

Podoplanin-expressing cancer-associated fibroblasts lead and enhance the local invasion of cancer cells in lung adenocarcinoma

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Cancer-associated fibroblasts (CAFs) communicate with cancer cells and play important roles in cancer invasion. We previously reported that local invasion of cancer cells was frequently observed in lung adenocarcinoma patients with podoplanin (PDPN)-expressing CAFs. However, the underlying mechanisms of this phenomenon have remained unclear. In this study, we established a novel collagen invasion assay model in which cancer cells and CAFs were cocultured; we analyzed the mechanisms governing how cancer cell invasion was promoted by PDPN(+)CAF. By observing the dynamic movement of both CAFs and cancer cells in the collagen matrix, we found that PDPN(+)CAF invaded the matrix to a greater extent, with more cancer cells invading within the “tracks” created by the CAFs, compared with control CAFs. The knockdown of PDPN in CAFs decreased the invasion of both the CAFs and the cancer cells. PDPN(+)CAF displayed a higher RhoA activity and treatment with a ROCK inhibitor cancelled the increased invasion ability of PDPN(+)CAF and subsequently decreased the number of invaded cancer cells. After intravenous injection in the mouse tail vein, PDPN(+)CAF invaded and promoted cancer cell invasion into the lung parenchyma, compared with control CAFs. Among the patients with lung adenocarcinoma, we observed some cases with PDPN(+)CAF at the invasive front of the tumor. These cases predominantly exhibited pleural invasion of cancer cells, known as pathological invasiveness. Our results indicated that PDPN(+)CAF were tumor-promoting CAFs that lead and enhance the local invasion of cancer cells, suggesting that the invasion activity of CAFs themselves could be rate-determining for cancer cell invasion.

Cancer tissue is composed of not only cancer cells but also different kinds of stromal cells including fibroblasts, immune cells and endothelial cells.^{1–3} These components create and orchestrate a favorable microenvironment for cancer cells and influence the proliferation, survival and invasion of cancer cells.^{4–6} Fibroblasts that are found in association with cancer cells are known as cancer-associated fibroblasts (CAFs); these cells acquire a specific biological phenotype *via* interactions with cancer cells. The biological importance of CAFs in tumor progression has been supported by extensive

clinical evidence and the use of experimental mouse models.^{2,7–9} However, CAFs are heterogeneous and different types of CAFs make distinct functional contributions to cancer progression.^{7,10–16} Our previous studies have documented the impact of podoplanin (PDPN) expression in CAFs on the malignant biological behavior of cancer cells and clinically poor prognosis.^{17–21}

Human PDPN is a 38 kDa type-1 transmembrane glycoprotein consisting of 162 amino acids, 9 of which form the intracellular domain. The extracellular domain is highly O-

Key words: cancer-associated fibroblasts, podoplanin, cancer invasion, tumor microenvironment

Additional Supporting Information may be found in the online version of this article.

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What's new?

The protein podoplanin helps tumors push into the surrounding tissue, and thus may be a useful target for slowing down the disease. Cancer-associated fibroblasts (CAFs) communicate with cancer cells and assist with invasion. In this paper, the authors report that CAFs expressing podoplanin boosted the efficiency of a lung tumor's invasion of the matrix. Knocking down podoplanin, furthermore, reduced the invasiveness of both the CAFs and the tumor cells. Finally, the authors showed that this activity relied on the Rho-ROCK pathway, and treatment with a ROCK inhibitor stifled the cells' ability to invade.

glycosylated, with sialic acid, α -2, 3 linked to galactose, forming the main part of the protein's carbohydrate moieties.²² We have reported that the recruitment of PDPN-positive CAFs is correlated with a poor outcome among lung adenocarcinoma patients.^{18,20,21} Furthermore, *in vitro* and *in vivo* studies have revealed that PDPN molecule on these CAFs promote lung adenocarcinoma engraftment into SCID mice.¹⁷ These studies suggest that PDPN-expressing CAFs create a supportive microenvironment that enhances tumor progression. Moreover, in other types of malignant tumors including intrahepatic cholangiocarcinoma, breast cancer, esophageal cancer and melanoma, a higher level of PDPN expression in CAFs is reportedly correlated with a poor outcome.^{23–27}

The molecular mechanism explaining how PDPN-expressing CAFs can contribute to generating a supportive tumor microenvironment has not been clarified. Clinicopathological findings show that PDPN-positive CAFs are significantly correlated with local invasiveness, such as vascular, lymphatic and pleural invasions, in lung adenocarcinoma patients.^{18,20} Therefore, we hypothesized that PDPN-expressing CAFs facilitate the local invasion of cancer cells.

In this study, we established an *in vitro* collagen invasion assay model; we then analyzed how CAFs expressing PDPN enhance cancer cell invasion in the extracellular matrix and investigated the molecular mechanisms. By observing the dynamic movement of CAFs and cancer cells cocultured in this invasion assay model, we found that CAFs expressing PDPN invaded the collagen matrix to a greater extent and more cancer cells invaded within the tracks created by the PDPN-expressing CAFs. We further found that CAFs expressing PDPN generate tracks in the matrix through the high activity of the Rho-ROCK pathway, thereby promoting cancer cell invasion.

Material and Methods**Cell culture**

CAFs were obtained from the surgically resected tumors of lung adenocarcinoma patients. Each tumor was cut into pieces and the pieces were plated on a culture dish containing Minimum Essential Medium α (MEM α , Life Technologies) with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin and streptomycin (Sigma-Aldrich). The medium was changed every other day until the tissue was surrounded by adherent fibroblasts. The tissue was then

removed and cultured for two more days. When the cells reached 80% confluence, they were harvested with 0.25% trypsin and 1 mM EDTA and then replated at a density of 1×10^4 cells/cm². All the specimens were collected after the subjects had provided their written informed consent and the specimen collection was approved by the Institutional Review Board of our institution. We analyzed two primarily cultured CAFs from lung cancer patients. The positive percentage of the intrinsic PDPN expression by FACS analysis in these CAFs ranged 21.2 – 28.8% (Pt1-CAFs), 26.9 – 36.3% (Pt2-CAFs), respectively (data not shown). We used Pt1-CAFs for PDPN-overexpressing experiments and Pt2-CAFs for PDPN-knockdown experiments. The human lung adenocarcinoma cell lines, A549 (American Type Culture Collection) and PC-9 (Public Health England), were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12, Sigma-Aldrich) containing 10% FBS and 1% penicillin and streptomycin and RPMI-1640 Medium (Sigma-Aldrich) containing 2 mM glutamine, 10% FBS and 1% penicillin and streptomycin, respectively. All the CAFs and cell lines were incubated at 37 °C in an atmosphere containing 5% CO₂.

Expression of DsRed2 fluorescent proteins in cancer cell lines

A549 and PC9 cancer cells were transfected with the pDsRed2-Nuc vector (Clontech Laboratories Inc.) using LipofectAMINE 2000 reagent (Life Technologies) according to the manufacturer's instructions. Stable transformants were selected using G418 (Sigma-Aldrich) and FACSaria II cell sorting. The localization of pDsRed2-Nuc in the nucleus of cancer cells facilitated the measurement of the number of cancer cells for the *in vitro* experiments.

Western blot analysis

The cells were lysed in RIPA buffer (Thermo Scientific) containing complete, a protease inhibitor cocktail tablet and PhosSTOP, a phosphatase inhibitor cocktail tablet (Roche). The proteins were separated on a 7.5 or 12% SDS-polyacrylamide gel and were transferred to an Immobilon-P, PVDF membrane (Millipore). The blots were incubated overnight at 4 °C with anti-phospho-ERM antibody (Cell Signaling Technology, #3149), antiphospho-Cofilin antibody (Cell Signaling Technology, #3311), antiphospho-myosin light chain 2 antibody (Cell Signaling Technology, #3675) or anti-

α -tubulin antibody (Cell Signaling Technology, #2144). After washing in TBS-T, the membranes were incubated with anti-rabbit IgG, HRP-linked whole antibody from donkey or anti-mouse IgG, HRP-linked whole antibody from sheep (GE Healthcare). ECL Western Blotting Detection Reagents (GE Healthcare) were used to develop the high-performance chemiluminescence film (GE Healthcare).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells were suspended in 1 ml of TRIzol (Life Technologies) and stored at -80°C . Total RNA was purified from thawed samples using standard techniques and cDNA was synthesized using the PrimeScript RT reagent Kit (TaKaRa), according to the manufacturer's instructions. RT-PCR was performed in a Smart Cycler System (TaKaRa) using SYBR Premix Ex Taq II (TaKaRa) according to the manufacturer's instructions. The PDPN primers were described in our previous study.¹⁷

Flow cytometry and cell sorting

The cells were incubated with anti-PDPN (clone 18H5, Abcam) antibody and excess antibody was removed by washing with PBS (containing 1% FBS). Goat anti-mouse IgG F(ab')₂-APC (Santa Cruz Biotechnology) was added as a secondary antibody. The cells were rinsed with PBS and a FACS analysis was performed using FACSCalibur (BD Biosciences). Sorting was performed using a FACSaria II (BD Biosciences).

Lentiviral vectors

Lentivirus vectors containing human wild-type PDPN (WT-PDPN) or mutant PDPN devoid of the cytoplasmic region (PDPN-del.IC) were generated as described previously.^{17,19} Briefly, cDNAs for WT-PDPN (residues 1–162) and PDPN-del.IC (residues 1–153) were obtained by PCR amplification using the pcDNA3/human-WT-PDPN vector (kindly provided by Dr. N. Fujita, Japanese Foundation for Cancer Research) and were inserted into pENTR2B (Life Technologies). Thereafter, LR recombination reaction was performed between the entry clones and CSII-CMV-RfA-IRES2-Venus (RIKEN BioResource Center), using Gateway LR Clonase (Life Technologies) according to the manufacturer's instruction. For the PDPN short hairpin RNA (shRNA) experiments, the oligonucleotides described in our previous study were chemically synthesized.¹⁷ To create entry clones, the top and bottom strands of the oligonucleotide were annealed and ligated into pENTR4-H1 (RIKEN BioResource Center). Similar to the generation of the lentiviral vectors expressing cDNAs, an LR recombination reaction was performed between the entry clones and CS-RfA-EG (RIKEN BioResource Center) using Gateway LR Clonase. The oligonucleotides used for the amplification of all these constructs are described in our previous studies.^{17,19}

The lentiviruses were produced using 293T cells transfected with PCAG-HIV, pCMV-VSV-G-RSV-Rev and either a WT-PDPN, PDPN-del.IC, PDPN shRNA or mRFP (CSII-

CMV-mRFP1; RIKEN BioResource Center). Transfection was achieved using the LipofectAMINE 2000 reagent, according to the manufacturer's instructions. Vector-containing medium was filtered through a $0.45\text{-}\mu\text{m}$ filter and $8\text{ }\mu\text{g/ml}$ of polybrene (Santa Cruz Biotechnology) was added for target cell transduction.

Collagen invasion assays

CAFs (2.5×10^5 cells), unless stated otherwise, were cultured on type I collagen gel in a 35 mm dish at 37°C . Cellmatrix Type I-A (3 mg/ml Type I collagen, Nitta Gelatin) was mixed together with $10\times$ DF medium and reconstitution buffer in ice water at a ratio of 8:1:1, according to the manufacturer's instructions. When the cells reached confluence, 6×10^5 cancer cells were plated on top. After 5 hr of incubation, the cells were scratched with a pipette tip, washed with PBS and embedded in collagen gel. After 1 hr of incubation, fresh medium was added and fluorescence images were obtained using a BIOREVO BZ-9000 microscope (KEYENCE; Supporting Information Fig. 1a). Time-lapse fluorescence and phase-contrast images were taken using a BIOREVO BZ-9000 at 60 min intervals for 48–72 hr; the device was equipped with an incubation chamber that was regulated at 37°C in a 5% CO_2 humidified atmosphere (Tokai Hit). Each data element was measured in at least 10 fields (measurable invasion area: $9.2 \times 10^5\text{ }\mu\text{m}^2$ per field) in two or more independent experiments. The invasion index was determined as the average $\times 100$ of the invaded area/total measurable area using fluorescence images. When cancer cells were cocultured with CAFs, the number of invaded cancer cells was measured using fluorescence images. The ratio of invaded cancer cells was calculated by the division of the number of invaded cancer cells to the number of invaded cancer cells cocultured with control CAFs. These images were analyzed using standard software for BIOREVO BZ-9000. Y27632 (Sigma-Aldrich) was used at $10\text{ }\mu\text{M}$.

Measurement of matrix metalloproteinase (MMP) activity

MMP activity was measured in the supernatant of CAFs cultured for 24 hr on collagen gel using the SensoLyte 520 Generic MMP assay kit (AnaSpec). This kit can simultaneously detect the activities of MMP-1, 2, 7, 8, 9, 12, 13 and 14. Briefly, cell culture supernatants were incubated with the 5-FAM/QXL 520 FRET peptide at 37°C for 1 hr in a 96-well plate and the fluorescence intensity was measured at a wavelength of 490/520 nm (excitation/emission). 4-Aminophenylmercuric acetate-treated samples were used as positive controls. The performance of the kit, which was used within its linear range, was validated in preliminary studies using recombinant active MMP-1 (data not shown).

Measurement of RhoA activity

Direct RhoA activation was measured using the G-LISA assay (Cytoskeleton) according to the manufacturer's instructions, as described in our previous report.¹⁹ Briefly, the RhoA G-

LISA kit used 96-well plates coated with the Rho-binding domain of the RhoA effector Rhotekin. Rho-GDP was removed during the washing steps and Rho-GTP was detected using a RhoA-specific antibody and chemiluminescence.

Animal studies

Either Venus-labeled CAFs or mRFP-labeled A549 cells or both CAFs and A549 cells (2.5×10^5 cells each) were injected intravenously into the tail vein of SCID mice (7 weeks old; CLEA Japan). When the CAFs and cancer cells were both injected, the cancer cells were injected 2 hr after the injection of the CAFs. Ten minutes or 24 hr after the last injection, mice were killed and their organs were removed and fixed in 10% formalin.

Immunohistochemistry

Immunostaining was performed using 4- μ m paraffin-embedded serial tissue sections. The slides were deparaffinized in xylene and rehydrated in a graded ethanol series. After antigen retrieval, individual slides were then incubated overnight at 4 °C using rat monoclonal anti-mouse CD31 antibody (dianova) at a final dilution of 1:20 and rabbit polyclonal anti-GFP antibody that cross-reacts with Venus (MBL) at a final dilution of 1:1000 or rabbit polyclonal anti-RFP antibody (MBL) at a final dilution of 1:500. After washing with PBS, the slides were incubated with Rat HRP-Polymer (Biocare Medical) and Rabbit-on-Rodent AP-Polymer (Biocare Medical) for 30 min at room temperature, respectively. After washing with PBS, the color reaction using HRP was developed for 3 min in 2% 3,3'-diaminobenzidine in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen peroxidase. After washing with TBS, the AP color reaction was developed using the Warp Red Chromogen Kit (Biocare Medical) with 0.2 mM levamisole (Dako), according to the manufacturer's protocol. Finally, the sections were counterstained with Meyer's hematoxylin, dehydrated and mounted. In the invasion analysis of CAFs and cancer cells in mouse lung parenchyma, we defined invaded cells as those in which more than half of the invading cell surface faced the alveolar cavity. We counted the number of CAFs and cancer cells per 50 mm² in the lung and compared the index ratio.

Immunofluorescence and confocal microscopy

After incubating the sections with chicken polyclonal anti-GFP antibody (Novus Biologicals; dilution 1:1000), rabbit polyclonal anti-RFP antibody and rat monoclonal anti-mouse CD31 antibody, the sections were washed and Alexa Fluor 546 goat anti-chicken IgG, Alexa Fluor 633 goat anti-rabbit IgG and Alexa Fluor 488 donkey anti-rat IgG (Life Technologies; dilution 1:500) were used as secondary antibodies. After mounting, the sections were examined using an LSM 710 confocal microscope system (Zeiss) and then with an inverted microscope at an excitation wavelength of 488 nm for Alexa Fluor 488, 543 nm for Alexa Fluor 546 and 633 nm for Alexa

Fluor 633. Confocal images were analyzed using the standard software for the LSM 710 and Photoshop software (Adobe).

Clinicopathological studies

All the surgical specimens were fixed with 10% formalin and were embedded in paraffin. Serial 4- μ m sections were stained with hematoxylin and eosin and the Verhoeff-van-Gieson method to visualize elastic fibers. PDPN staining of the human surgical specimens was performed using anti-human mouse monoclonal PDPN antibody (D2-40; Acris Antibodies). According to a previously reported definition,²⁰ positive stained, spindle-shaped cells in the cancer stroma were identified as fibroblasts. When at least 10% of the area of the stromal fibroblasts in the stroma showed an unequivocally strong reaction that was the same as the strength of the reaction in lymphatic endothelial cells, the case was classified as PDPN positive.²⁰ The internal positive control for PDPN staining was lymphatic endothelial cells. We performed immunohistochemical staining for PDPN in 78 consecutive lung adenocarcinoma patients with tumor sizes of 3–5 cm and no lymph node metastasis who had undergone a complete resection between January 2006 and December 2008. Two observers (S.N. and G.I.) who were unaware of the clinical data independently reviewed all the pathological slides.

Statistical analysis

All the data were analyzed using the Dunnett test for comparisons against a single control. Correlations between PDPN expression in the CAFs and the clinicopathological factors were evaluated using the Fisher exact test. The disease-free survival time was measured from the date of surgery until the date of first relapse or the last follow-up. The survival rates were estimated using the Kaplan–Meier method and differences in the survival curves were compared using a log-rank test. A *p* values of <0.05 was considered statistically significant. All the statistical analyses were performed using statistical software SPSS 11.0 (SPSS, Chicago, IL) and JMP 9 (SAS Institute, Cary, NC).

Results

CAFs lead and enhance cancer cell invasion in the collagen matrix

In this study, we established an *in vitro* collagen invasion assay model that enabled time-lapse observation (Supporting Information Fig. 1a) and evaluated whether CAFs enhance cancer cell invasion in the extracellular matrix. First, we examined primarily cultured CAFs and lung adenocarcinoma cells (A549 and PC-9 cell lines) separately using the collagen invasion assay and observed that CAFs invaded the collagen matrix to a significantly greater extent than the cancer cells (Figs. 1a–1c). Second, when the CAFs and cancer cells were cocultured, the leading cells in the collagen gel were always CAFs and the cancer cells moved within the tracks created by the CAFs when viewed using time-lapse imaging (Fig. 1d and Supporting Information Movie S1), similar to a

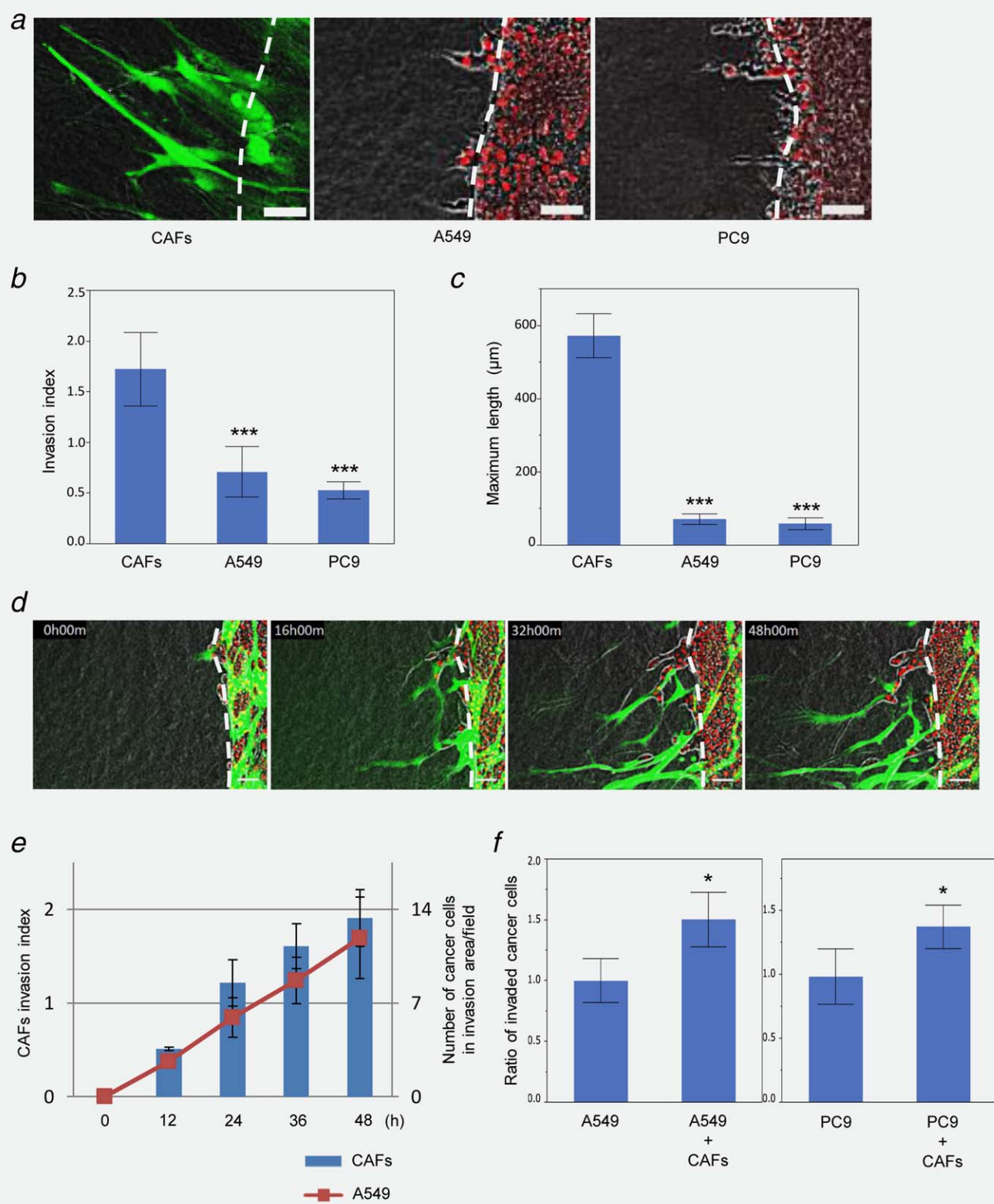


Figure 1. CAFs lead and enhance cancer cell invasion in the collagen matrix. (a–c) Examples (a) and quantification (b, c) of the invasion of CAFs, A549 cancer cells and PC9 cancer cells cultured alone for 48 hr using the collagen invasion assay model. The invaded areas of them were measured using phase images in this experiment. (d, e) Representative time-lapse images (d) and quantification (e) of the invasion of cocultures of A549 cancer cells and CAFs. (f) Quantification of the invasion of A549 or PC9 cancer cells cultured alone and cocultured with CAFs for 72 hr using the collagen invasion assay model. The error bars indicate $\pm$ SD. The scale bars represent 50 μm . * $p < 0.05$, *** $p < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

previously reported phenomenon.⁹ As the invasion area of the CAFs gradually increased, the number of invading A549 cells also subsequently grew larger (Figs. 1d–e). The difference in CAF invasion between CAFs cultured alone and those cocultured with cancer cells was not significant (Supporting Information Fig. 1b). The number of A549 or PC9 cancer cells cocultured with CAFs that were within the invasion area was significantly higher than the number of cancer cells cultured alone (Fig. 1f). Thus, we demonstrated that one form of cancer cell invasion depends on the leading CAFs in the collagen matrix.

CAFs expressing PDPN invade further in the collagen matrix and promote cancer cell invasion

We recently found that tumors with PDPN-positive CAFs frequently exhibited pathological local invasiveness (such as vascular, lymphatic and pleural invasions) in lung adenocarcinoma patients.^{18,20} Hence, we verified the hypothesis that CAFs expressing PDPN facilitate the local invasion of cancer cells using the *in vitro* collagen invasion assay. As a result, CAFs overexpressing WT-PDPN invaded the collagen matrix to a greater extent than the control CAFs (Fig. 2a and Supporting Information Figs. 2a and 2b). The invasion area of CAFs overexpressing WT-PDPN was significantly larger (Fig. 2b). Similarly, when CAFs overexpressing WT-PDPN were cocultured with cancer cells, the CAF invasion was significantly greater than the invasion of control CAFs cocultured with cancer cells (Figs. 2c–2d and Supporting Information Fig. 2c). The number of A549 cancer cells cocultured with CAFs overexpressing WT-PDPN that were within the invasion area was significantly higher than the number of cancer cells in the invasion area when the cancer cells were cocultured with control CAFs (Fig. 2e). Similar findings tended to be observed in PC9 cell lines (Supporting Information Fig. 2d, $p = 0.067$). The knockdown of PDPN significantly reduced the invasion of CAFs within the collagen matrix when the cells were both cultured alone and when they were cocultured with cancer cells (Figs. 1f–1i, Supporting Information Figs. 2e and 2f). The number of A549 cancer cells in the invasion area also decreased (Fig. 2j– $p = 0.169$ for shPDPN 1, $p < 0.05$ for shPDPN 2).

We observed A549 cancer cell division using an *in vitro* collagen invasion assay with time-lapse imaging because we wondered whether enhanced mitotic activity would increase the number of A549 cancer cells in the invasion area when the cells were cocultured with CAFs overexpressing WT-PDPN. However, no difference in A549 cell division was seen when the cancer cells were cocultured with CAFs overexpressing WT-PDPN and when they were cocultured with control CAFs (Supporting Information Fig. 2g).

Rho-ROCK function is required in invasion of CAFs expressing PDPN in the collagen matrix

PDPN cytoplasmic domain reportedly regulates RhoA activity *via* the ERM binding motif.^{19,28} To explore whether the

intracellular domain of PDPN has a functional role in the invasion of CAFs, we generated CAFs expressing mutant PDPN devoid of the cytoplasmic region (PDPN-del.IC). First, we analyzed the general MMP activity in CAFs, since MMPs affect the invasiveness of cancer cells.² We measured the active MMP level of CAFs (control, PDPN-del.IC and WT-PDPN) using an MMP FRET assay, but observed no significant differences (Supporting Information Fig. 3a).

Whereas CAFs overexpressing WT-PDPN invaded the collagen matrix to a greater extent than the control CAFs, no significant difference in the invasion area was seen between CAFs overexpressing PDPN-del.IC and control CAFs (Fig. 3a and Supporting Information Fig. 3b). When CAFs were cocultured with A549 cancer cells, both the invasion area of the CAFs overexpressing PDPN-del.IC and the number of cancer cells with CAFs overexpressing PDPN-del.IC were the same as the values observed for control CAFs (Figs. 3b–3d). These findings suggested that the cytoplasmic domain of PDPN in CAFs might be important for the initial invasion of CAFs followed by cancer cell invasion.

Since the cytoplasmic domain of PDPN reportedly regulates RhoA activity *via* the ERM binding motif,²⁸ we measured the RhoA activity of CAFs (control, PDPN-del.IC and WT-PDPN) using G-LISA. CAFs overexpressing WT-PDPN significantly increased the RhoA activity by 2.2-fold, compared with the control CAFs (Fig. 3e). The phosphorylation of ERM, myosin light chain and cofilin in CAFs overexpressing WT-PDPN was activated (Fig. 3f). Conversely, the knockdown of PDPN in CAFs decreased the phosphorylation of these molecules (Supporting Information Fig. 3c). In the monoculture *in vitro* invasion assay, the inhibition of ROCK using Y27632 prevented the invasion of CAFs into the collagen matrix, whereas Y27632 did not affect A549 cancer cell invasion (Fig. 3g). Y27632-treated CAFs overexpressing WT-PDPN exhibited a significantly reduced extent of invasion, compared with nontreated cells ($p < 0.05$). When CAFs were cocultured with A549 cancer cells, the Y27632-treated CAFs (WT-PDPN and control) also exhibited a significantly reduced extent of invasion, compared with nontreated cells (Fig. 3h). Both in monoculture and coculture experiments, the decrement in the invasion area of CAFs overexpressing WT-PDPN that were treated with Y27632 was greater than that of the control CAFs. Interestingly, when cocultured, the number of A549 cancer cells treated with Y27632 decreased, compared with non-treated cells ($p = 0.07$ in control CAFs, $p < 0.05$ in WT-PDPN CAFs; Fig. 3i). Taken together, these data demonstrate that PDPN in CAFs might promote cancer cell invasion, as well as the invasiveness of CAFs, *via* RhoA activation.

CAFs expressing WT-PDPN invade the mouse lung parenchyma and promote cancer cell invasion

To investigate whether the results obtained from the *in vitro* studies could be confirmed, labeled CAFs and/or cancer cells were injected *via* the tail vein into SCID mice. After

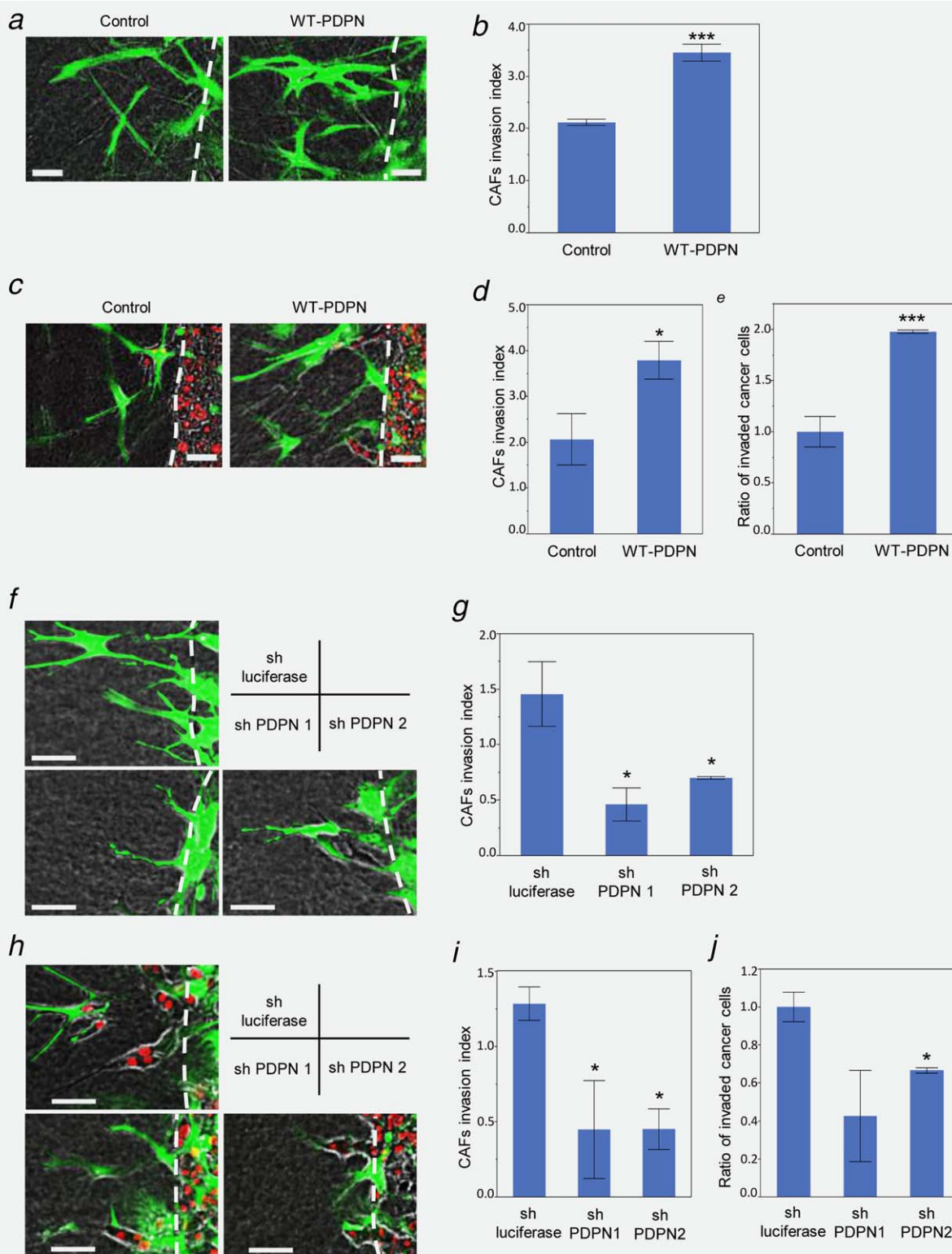


Figure 2. CAFs expressing PDPN invade further in the collagen matrix and promote cancer cell invasion. (a, b) Examples (a) and quantification (b) of the invasion of CAFs overexpressing WT-PDPN cultured alone for 72 hr using the collagen invasion assay model. (c–e) Examples (c) and quantification (d, e) of the invasion of cocultures of A549 cancer cells and CAFs overexpressing WT-PDPN for 72 h using the collagen invasion assay model. (f, g) Examples (f) and quantification (g) of the invasion of CAFs with the knockdown of PDPN that were cultured alone for 48 hr using the collagen invasion assay model. (h–j) Examples (h) and quantification (i, j) of the invasion of cocultures of A549 cancer cells and CAFs with the knockdown of PDPN for 48 hr using the collagen invasion assay model. The error bars indicate $\pm$ SD. The scale bars represent 50 μ m. * p < 0.05, *** p < 0.001. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

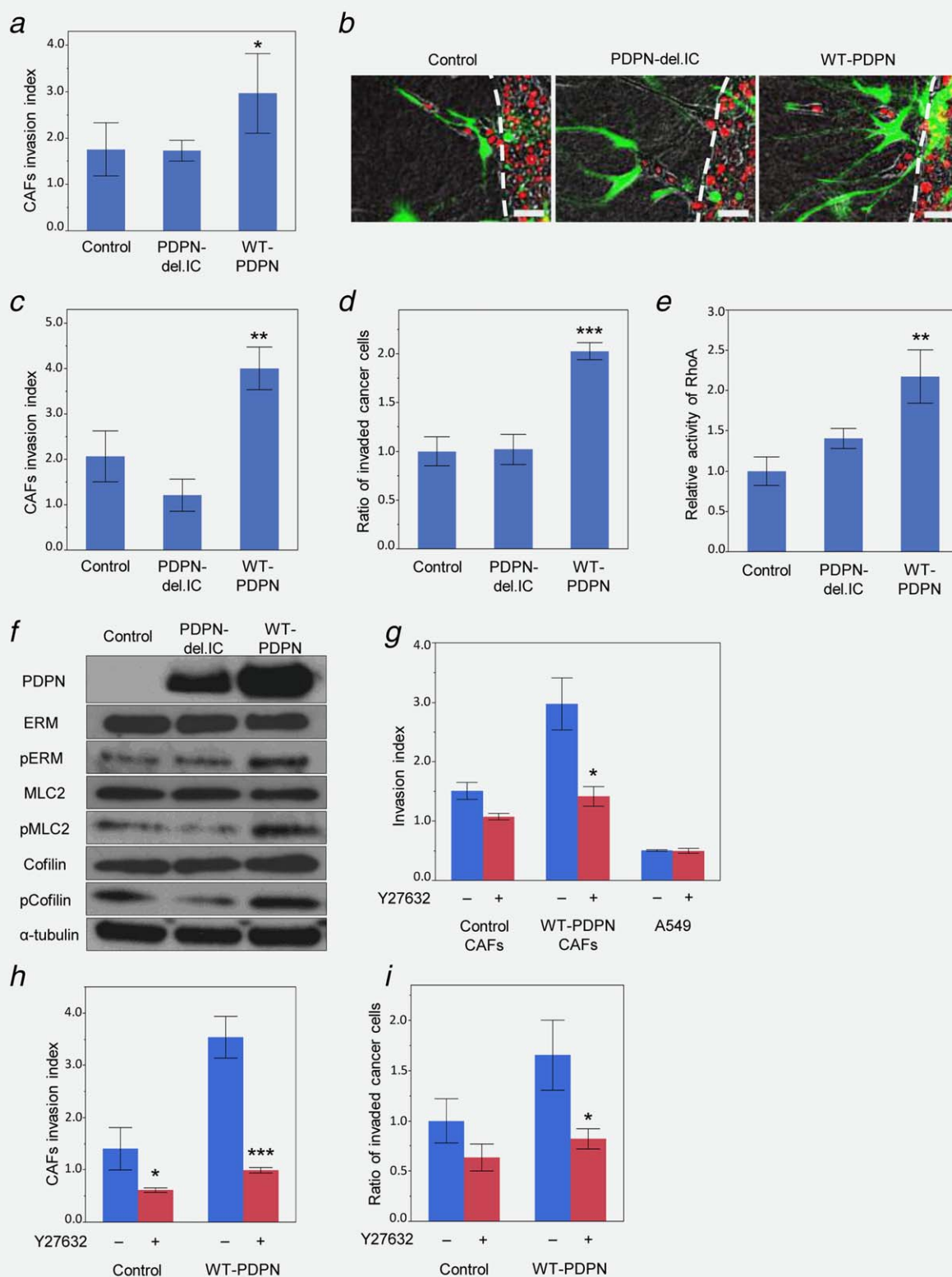


Figure 3. Rho-ROCK function is required for the invasion of CAFs expressing PDPN in the collagen matrix. (a) Quantification of the invasion of CAFs overexpressing PDPN-del.IC or WT-PDPN cultured alone for 72 hr using the collagen invasion assay model. (b–d) Examples (b) and quantification (c, d) of the invasion of cocultures of A549 cancer cells and CAFs overexpressing PDPN-del.IC or WT-PDPN for 72 hr using the collagen invasion assay model. (e) Relative activity of RhoA using the G-LISA assay in CAFs overexpressing PDPN-del.IC or WT-PDPN. (f) Western blot analysis showing the phosphorylation of ERM, myosin light chain and cofilin in CAFs overexpressing PDPN-del.IC or WT-PDPN. The expression of α -tubulin was used as a control for protein loading. (g) Quantification of the invasion of A549 cancer cells and CAFs overexpressing WT-PDPN cultured alone with the ROCK inhibitor Y27632 for 72 hr using the collagen invasion assay model. (h, i) Quantification of the invasion of co-cultures of A549 cancer cells and CAFs overexpressing WT-PDPN with Y27632 treatment for 72 hr using the collagen invasion assay model. The error bars indicate $\pm$ SD. The scale bars represent 50 μ m. * p < 0.05, ** p < 0.01, *** p < 0.001. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

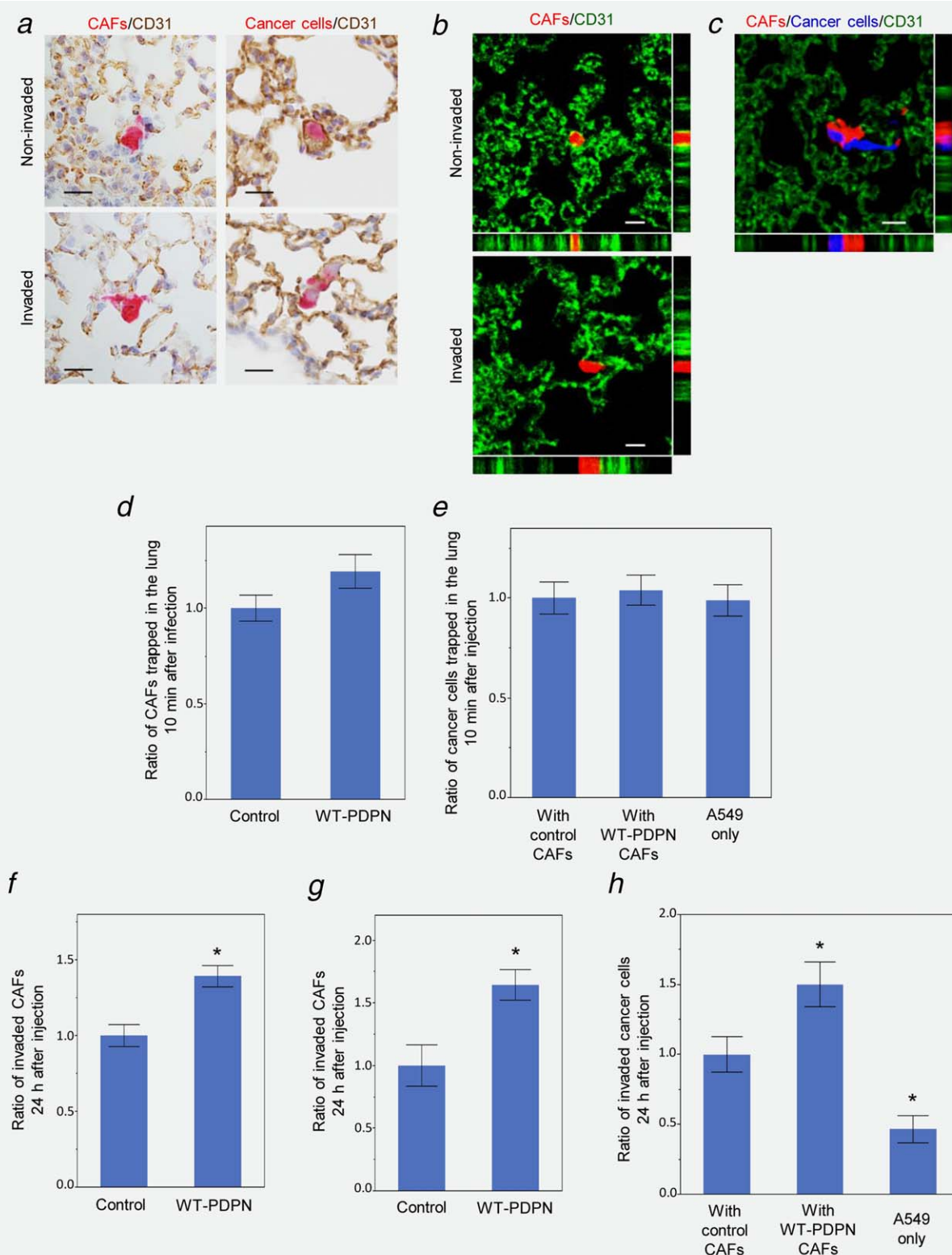


Figure 4. CAFs expressing WT-PDPN invade the mouse lung parenchyma and promote cancer cell invasion. (a) Examples of non-invaded (upper) and invaded (lower) CAFs (left) and A549 cancer cells (right) in the mouse lung parenchyma at 24 hr after injection with immunohistochemical staining for Venus-CAFs or A549 cancer cells (red) and CD31 (brown). (b) Examples of noninvaded (left) and invaded (right) CAFs in the lung parenchyma at 24 hr after injection with immunofluorescence staining for Venus-CAFs (red) and CD31 (green). (c) Examples of invaded CAF and A549 cancer cells in the lung parenchyma at 24 hr after injection with immunofluorescence staining for Venus-CAF (red), mRFP-A549 cells (blue) and CD31 (green). (d) Quantification of CAFs overexpressing WT-PDPN injected with A549 cancer cells just after injection ($n = 5$, each). (e) Quantification of A549 cancer cells injected alone and with control CAFs or CAFs overexpressing WT-PDPN just after injection ($n = 5$, each). (f) Quantification of invaded CAFs overexpressing WT-PDPN injected alone 24 hr after injection ($n = 5$, each). (g) Quantification of invaded control CAFs and CAFs overexpressing WT-PDPN injected with A549 cancer cells 24 hr after injection ($n = 5$ and 6, respectively). (h) Quantification of invaded A549 cancer cells injected with control CAFs or CAFs overexpressing WT-PDPN and injected alone 24 hr after injection ($n = 5$, 6 and 5, respectively). The error bars indicate $\pm$ SEM. The scale bars represent 20 μ m. * $p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

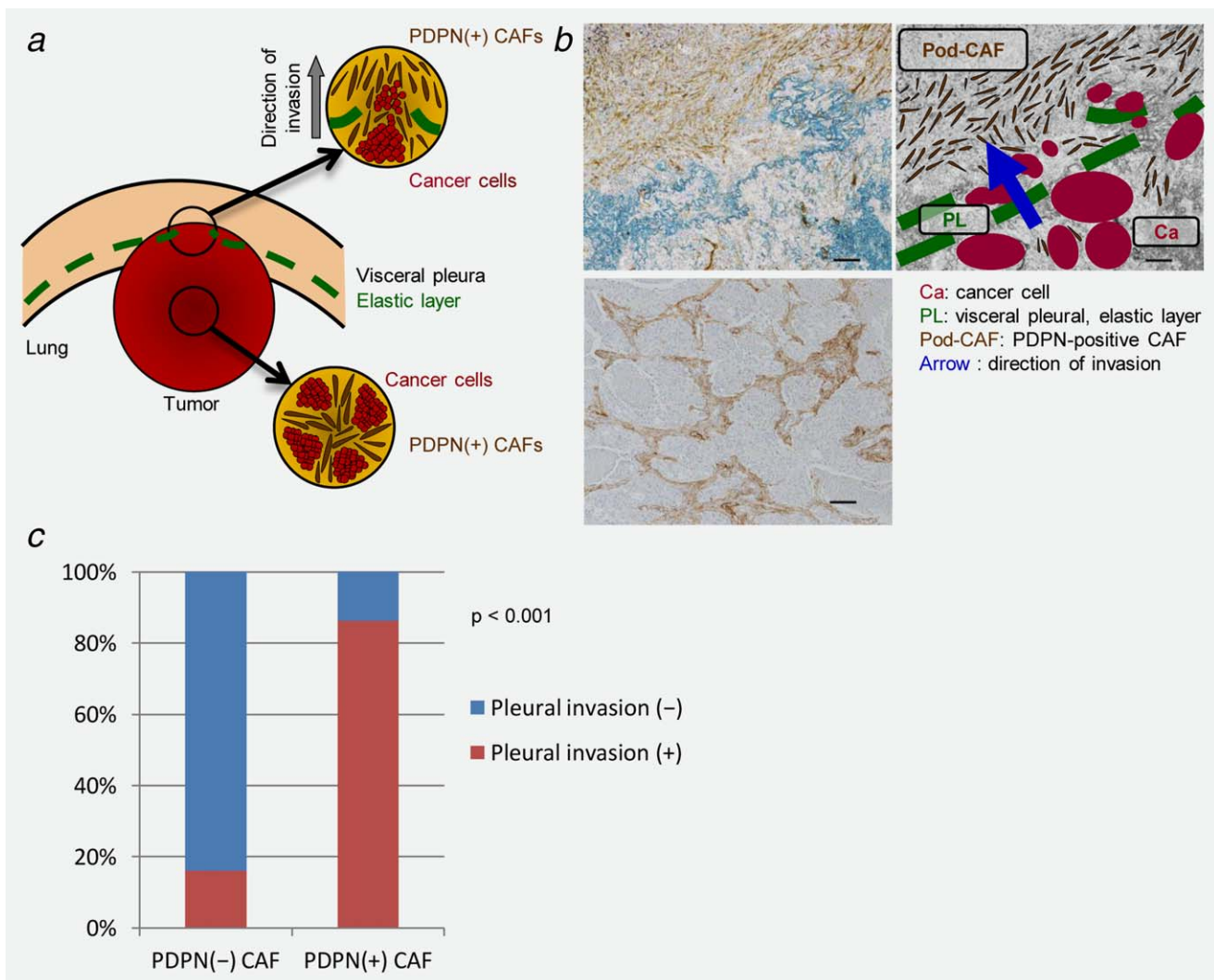


Figure 5. PDPN-positive CAFs in clinical samples of lung adenocarcinoma. (a) Scheme showing lung adenocarcinoma invasion into the visceral pleura in clinical samples. While the lung tumor is growing up, it invades the outside of the lung, which is called the pleural invasion. In the central area of the tumor, PDPN-expressing CAFs were observed. At the invasive front of the tumor, PDPN-expressing CAFs were also found. (b) Examples of PDPN expression (brown) in CAFs and the elastic layer in the visceral pleura (green) at the invasive front (upper left; scheme, upper right) and in the central area (lower left) using immunohistochemical staining. (c) Correlation between PDPN-expressing CAFs and pleural invasion at the invasive front. (PDPN(+)CAF/pleural invasion: -/-, $n = 47$; -/+, $n = 9$; +/-, $n = 3$; and +/+, $n = 19$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

intravenous injection, the CAFs and cancer cells were trapped in the lung vasculature and thereafter invaded the lung parenchyma within 24 hr, similar to previously reported findings (Figs. 4a–4c).^{29,30} First, we analyzed the number of CAFs and A549 cancer cells that were trapped in the lung vasculature at 10 min after injection into the tail vein. No difference between the number of CAFs overexpressing WT-PDPN and the number of control CAFs that were trapped in the lung vasculature was observed (Fig. 4d) and no differences among the number of trapped A549 cancer cells injected alone, the number of trapped A549 cells injected with CAFs overexpressing WT-PDPN or the number of trapped A549 cells injected with control CAFs were seen (Fig. 4e). Next, we injected CAFs alone into the mice intravenously and analyzed

the number of CAFs that had invaded the lung parenchyma from the vasculature at 24 hr after the injection. To analyze the extravasation, the number of cells inside or outside of the vasculature was counted.³¹ CAFs overexpressing WT-PDPN exhibited a significantly greater invasion, compared with control CAFs (Fig. 3f). When CAFs and A549 cells were injected simultaneously into the mice, CAFs overexpressing WT-PDPN also exhibited a significantly greater invasion of the lung tissue, compared with control CAFs (Fig. 4g). When injected with CAFs overexpressing WT-PDPN, the number of A549 cancer cells that invaded the lung parenchyma was significantly higher, compared with when they were injected with control CAFs (Fig. 4h). In contrast, the number of A549 cells injected alone and that invaded the lung parenchyma

was significantly lower than the number after injection with control CAFs (Fig. 4h). These findings suggested that CAFs enhanced the local invasion of cancer cells into the lung parenchyma and CAFs expressing PDPN might invade further into the lung tissue and promote cancer cell invasion into the lung parenchyma.

PDPN-positive CAFs in clinical samples of lung adenocarcinoma

To investigate whether the results obtained from the *in vitro* and *in vivo* studies could be confirmed using human clinical specimens, we examined the presence and location of PDPN-positive CAFs in surgically resected pStage I lung adenocarcinoma with a tumor size of 3–5 cm and no lymph node metastasis (Fig. 5a). While the lung tumor is growing up, it invades the outside of the lung, which is called the pleural invasion. In lung cancer, the pleural invasion is one of the poor prognostic factors.³² We analyzed whether PDPN-expressing CAFs exist in the central area of the tumor and at the invasive front of the tumor.²⁰ In the central area of the tumor, 32 (41.0%) of the 78 specimens were positive for PDPN-positive CAFs (Fig. 5b, left lower). The disease-free survival time of the patients with an abundance of PDPN-positive CAFs located in the center of the tumor was significantly shorter than that of patients whose CAFs were PDPN-negative, similar to previously reported findings ($p = 0.012$; Supporting Information Fig. 4a).^{18,20} Moreover, at the invasive front of the tumor, PDPN-positive CAFs were observed in 22 (28.2%) of the 78 cases. In most of these cases (19/22), the cancer cells had invaded the visceral pleura, indicating an invasive cancer (Fig. 5b, upper). Pleural invasion was observed more frequently in the PDPN-positive CAFs at the invasive front than in the PDPN-negative CAFs (Fig. 5c). These results suggested that cancer cells might invade further when PDPN-positive CAFs existed at the invasive front of the tumor.

Discussion

The mechanisms of CAFs that stimulate proliferation, invasion and the epithelial-mesenchymal transition of cancer cells have been reported to involve the over-secretion of growth factors such as transforming growth factor- β and stromal-cell-derived factor 1 or MMPs.^{2,33,34} In general, the well-known types of cancer cell invasion include mesenchymal movement, which depends on MMPs secreted by cancer cells and amoeboid movement, which upregulates their motor activity.^{35–37} However, Gaggioli *et al.* have studied another mechanism in which CAFs facilitate the local invasion of cancer cells by physically remodeling the extracellular matrix.⁹ They have illustrated that cancer cells invade by following the CAFs into the matrix. However, these studies have assayed CAFs as a uniform population, while a diversity of phenotypes and functions in CAFs have been reported in other studies.^{7,13,15,16,38} In this study, we demonstrated that CAFs expressing PDPN invaded a collagen matrix to a

greater extent and enhance the local invasion of cancer cells. The knockdown of PDPN in the CAFs and the inhibition of ROCK as a downstream factor of PDPN decreased the invasion ability of the CAFs and the number of invaded cancer cells. These findings suggested the possibility that the local invasion of cancer cells might depend on the invasion ability of a certain subtype of recruited CAFs in the tumor tissue.

The overexpression of WT-PDPN in CAFs increased RhoA activity and treatment with a ROCK inhibitor significantly decreased the invasion area and the number of invaded cancer cells. The Rho kinase signaling pathway in CAFs has been reported to be upregulated³⁹ and to be important for the generation of the tracks of CAFs in the matrix.^{9,40} These data collectively suggest that a high RhoA activity level in CAFs could enhance the local invasion of cancer cells by increasing the track area of the CAFs. This suggestion was also supported by the result that a ROCK inhibitor did not affect the invasiveness of cancer cells that were cultured alone in the collagen matrix. The tracks created by PDPN-expressing CAFs with their high RhoA activity level could have properties that make them favorable to the local invasion of cancer cells.

In our *in vivo* study in mice, CAFs overexpressing WT-PDPN had invaded the lung parenchyma to a significantly greater extent, compared with control CAFs, at 24 hr after their injection into the tail vein. We found that both CAFs and A549 cells invaded the lung tissue together when they were injected simultaneously. The number of invaded A549 cells injected with CAFs overexpressing WT-PDPN was larger than the number of invaded A549 cells injected with control CAFs. Because the numbers of A549 cells and CAFs were not different immediately after the injection (Figs. 4d–4e), the invasion of the cancer cells into the lung parenchyma was considered to have been enhanced by the increased invasion ability of CAFs overexpressing PDPN. As for cancer cell invasion into the lung tissue depending on fibroblasts, Oskarson *et al.* reported that tenascin C, which is secreted by stromal active fibroblasts promotes the growth of lung lesions.⁴¹ However, stromal derived tenascin C contributes to tumor growth during the late phase (one month after injection), not the early phase. Our results demonstrated that during the early phase of invasion (24 hr after injection), PDPN-expressing CAFs could promote cancer cell invasion into the lung parenchyma by increasing their own invasiveness. Therefore, various subtypes of CAFs might be recruited and contribute to tumor invasion during different phases, thereby orchestrating events critical to tumor evolution toward metastasis.

Many studies have reported that the expression of PDPN in CAFs is a poor prognostic factor in various malignant tumors such as intrahepatic cholangiocarcinoma, breast cancer, esophageal cancer and melanoma.^{23–27} We previously reported that lung adenocarcinoma patients with PDPN-positive CAFs clinically have poor outcomes.^{18,20,21} Similar to the present *in vitro* and *in vivo* analyses, PDPN-expressing

CAFs in the center of the tumor might be capable of enhancing cancer cell invasion. However, whether these CAFs lead to the local invasion of cancer cells is difficult to determine. In this study, we analyzed PDPN expression in CAFs in the pleural invasion area of the tumor, which is clinically known as the invasive front of lung cancers. We found a correlation between pleural invasion and PDPN-expressing CAFs in the invasive front; these findings suggested that PDPN-expressing CAFs in the invasive front might promote the local invasion of cancer cells, similar to the *in vitro* and *in vivo* results.

In conclusion, we demonstrated that PDPN-expressing CAFs invade the collagen matrix to a greater extent and enhance cancer cell invasion. PDPN expression in CAFs activates the Rho-ROCK pathway *via* its cytoplasmic domain, thereby facilitating cancer cell invasion. These findings sug-

gest that the properties of track areas that have been remodeled by CAFs might determine cancer cell invasion. Another study of scirrhous gastric cancer has reported that asporin-secreting CAFs activate co-invasion by CAFs and cancer cells.⁴² This result also indicates that a certain subtype of CAFs might contribute to cancer cell invasion. To investigate this speculation, we need to determine which unique subtypes of CAFs might determine cancer cell invasion in a manner that is dependent on their own properties. Understanding the effect of CAF subtypes on tumor invasiveness could be helpful for studies on the local invasion of cancer cells.

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