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Organoid culture containing cancer cells and stromal cells reveals that podoplanin-positive cancer-associated fibroblasts enhance proliferation of lung cancer cells

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ABSTRACT

Objective: Podoplanin-positive cancer-associated fibroblasts (CAFs) play an important role in tumor progression. The aim of this study was to evaluate the effect of podoplanin (+) CAFs on the proliferation of cancer cells using a three-dimensional (3D) organoid model.

Materials and methods: We examined the success rate of organoid culture containing PC-9 cancer cells and CAFs. Thereafter, we compared the proliferating index (MIB-1 index) of PC-9 cells co-cultured with podoplanin-overexpressing CAFs and control CAFs using organoid specimens. Furthermore, we compared the MIB-1 labeling index of cancer cells in podoplanin (+) CAFs cases (n = 13) and podoplanin (-) CAFs cases (n = 14) using surgically resected adenocarcinoma specimens.

Results: Without CAFs, PC-9 cells did not form any organoid (success rate: 0%). When PC-9 cells were mixed with CAFs (1:10), the mixed cells generated round and steric aggregates (hybrid cancer organoids, success rate: 100%). In three independent experiments, the MIB-1 index of PC-9 cells in hybrid cancer organoids containing podoplanin-overexpressing CAFs was significantly higher than that of PC-9 cells in organoids containing control CAFs (Exp. 1: 40.4% vs. 24.4%; Exp. 2: 40.0% vs. 24.5%; Exp. 3: 40.3% vs. 25.2%; $p < 0.001$). Surgically resected human tumors revealed that the MIB-1 index of adenocarcinoma cells was significantly higher in the case of podoplanin (+) CAFs than in the case of podoplanin (-) CAFs (34.8% vs. 16.2%; $p < 0.01$).

Conclusion: Our data suggested that the hybrid cancer organoid model might reflect the growth-promoting effect of podoplanin (+) CAFs in cancer cells, and this new system can be a useful tool for evaluating the tumor microenvironment.

1. Introduction

A variety of culture systems have been developed previously to examine cancer biology, among which two-dimensional (2D) systems are the most frequently used models. However, it is well known that these systems do not reflect the complexity, heterogeneity, and plasticity of the human tumor microenvironment. Cancer tissues consist of various cell components with highly complex three-dimensional (3D) microstructures. Recent studies have indicated that 3D culture systems, such as spheroid and/or organoid cultures, can provide better insight on the physiological characteristics of cancer cells in comparison to 2D culture systems [1–3].

Fibroblasts in cancer tissues, also known as cancer-associated fibroblasts (CAFs), are one of the crucial components of stromal cells. Growing evidence has shown that the proliferation, invasion, and metastasis of cancer cells are influenced by their interaction with CAFs [4–7]. Together, CAFs and other stromal cells can create specific microenvironments for tumor progression. However, CAFs exhibit

Abbreviations: CAFs, cancer associated fibroblasts; 2D, two dimensional; 3D, three dimensional; EGFR, epidermal growth factor receptor; mRFP, monomeric red fluorescent protein; hTERT, human telomerase reverse transcriptase; CDK4, cyclin dependent kinase 4

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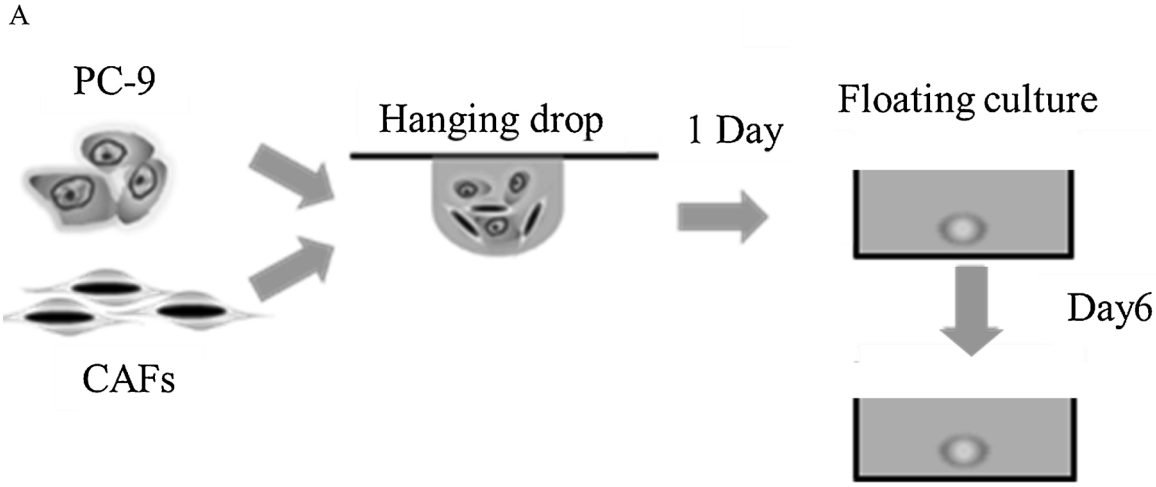

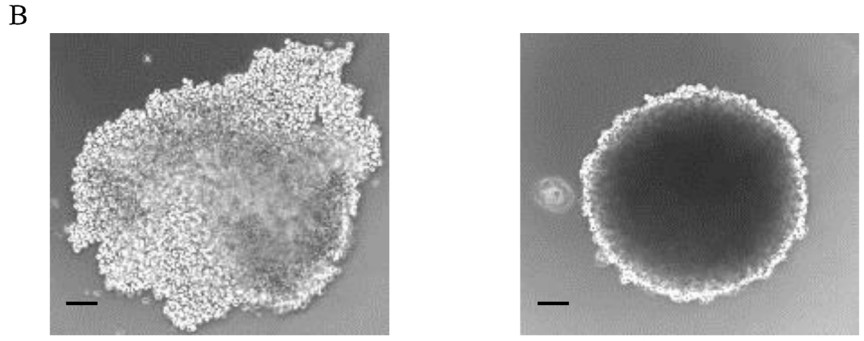

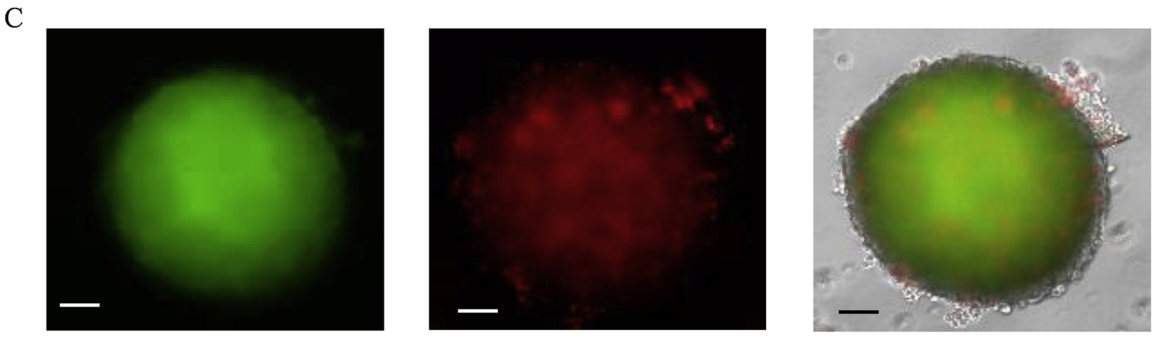

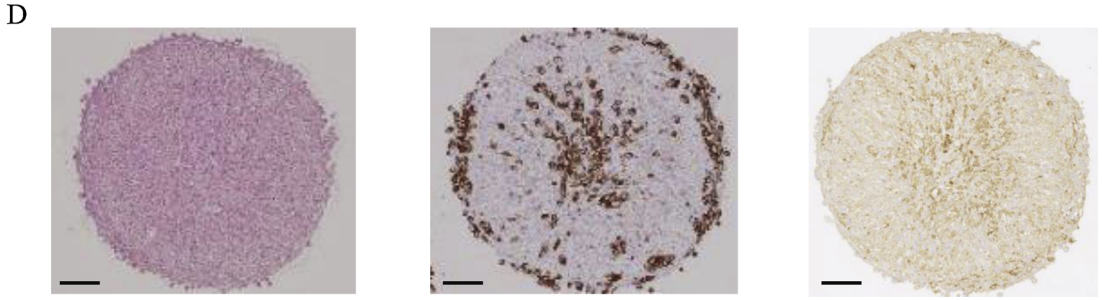

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Fig. 1. Macroscopic and microscopic features of hybrid cancer organoid.
A. The scheme of the method of 3D culture.
B. Macroscopic feature of hybrid cancer organoid — : 100μm.
left: 3D culture containing only PC-9 cells (1.0×10^4 cells).
right: 3D culture containing both PC-9 cells (1.0×10^4 cells) and CAFs (1.0×10^5 cells).
C. Phase contrast microscope feature of hybrid cancer organoid containing of both PC-9 cells (1.0×10^4 cells) and CAFs. (1.0×10^5 cells) — : 100 μm.
left: green fluorescence image(Venus-expressing CAF).
middle: red fluorescence image (mRFP-expressing PC-9)
right: overlay images.
D. Microscopic features of hybrid cancer organoid — : 100 μm.
left: HE staining image of hybrid cancer organoid.
middle: CK7 immunostaining (cancer cells).
right: Venus immunostaining (CAFs).

Table 1
Success rate of 3D organoid culture.

Cell number	Exp.1	Exp.2	Exp.3	total
A. PC-9 1.0×10^4	0/20	0/20	0/20	0/60(0%)
B. PC-9 1.0×10^4 + CAFs 1.0×10^4	8/20	6/20	10/20	24/60(40%)
C. PC-9 1.0×10^4 + CAFs 1.0×10^5	20/20	20/20	20/20	60/60(100%)

heterogeneous phenotypes and functions within the same tumor [8–11]. Furthermore, the properties of CAFs vary widely from case to case. Thus, an analysis of CAF subpopulations is required in order to gain a functional assessment of the tumor microenvironment.

Human podoplanin is a transmembrane glycoprotein that contributes to cancer progression and is upregulated on cancer cells, CAFs, and inflammatory macrophages. Recruitment of podoplanin (+) CAFs is considered as a prognostic indicator of lung adenocarcinoma, lung squamous cell carcinoma, and breast carcinoma [12–15]. Moreover, *in vivo* studies have revealed that podoplanin (+) CAFs promoted lung adenocarcinoma cell engraftment into SCID mice [16]. Using an *in vitro* model, we demonstrated that podoplanin (+) CAFs enhanced fibroblast-dependent cancer cell invasion. These studies suggested that podoplanin (+) CAFs are a special subpopulation of CAFs with tumor-promoting functions [17]. Generally, the high proliferative capacity of cancer cells is associated with their high malignant potential. It is important to elucidate whether podoplanin (+) CAFs are capable of enhancing the proliferative activity of cancer cells to further clarify their biological functions. In the current study, we examined the effect of podoplanin (+) CAFs on the proliferation of cancer cells using a 3D organoid model containing both CAFs and cancer cells, instead of common 2D culture systems. In order to confirm the results obtained from 3D culture, we examined specimens of surgically resected lung adenocarcinoma and evaluated the MIB-1 index of cancer cells in cases involving podoplanin (+) and podoplanin (−) CAF.

2. Materials and methods

2.1. Cell culture and reagents

The Epidermal growth factor receptor (EGFR) mutant human lung adenocarcinoma cell lines PC-9 (del E746_A750), was purchased from the European Collection of Cell Culture. The PC-9 cells and PC-9 cells labeled with monomeric red fluorescent protein (mRFP) were maintained in RPMI 1640(Thermo Fisher Scientific, Waltham, MA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO), 1% glutamine, and antibiotics (1% penicillin and streptomycin; Sigma) [18]. CAFs were prepared from human lung adenocarcinoma tissues as previously reported [8,17]. The cultures were incubated at 37 °C in an atmosphere containing 5% CO_2. IRB approval number of this study is 2017-43.

2.2. Life time extension of CAFs

To extend the lifetime of CAFs, we used a primary CAFs culture (CAFs-621) from lung adenocarcinoma. Transduction was done with human telomerase reverse transcriptase (hTERT) and mutant forms of cyclin dependent kinase 4 (CDK4R24C) in combination (hTERT/CDK4R24C) [8]. hTERT cDNA and CDK4R24C cDNA were kindly gifted from Dr. Masutomi K (Division of Cancer Stem Cell, National Cancer Center Research Institute) and Dr. Kiyono T (Division of Carcinogenesis and Cancer Prevention, National Cancer Center Research Institute), respectively.

2.3. Overexpression of podoplanin

Lentivirus vectors containing human wild-type podoplanin were generated as described previously [16,19]. The lentiviruses were produced using 293 T cells transfected with PCAG-HIV, pCMV-VSV-G-RSV-Rev (RIKEN Bioresource Center, Ibaraki, Japan), and a wild-type podoplanin. Transfection was achieved using the Lipofectamine 2000 reagent (ThermoFisher Scientific), according to the manufacturer's instructions. Vector-containing medium was filtered through a 0.45 mm filter and 8 mg/ml of polybrene (Santa Cruz Biotechnology, Santa Cruz, CA) was added to CAFs expressing hTERT/CDK4R24C for transduction.

2.4. Flow cytometry and cell sorting

CAFs were incubated with anti-podoplanin antibody (clone 18H5, Abcam, Cambridge, UK) 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 podoplanin positive cells were sorted using a FACSAria II (BD Biosciences, Sun Jose, CA).

2.5. Generation of 3D hybrid cancer organoids

Hybrid cancer organoids were created via the following procedure (Fig. 1A). First, the cell mixtures A, B, and C were prepared (Supplementary Table 1 Table 1). The lid of the dish was inverted and 20 μL of cell mixture A, B, and C was deposited and incubated for 24 h in a hanging drop culture. Successfully created spheroids were cultured in 1.0 mL of Gibco StemPro human embryonic stem cell serum-free medium (ThermoFisher Scientific) in each well of a Nunclon Sphera 24-well plate. The cultures were maintained for six days and organoid formation was considered successful if the organoids did not break

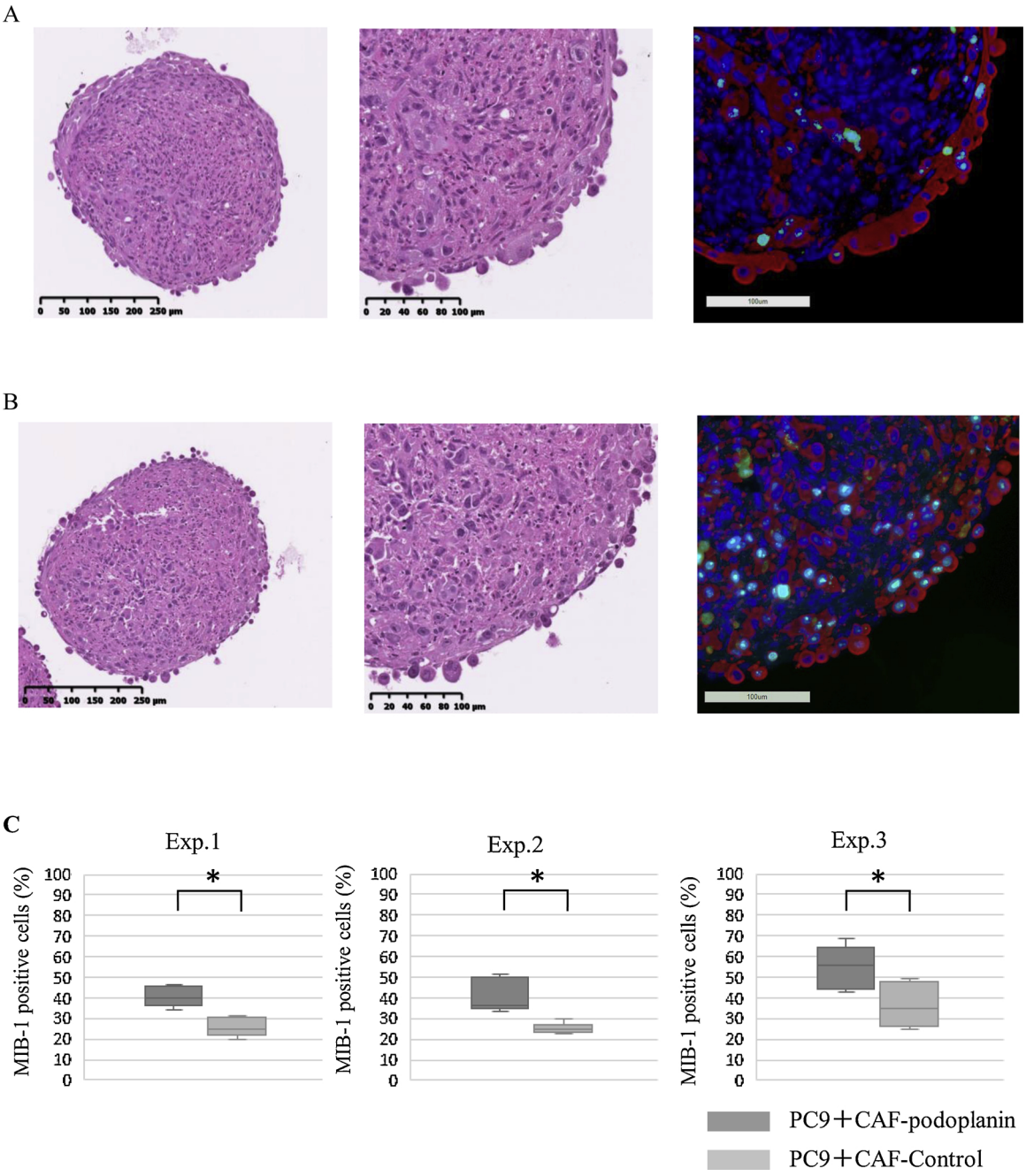

Fig. 2. The effect of podoplanin (+) CAFs on cancer cell proliferation in 3D culture (hybrid cancer organoid).
Microscopic images of hybrid cancer organoid containing control CAFs.
left: HE staining images with lower magnification.
middle: HE staining image with higher magnification.
right: Immunofluorescence images (Green: MIB-1, Red: CK7, Blue: nucleus).
B. Microscopic images of hybrid cancer organoid containing podoplanin-overexpressing CAFs.
left: HE staining images with lower magnification.
middle: HE staining image with higher magnification.
right: Immunofluorescence images (Green: MIB-1, Red: CK7, Blue: nucleus).
C. Results of MIB-1 index of PC-9 cells in hybrid cancer organoid * $p < 0.001$: *t*-test.

during the transfer to the well plate.

2.6. Immunofluorescence studies of hybrid cancer organoids

Hybrid cancer organoids were fixed with 10% formalin and embedded in paraffin. The serial 4 μm sections were then deparaffinized in xylene. After washing with distilled water, they were placed in pH 9.0 Tris-EDTA buffer (Agilent Technologies, Palo Alto, CA). Antigen retrieval was performed by placing and heating the slides at 95 °C for 20 min in a microwave oven, and allowing them to cool for 1 h at 25 °C. Slide glasses were incubated with primary antibodies (MIB-1, Agilent Technologies and CK7, GeneTex, San Antonio, TX) at room temperature for 1 h. After PBS washing, cells were incubated with secondary immunofluorescence anti-bodies (Alexa Fluor 488 and 633, respectively,

Table 2
Clinicopathological characteristics of podoplanin (+) CAFs cases.

Characteristics	Podoplanin CAF		
	(+) n = 13	(-) n = 14	*P*
total			
Age			0.30
< 65years	4	2	
≧65years	9	12	
Sex			0.90
Male	4	4	
Female	9	10	
Smoking			0.34
Never	7	10	
Current/Former	6	4	
Tumor size (invasive size)			0.92
≦3cm	10	11	
> 3cm	3	3	
Lymph node metastasis			0.34
Absent	7	10	
Present	6	4	
Vascular invasion			0.03
Absent	3	9	
Present	10	5	
Lymphatic permeation			0.94
Absent	11	12	
Present	2	2	
Pleural invasion			0.23
Absent	2	5	
Present	11	9	

Thermo Fisher Scientific) at room temperature for 1 h. After PBS washing, cells were incubated with Hoechst® 33,342 (Dojindo) at room temperature for 3 min. After PBS washing, they were mounted Perma Fluor Aqueous Mounting Medium (Thermo Fisher Scientific).

2.7. Evaluation of CK7- and MIB-1-positive cells in hybrid cancer organoids

All stained slides were scanned and captured using an Aperio VERSA SL200 digital slide scanner (Leica Biosystems, Nußloch, Germany). In each independent experiment, we evaluated six hybrid cancer organoids. The maximum split surface of each organoid, where the cancer cells and CAFs intermingled, were observed at high-power fields (0.196 mm^2/field). The number of CK7-positive cells was counted, and both MIB-1- and CK7-positive cells were averaged. Ki-67 is known to indicate the degree of malignancy in many tumors [20]. In the current study, we used MIB-1, a clone of Ki-67, to represent proliferation index.

2.8. Patient cohort

Between 2016 and December 2017, pathological T2a and EGFR mutation positive lung adenocarcinoma cases that underwent surgery with curative intent, were selected. In total, 27 samples were enrolled in this study. All specimens were collected after obtaining written comprehensive informed consent from the patient, and the study was conducted with the approval of the Institutional Review Board of the National Cancer Center. IRB approval number of this study is 2017-462.

The diagnosis of histological type was based on the 4th edition of World Health Organization classification of the lung, pleura, thymus, and heart; and their pathological stages were determined according to the 8th edition TNM classification.

2.9. Immunohistochemical studies of surgically resected specimens

Surgically resected specimens were fixed with 10% formalin and embedded in paraffin. Serial 4 mm sections were used for the immunohistochemical studies. Immunohistochemical staining was performed according to the methods as previously reported [12].

Podoplanin (+) cases were identified as those in which the ratio of the podoplanin-positive CAF area to the stromal area was ≥5%.

In terms of MIB-1 labeling index, we selected five areas of cancer nests closely associated with podoplanin (+) or podoplanin (-) CAFs, observed them in high-power fields (0.0625 mm^2 /field), and counted and averaged the number of cells for each case.

Hematoxylin and eosin images and immunohistochemical images were captured using a NanoZoomer 2.0-HT (Hamamatsu Photonics, Shizuoka, Japan) and the images were analyzed with NDPview2 (Hamamatsu Photonics).

3. Result

3.1. Success rate of 3D cancer organoid culture

First, we attempted to generate organoids from PC-9 cells (1.0×10^4 cells) without CAFs. We defined "successfully created organoids" as those that did not break when aspirated by a yellow tip. Without CAFs, PC-9 cells formed irregular and flat fragile aggregates and failed to form organoids (Fig. 1B left, Supplementary Fig. 1 left). When 1.0×10^4 PC-9 cells were mixed with 1.0×10^5 CAFs, round and steric aggregates were generated and the success rate of cancer organoid formation was 100% (Fig. 1B right, Supplementary Fig. 1 right, and Table 1). On the other hand, the addition of an equal number of CAFs (1.0×10^4 cells) to PC-9 cells decreased the success rate to 40%. Therefore, we conducted the subsequent experiments using a 1:10 cell mixture (1.0×10^4 PC-9 cells and 1.0×10^5 CAFs).

3.2. Microscopic features of hybrid cancer organoids

We evaluated the hybrid cancer organoids with phase-contrast microscopy and found that the cancer cells (red fluorescence) were mixed with the CAFs (green fluorescence). We did not find any biased pattern in cell distribution (Fig. 1C).

Using thin sliced sections of specimens, we also confirmed that CK7-positive cancer cells and Venus-positive CAFs were intermingled with each other, mimicking human cancer tissue (Fig. 1D).

3.3. Generation of podoplanin-overexpressing CAFs

We have previously reported that podoplanin-positive CAFs promoted tumor cell engraftment *in vivo* and local invasion of cancer cells *in vitro* [9,16]. In the current study, we investigated the effect of podoplanin on cancer cell proliferation using a hybrid cancer organoid model. For this purpose, CAFs with an extended life span that stably overexpress podoplanin were required. To this end, we first transduced both human telomerase reverse transcriptase and mutant forms of CDK4R24C. The original lifetime-prolonged CAFs had a podoplanin positivity rate of 24%. We transduced podoplanin into lifetime-prolonged CAFs to generate podoplanin-overexpressing CAFs at a positivity rate of 86.9% (Supplementary Fig. 2).

3.4. The effect of podoplanin (+) CAFs on cancer cell proliferation in hybrid cancer organoids

Next, we examined the effect of podoplanin (+) CAFs on the proliferation of cancer cells in the hybrid cancer organoids. Fig. 2A and B display the typical features of each type of organoid. The morphological features of cancer cell nests were identical between hybrid cancer organoids containing podoplanin-overexpressing CAFs and control CAFs

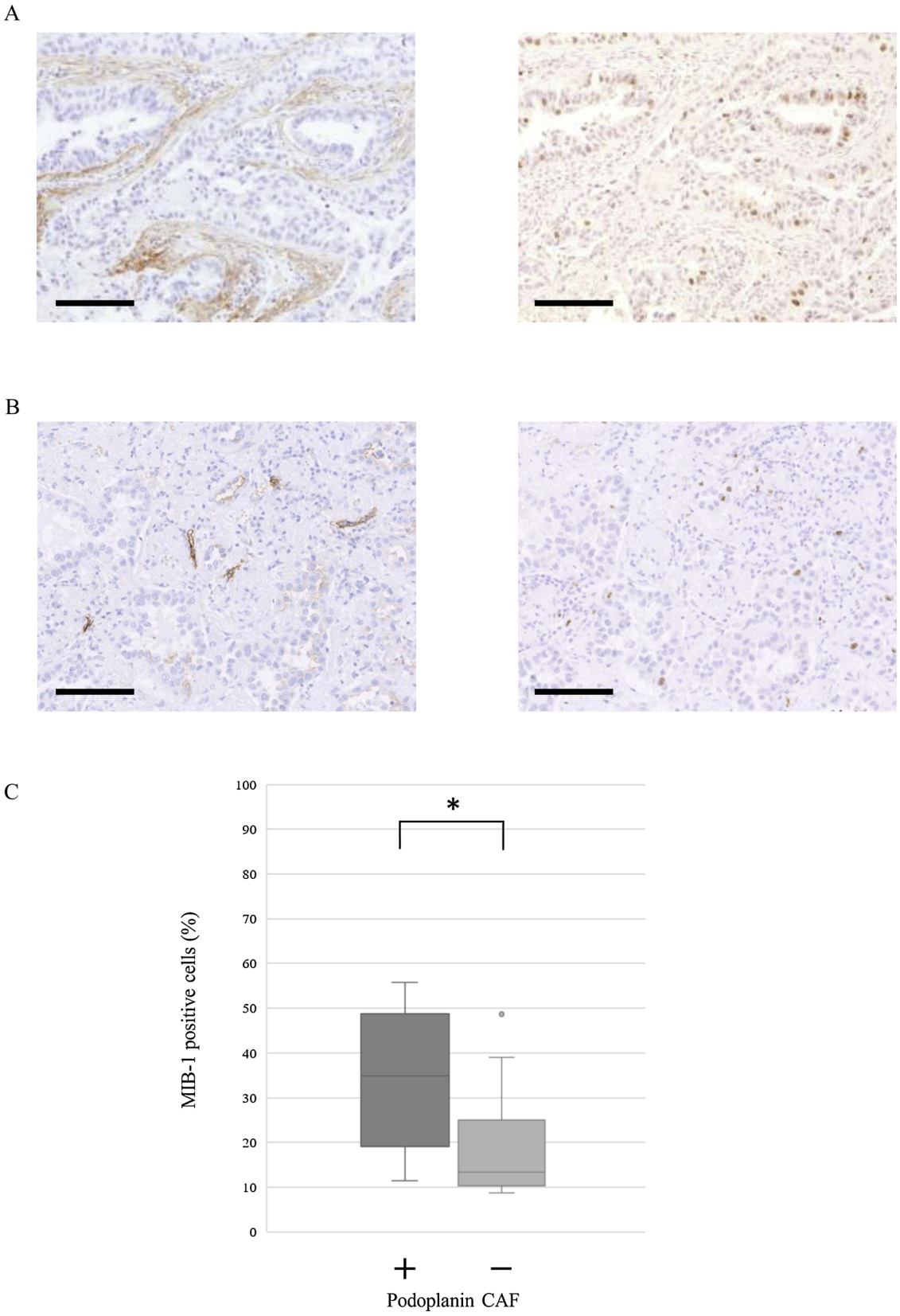

Fig. 3. MIB-1 index of cancer cells in surgically resected 27 adenocarcinoma cases —: 100μm.
Microscopic images of Podoplanin (+) CAF cases.
left: Podoplanin expression of CAFs, right: MIB-1 staining of cancer cells.
Microscopic images of Podoplanin (-) CAF cases.
left: Podoplanin expression of CAFs, right: MIB-1 staining of cancer cells.
C. Comparison of MIB-1 index of cancer cells between podoplanin (+) CAF cases and negative cases.
* $p < 0.01$: *t*-test.

(Fig. 2A, left and middle; Fig. 2B, left and middle). The size of hybrid cancer organoids containing podoplanin-overexpressing CAFs was not different from that of organoids containing control CAFs (Supplementary Fig. 3 Fig. 3). We evaluated the percentage of MIB-1-positive cancer cells in the hybrid cancer organoids (Fig. 2A right and B right). In all three independent experiments, the percentage of MIB-1-positive cancer cells in the hybrid cancer organoids consisting of podoplanin-overexpressing CAFs was significantly higher than that in organoids containing control CAFs (Exp. 1: 40.4% vs. 24.4%, Exp. 2: 40.0% vs. 24.5%, Exp. 3: 40.3% vs. 25.2%, Fig. 2C; each $p < 0.001$)

3.5. Comparison of MIB-1 labeling index between lung adenocarcinoma with podoplanin (+) and podoplanin (−) CAFs

In order to confirm the results obtained from hybrid cancer organoids, 27 cases of surgically resected adenocarcinoma were examined. The clinicopathological characteristics of the podoplanin (+) CAF cases are shown in Table 2. There were 13 cases of adenocarcinoma with podoplanin (+) CAFs and 14 cases with podoplanin (−) CAFs. The frequency of vascular invasion was significantly higher in cases of podoplanin (+) CAFs ($p < 0.05$), which was consistent with the previous reports [12].

The MIB-1 labeling index of cancer cells in cases with podoplanin (+) CAFs was 34.8% (11.3–55.7%, Fig. 3A). In cases with podoplanin (−) CAFs, the MIB-1 index was 16.2% (8.6–48.7%, Fig. 3B). The presence of podoplanin (+) CAFs was significantly associated with higher proliferative activity in cancer cells ($p < 0.01$, Fig. 3C).

4. Discussion

Recent studies have indicated that 3D culture systems, such as organoid cultures, could reflect the physiological complexity, heterogeneity, and plasticity of human tumor microenvironments better than conventional 2D culture systems [21–23]. However, most studies involving 3D systems use organoid models that contain only cancer cells. As CAFs are a major component of cancer stromal cells, 3D culture systems containing both cancer cells and CAFs can be considered as a better model for cancer research. In the current study, we first generated hybrid cancer organoids containing both cancer cells and podoplanin (+) CAFs, a subpopulation of tumor-promoting CAFs. Using this system, we analyzed the influence of podoplanin (+) CAFs on cancer cell proliferation.

In our previous study, we found that in CAFs, podoplanin enhanced tumor formation in lung adenocarcinoma cell lines [16]. Furthermore, during fibroblast-dependent cancer cell invasion, podoplanin (+) CAFs enhanced local invasion of cancer cells [17]. In this study, we clarified that podoplanin (+) CAFs increased cancer cell proliferation using a novel hybrid cancer organoid model and surgically resected samples. The molecular mechanism explaining how podoplanin-overexpressing CAFs enhance the proliferative capacity of PC-9 cells remains unclear. One possibility is that podoplanin in CAFs interacts directly with PC-9 cells through C-type lectin-like receptor 2 (CLEC-2), the known receptor for podoplanin [24]. However, PC-9 cells were negative for CLEC-2 when examined using quantitative reverse transcription polymerase chain reaction, suggesting that podoplanin-positive CAFs did not bind directly to cancer cells through CLEC-2 [25]. Identifying extracellular binding proteins that can interact with podoplanin will be an important topic of research in the future.

In the current study, PC-9 cells alone did not form spheroids, but the addition of CAFs successfully promoted spheroid/organoid formation (Table 1). The success rate of spheroid/organoid formation was 40% when equal numbers of CAFs and PC-9 cells (1×10^4) were mixed. However, the addition of 1×10^5 CAFs increased the success rate to up to 100%. These results supported the possibility that the CAFs provided a favorable environment in which PC-9 cancer cells could form organoids.

Several reports have demonstrated that epidermal growth factor receptor (EGFR) mutation-positive adenocarcinomas displayed a more non-solid histology than that of adenocarcinomas expressing wild-type EGFR [26,27]. In order to eliminate such bias, we used only EGFR mutation-positive adenocarcinoma specimens in the surgically resected samples.

To analyze genetic or protein functions, gene knockdown and overexpression are widely applied. We performed podoplanin knockdown in CAFs using short hairpin RNA technique and created hybrid cancer organoids using CAFs with reduced podoplanin expression [16,25]. However, the survival rate of both CAFs and cancer cells was markedly reduced in the hybrid cancer organoids (Supplementary Fig. 4A and B). The reason behind this phenomenon is unclear, and knockdown experiments could not be used for further analysis.

In the current study, we examined the effect of podoplanin (+) CAFs on the proliferation of cancer cells using 2D culture. We performed a mixed culture (PC-9 cells:CAFs = 1:10), but in all three independent experiments, there were no significant differences in the proportion of MIB-1-positive PC-9 cells between co-culture with podoplanin (+) and control CAFs (Supplementary Fig. 5). Although it is difficult to ensure that the conditions of 2D culture, such as cell density and/or cell-cell contact, are completely identical to those of 3D culture, this result supports the concept that organoid cultures can provide better insight regarding the physiological tumor characteristics in comparison to 2D culture systems.

In conclusion, we reported the generation of hybrid cancer organoids containing both cancer cells and podoplanin (+) CAFs and observed that podoplanin (+) CAFs exerted cancer cell growth-promoting effects. The development of this technique has the potential to become an innovative cancer research tool. The generation of such patient-derived hybrid cancer organoids that consist of both cancer cells and matched CAFs will boost our understanding of individual tumor microenvironments.

Conflict of interest

The authors have no conflict of interests to disclose.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Comprehensive informed consent was obtained in the study. IRB approval number of this study is 2017-43.

Acknowledgment

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.04.007.

References

[1] M. van de Wetering, H.E. Francies, J.M. Francis, G. Bounova, F. Iorio, A. Pronk, W. van Houdt, J. van Gorp, A. Taylor-Weiner, L. Kester, A. McLaren-Douglas, J. Blokker, S. Jaksani, S. Bartfeld, R. Volckman, P. van Sluis, V.S. Li, S. Seepo, C. Sekhar Pedamallu, K. Cibulskis, S.L. Carter, A. McKenna, M.S. Lawrence,

L. Lichtenstein, C. Stewart, J. Koster, R. Versteeg, A. van Oudenaarden, J. Saez-Rodriguez, R.G. Vries, G. Getz, L. Wessels, M.R. Stratton, U. McDermott, M. Meyerson, M.J. Garnett, H. Clevers, Prospective derivation of a living organoid biobank of colorectal cancer patients, Cell 161 (4) (2015) 933–945.
[2] F. Pampaloni, E.G. Reynaud, E.H. Stelzer, The third dimension bridges the gap between cell culture and live tissue, nature reviews, Molecular cell Boil. 8 (10) (2007) 839–845.
[3] K.M. Yamada, E. Cukierman, Modeling tissue morphogenesis and cancer in 3D, Cell 130 (4) (2007) 601–610.
[4] R. Kalluri, M. Zeisberg, Fibroblasts in cancer, Nat. Rev. Cancer 6 (5) (2006) 392–401.
[5] M.J. Bissell, D. Radisky, Putting tumours in context, Nat. Rev. Cancer 1 (1) (2001) 46–54.
[6] A. Orimo, P.B. Gupta, D.C. Sgroi, F. Arenzana-Seisdedos, T. Delaunay, R. Naeem, V.J. Carey, A.L. Richardson, R.A. Weinberg, Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion, Cell 121 (3) (2005) 335–348.
[7] C.L. Chaffer, R.A. Weinberg, A perspective on cancer cell metastasis, Science 331 (6024) (2011) 1559–1564.
[8] H. Hashimoto, Y. Suda, T. Miyashita, A. Ochiai, M. Tsuboi, K. Masutomi, T. Kiyono, G. Ishii, A novel method to generate single-cell-derived cancer-associated fibroblast clones, J. Cancer Res. Clin. Oncol. 143 (8) (2017) 1409–1419.
[9] S. Neri, H. Hashimoto, H. Kii, H. Watanabe, K. Masutomi, T. Kuwata, H. Date, M. Tsuboi, K. Goto, A. Ochiai, G. Ishii, Cancer cell invasion driven by extracellular matrix remodeling is dependent on the properties of cancer-associated fibroblasts, J. Cancer Res. Clin. Oncol. 142 (2) (2016) 437–446.
[10] M. Ishibashi, S. Neri, H. Hashimoto, T. Miyashita, T. Yoshida, Y. Nakamura, H. Udagawa, K. Kirita, S. Matsumoto, S. Umemura, K. Yoh, S. Niho, M. Tsuboi, K. Masutomi, K. Goto, A. Ochiai, G. Ishii, CD200-positive cancer associated fibroblasts augment the sensitivity of Epidermal Growth Factor Receptor mutation-positive lung adenocarcinomas to EGFR Tyrosine kinase inhibitors, Sci. Rep. 7 (2017) 46662.
[11] G. Ishii, A. Ochiai, S. Neri, Phenotypic and functional heterogeneity of cancer-associated fibroblast within the tumor microenvironment, Adv. Drug Deliv. Rev. 99 (Pt B) (2016) 186–196.
[12] A. Kawase, G. Ishii, K. Nagai, T. Ito, T. Nagano, Y. Murata, T. Hishida, M. Nishimura, J. Yoshida, K. Suzuki, A. Ochiai, Podoplanin expression by cancer associated fibroblasts predicts poor prognosis of lung adenocarcinoma, Int. J. Cancer 123 (5) (2008) 1053–1059.
[13] S. Ono, G. Ishii, K. Nagai, T. Takuwa, J. Yoshida, M. Nishimura, T. Hishida, K. Aokage, S. Fujii, N. Ikeda, A. Ochiai, Podoplanin-positive cancer-associated fibroblasts could have prognostic value independent of cancer cell phenotype in stage I lung squamous cell carcinoma: usefulness of combining analysis of both cancer cell phenotype and cancer-associated fibroblast phenotype, Chest 143 (4) (2013) 963–970.
[14] M. Ito, G. Ishii, K. Nagai, R. Maeda, Y. Nakano, A. Ochiai, Prognostic impact of cancer-associated stromal cells in patients with stage I lung adenocarcinoma, Chest 142 (1) (2012) 151–158.
[15] B. Pula, A. Jethon, A. Piotrowska, A. Gomulkiewicz, T. Owczarek, J. Calik, A. Wojnar, W. Witkiewicz, J. Rys, M. Ugorski, P. Dziegiel, M. Podhorska-Okolow, Podoplanin expression by cancer-associated fibroblasts predicts poor outcome in invasive ductal breast carcinoma, Histopathology 59 (6) (2011) 1249–1260.
[16] A. Hoshino, G. Ishii, T. Ito, K. Aoyagi, Y. Ohtaki, K. Nagai, H. Sasaki, A. Ochiai, Podoplanin-positive fibroblasts enhance lung adenocarcinoma tumor formation: podoplanin in fibroblast functions for tumor progression, Cancer Res. 71 (14) (2011) 4769–4779.
[17] S. Neri, G. Ishii, H. Hashimoto, T. Kuwata, K. Nagai, H. Date, A. Ochiai, Podoplanin-expressing cancer-associated fibroblasts lead and enhance the local invasion of cancer cells in lung adenocarcinoma, Int. J. Cancer 137 (4) (2015) 784–796.
[18] S. Yamazaki, Y. Higuchi, M. Ishibashi, H. Hashimoto, M. Yasunaga, Y. Matsumura, K. Tsuchihara, M. Tsuboi, K. Goto, A. Ochiai, G. Ishii, Collagen type I induces EGFR-TKI resistance in EGFR-mutated cancer cells by mTOR activation through Akt-independent pathway, Cancer Sci. 109 (6) (2018) 2063–2073.
[19] S. Ito, G. Ishii, A. Hoshino, H. Hashimoto, S. Neri, T. Kuwata, M. Higashi, K. Nagai, A. Ochiai, Tumor promoting effect of podoplanin-positive fibroblasts is mediated by enhanced RhoA activity, Biochem. Biophys. Res. Commun. 422 (1) (2012) 194–199.
[20] J.N. Jakobsen, J.B. Sorensen, Clinical impact of ki-67 labeling index in non-small cell lung cancer, Lung cancer (Amsterdam, Netherlands 79 (1) (2013) 1–7.
[21] L.A. Kunz-Schughart, J.P. Freyer, F. Hofstaedter, R. Ebner, The use of 3-D cultures for high-throughput screening: the multicellular spheroid model, J. Biomol. Screen. 9 (4) (2004) 273–285.
[22] I. Rizvi, U.A. Gurkan, S. Tasoglu, N. Alagic, J.P. Celli, L.B. Mensah, Z. Mai, U. Demirci, T. Hasan, Flow induces epithelial-mesenchymal transition, cellular heterogeneity and biomarker modulation in 3D ovarian cancer nodules, Proceedings of the National Academy of Sciences of the United States of America 110 (22) (2013) E1974–E1983.
[23] M. Zanoni, F. Piccinini, C. Arienti, A. Zamagni, S. Santi, R. Polico, A. Bevilacqua, A. Tesei, 3D tumor spheroid models for in vitro therapeutic screening: a systematic approach to enhance the biological relevance of data obtained, Sci. Rep. 6 (2016) 19103.
[24] K. Suzuki-Inoue, Y. Kato, O. Inoue, M.K. Kaneko, K. Mishima, Y. Yatomi, Y. Yamazaki, H. Narimatsu, Y. Ozaki, Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, Cancer Cells 282 (36) (2007) 25993–26001.
[25] T. Yoshida, G. Ishii, K. Goto, S. Neri, H. Hashimoto, K. Yoh, S. Niho, S. Umemura, S. Matsumoto, H. Ohmatsu, S. Iida, A. Niimi, K. Nagai, Y. Ohe, A. Ochiai, Podoplanin-positive cancer-associated fibroblasts in the tumor microenvironment induce primary resistance to EGFR-TKIs in lung adenocarcinoma with EGFR mutation, Clin. cancer Res.:off. J. Am. Assoc. Cancer Res. 21 (3) (2015) 642–651.
[26] N. Motoi, J. Szoke, G.J. Riely, V.E. Seshan, M.G. Kris, V.W. Rusch, W.L. Gerald, W.D. Travis, Lung adenocarcinoma: modification of the 2004 WHO mixed subtype to include the major histologic subtype suggests correlations between papillary and micropapillary adenocarcinoma subtypes, EGFR mutations and gene expression analysis, Am. J. Surg. Pathol. 32 (6) (2008) 810–827.
[27] T. Isaka, H. Nakayama, H. Ito, T. Yokose, K. Yamada, M. Masuda, Impact of the epidermal growth factor receptor mutation status on the prognosis of recurrent adenocarcinoma of the lung after curative surgery, BMC Cancer 18 (1) (2018) 959.