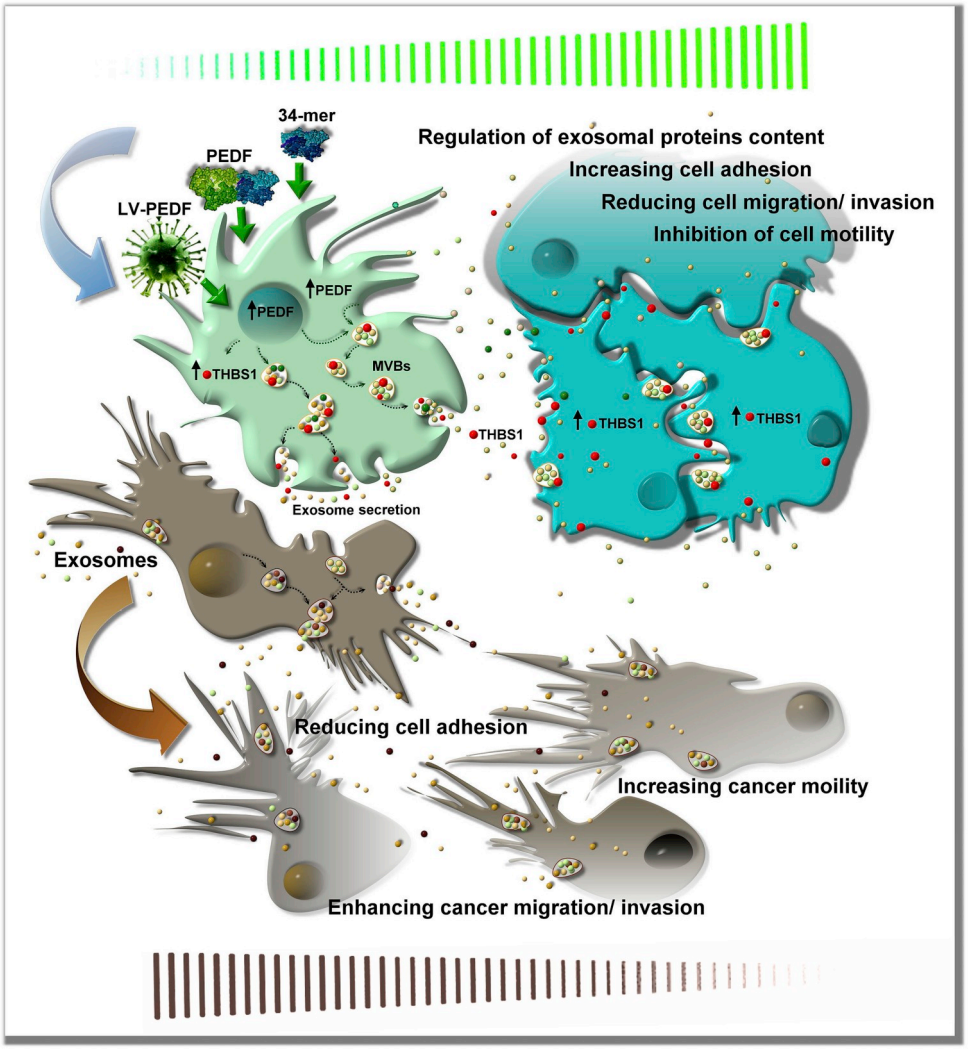

Fig. 8. Schematic representation of putative mechanisms underlying PEDF inhibition of lung cancer progression by cancer cell-derived exosomes. PEDF upregulates THBS1 levels in the cytoplasm and exosomes, thus promoting cell-cell adhesion, increasing cell surface area, and inducing the formation of lamellipodia and filopodia, which results in the inhibition of cancer cell migration, invasion, and motility.

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