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# Characterization of human lung cancer-associated fibroblasts in three-dimensional *in vitro* co-culture model

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ABSTRACT

Lung cancer is the most common cause of cancer-related death worldwide. Stromal cancer-associated fibroblasts (CAFs) play crucial roles in carcinogenesis, proliferation, invasion, and metastasis of non-small cell lung carcinoma, and targeting of CAFs could be a novel strategy for cancer treatment. However, the characteristics of human CAFs still remain to be better defined. In this study, we established patient-matched CAFs and normal fibroblasts (NFs), from tumoral and non-tumoral portions of resected lung tissue from lung cancer patients. CAFs showed higher α-smooth muscle actin (α-SMA) expression than NFs, and CAFs clearly enhanced collagen gel contraction. Furthermore, we employed three-dimensional co-culture assay with A549 lung cancer cells, where CAFs were more potent in inducing collagen gel contraction. Hematoxylin and eosin staining of co-cultured collagen gel revealed that CAFs had the potential to increase invasion of A549 cells compared to NFs. These observations provide evidence that lung CAFs have the tumor-promoting capacity distinct from NFs.



## 1. Introduction

Lung cancer is the most common cause of cancer-related death worldwide, both in men and women [1]. Despite the advance in lung cancer therapeutics, 5-years survival rate remains largely unaltered. Cancer progression depends on genetic alterations intrinsic to cancer cells as well as the tumor microenvironment. The tumor stroma is considered as the potential target of cancer therapy, and molecular targeted agents against tumor vessels have been recently applied in clinical use.

Cancer-associated fibroblasts (CAFs), a major constituent of the tumor stroma, are shown to be a key determinant in malignant progression of cancer [2–6]. Fibroblasts become activated in wound healing and fibrosis, and these cells are called myofibroblasts which differ morphologically and functionally from normal fibroblasts [7]. The activated fibroblasts surrounding cancer cells, termed as CAFs, are not removed by apoptosis as seen in normal wound healing. Instead, CAFs are prominent contributors in carcinogenesis and play key roles in promoting cancer progression via cell–cell interaction through growth factors and chemokines, or by modification of extracellular matrices (ECM). Thus direct targeting of CAFs attracts increasing attention as a novel strategy for cancer treatment [8–10]. Recent studies demonstrate that normal fibroblasts (NFs) and CAFs display distinct gene expression signatures [11–15]. However, it is also becoming recognized that CAFs are heterogeneous and are likely to be composed of subsets, still remaining to be better defined [16,17].

Two-dimensional (2D) culture system has been widely used for *in vitro* studies though cell–cell and cell–matrix interaction are largely lost under these simplified conditions. To fill in the gap between the monolayer cell culture and the environment *in vivo*, three-dimensional (3D) *in vitro* culture has emerged as a new approach that mimics the *in vivo* cell growth environment. In these models, cells are embedded within matrices resembling the natural ECM which allow cell–ECM interaction in a less complex and more well-defined condition. There have been several reports describing the co-culture of lung fibroblasts and human bronchial cells in 3D type I collagen gels [18–20], and these 3D models have shed light on the molecular mechanisms underlying cell–cell communication.

*Abbreviations:* CAFs, cancer-associated fibroblasts; ECM, extracellular matrices; NFs, normal fibroblasts; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NSCLC, non-small cell lung carcinoma; α-SMA, α-smooth muscle actin.

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Until now, several studies have shown the differences between CAFs and NFs, using 3D *in vitro* co-culture model. These model systems closely mirrored the architecture of human cancers and allowed the characterization of the tumor cell–stroma interactions morphologically as well as at the molecular level [21,22]. In regard to lung cancer, one study investigated epithelial–mesenchymal interaction of primary cultured CAFs or IMR90, a normal lung fibroblast cell line, with primary human bronchial epithelial cells (HBECs). They showed CAFs altered the HBEC phenotype and gene expression [23]. However, there has been no report which compared characteristics of patient-matched CAFs and NFs, using 3D *in vitro* model of lung cancer.

In the present study, we established three sets of patient-matched primary cultured fibroblasts from the resected lung cancer stroma (CAFs) and the normal lung tissue (NFs). Morphological, proliferative and contractile properties of CAFs and NFs were compared, and 3D *in vitro* co-culture model has been established using A549 lung cancer cells.

## 2. Materials and methods

### 2.1. Cell culture

Tissue culture media and supplements were purchased from GIBCO (Life Technologies, Grand Island, NY). A549 lung cancer cells were obtained from American Type Culture Collection. Cells were cultured in 100-mm tissue-culture dishes (FALCON; Becton-Dickinson Labware, Lincoln Park, NJ) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS, GIBCO) and 1% penicillin–streptomycin at 37 °C, 5% $CO_2$ incubator.

### 2.2. Primary culture of lung fibroblasts

Lung tissues were collected from three non-small cell lung carcinoma (NSCLC) patients who underwent surgical resection at the University of Tokyo Hospital. The study was approved by the Ethics Committee at the University of Tokyo. Written informed consent was obtained from all the subjects. Both the tumor and corresponding normal lung parenchyma, which was as far from the tumor as possible, were used to establish primary culture of CAFs and NFs. Harvested tissues were minced into small pieces and cultured in DMEM containing 10% FBS on 100-mm tissue culture plates. The clinical features of the subjects are summarized in Supplementary Table S1. Most cells were used after 3–7 passages following primary culture.

### 2.3. Gel contraction assay

CAFs and NFs were cast into collagen gels using previously reported methods [24]. Briefly, collagen gel (Cell matrix type IA; Nitta Gelatin, Osaka, Japan), distilled water, 4× concentrated DMEM and suspended cells were gently mixed. Volumes were adjusted so that the collagen concentration was 1.5 mg/ml and the cell density was $3 \times 10^5$ cells/ml, with a physiological ionic strength equaling 1× DMEM. The mixture (0.5 ml) was dispensed into each well of 24-well tissue culture plates (FALCON). Gelation occurred in 20 min at room temperature. Gel contraction was initiated by detaching the perimeter of the collagen gels from the side of the wells. Gels were then transferred to 60-mm tissue culture dishes containing 5 ml of medium and placed in the incubator. The collagen gel area was determined daily for 3 days using an image analysis system (Densitograph, version 3.00; ATTO, Tokyo, Japan).

### 2.4. Three-dimensional co-culture

Three-dimensional (3D) gel cultures were carried out according to the previously published protocol [25,26]. Briefly, collagen gels were prepared by mixing 0.5 ml cell suspension of CAFs or NFs ($2.5 \times 10^5$ cells) in FBS, 2.3 ml of type I collagen (Cell matrix type IA; Nitta Gelatin, Tokyo, Japan), 670 μl of 5× DMEM, and 330 μl of reconstitution buffer, following the manufacturer's recommendation. The mixture (3 ml) was cast into each well of a 6-well culture plate. The solution was then allowed to polymerize at 37 °C for 30 min. For co-culture, A549 cells ($2 \times 10^5$ cells) were seeded on the surface of each gel and cultured in DMEM with 10% FBS. After incubation overnight, each gel was detached and cultured with growth medium for five additional days. The surface area of the gels was measured using Densitograph for consecutive 5 days and the relative value compared with that of the initial size was determined. We then exposed the gels to air by placing it on a mesh in new plates with growth medium. After 5 days of the air–liquid interface culture, the gel was fixed in formalin solution and embedded in paraffin, and vertical sections (4 μm) were stained with hematoxylin and eosin. Immunostaining for Ki67 was performed using the streptavidin–biotin-peroxidase method (LSAB2 Kit/HRP; DAKO, Kyoto, Japan).

### 2.5. Reverse-transcriptase/quantitative PCR (RT-qPCR) assay

Total RNA was extracted from CAFs and NFs, using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). The cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Quantification of mRNA levels was performed using Mx-3000P (Stratagene, La Jolla, CA) and QuantiTect SYBR Green PCR (Qiagen) according to the manufacturer's instruction. Relative mRNA expression was calculated using $\Delta\Delta C_t$ method. Individual data were normalized against the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Specific primers for GAPDH, and α-smooth muscle actin (α-SMA) were as follows: GAPDH sense, (5′)-GGTGAAGGTCGGAGTCAACGGA-(3′), GAPDH antisense, (5′)-GAGGGATCTCGCTCCTGGAAGA-(3′), α-SMA sense, (5′)-GCACCCAGCACCATGAAGA-(3′), α-SMA antisense, (5′)-ACCGATCCAGACAGAGTATTT-(3′).

### 2.6. Western Blot analysis

Cells were lysed in lysis buffer solution (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), followed by SDS gel-electrophoresis and semi-dry transfer of the proteins to polyvinylidene difluoride membrane. Non-specific binding of proteins to the membrane was blocked by incubation in TBS-T buffer (50 mM Tris–HCl, pH 7.4 and 150 mM NaCl, and 0.1% Tween-20) with 2% ECL Prime Blocking Reagent (GE Healthcare, Buckinghamshire, UK) and the membrane was incubated with primary antibodies to human α-SMA (Sigma–Aldrich, St. Louis, MO) at a 1:5000 dilution. After incubations with anti-mouse HRP-linked antibody at a 1:20,000 dilution, immunodetection was performed with the ECL Prime Western Blotting Detection Kit (GE Healthcare). Equal protein loading was confirmed by probing the blot with antibody against α-tublin (Sigma–Aldrich) at a 1:5000 dilution. Pictures were taken by a cold CCD camera (EZ-Capture MG; ATTO, Tokyo, Japan).

### 2.7. Immunocytochemistry

CAFs and NFs were seeded in chamber slides and immunocytochemistry staining was performed using the LSAB2 Kit/HRP. Briefly, the acetone–methanol fixed cultured cells were treated with 3% $H_2O_2$ for 5 min. The cells were then incubated with

primary antibody for vimentin (Sigma–Aldrich) at a 1:100 dilution, for pan-keratin (Cell Signaling, Beverly, MA) at a 1:100 dilution. Subsequently, the cells were incubated with the anti-rabbit IgG-biotin conjugate and streptavidin-HRP for 60 min each at room temperature. After that, the cells were washed thoroughly with TBS-T buffer. The cells were then incubated for 10 min at room temperature in a substrate, consisting of 0.05% diaminobenzidine (DAB) chromogen. Finally, the cells were counterstained with Harris hematoxylin, dehydrated, and visualized under a microscope.

### 2.8. Statistics

Results were confirmed by performing experiments in triplicate. Data are expressed as the mean ± SEM. Analyses were performed using JMP (version 9; SAS Institute Inc., Tokyo, Japan). Significance of difference was analyzed using Student's $t$ tests. $p < .05$ was considered to be significant.

## 3. Results

### 3.1. Isolation of CAFs and NFs

Three sets of CAFs and NFs were established from lung cancer patients who underwent surgical resection of the lung (Case #1–3). CAFs were obtained from the tumor portion and NFs were from the non-tumoral part of the resected lung, that is, the margin apart from the tumor area. Cell morphology was determined by phase-contrast microscopy, which revealed that CAFs and NFs showed similar spindle-like appearances (Fig. 1A). To exclude the possible contamination of epithelial cells, we performed immunostaining of these cells. Cytokeratins are specific to epithelial cells whereas vimentin is normally expressed in fibroblasts and not epithelial cells.[27] The obtained CAFs and NFs were positively stained for vimentin (Fig. 1B), whereas we could not find any cell positively stained for pan-keratin in the cell populations of both CAFs and

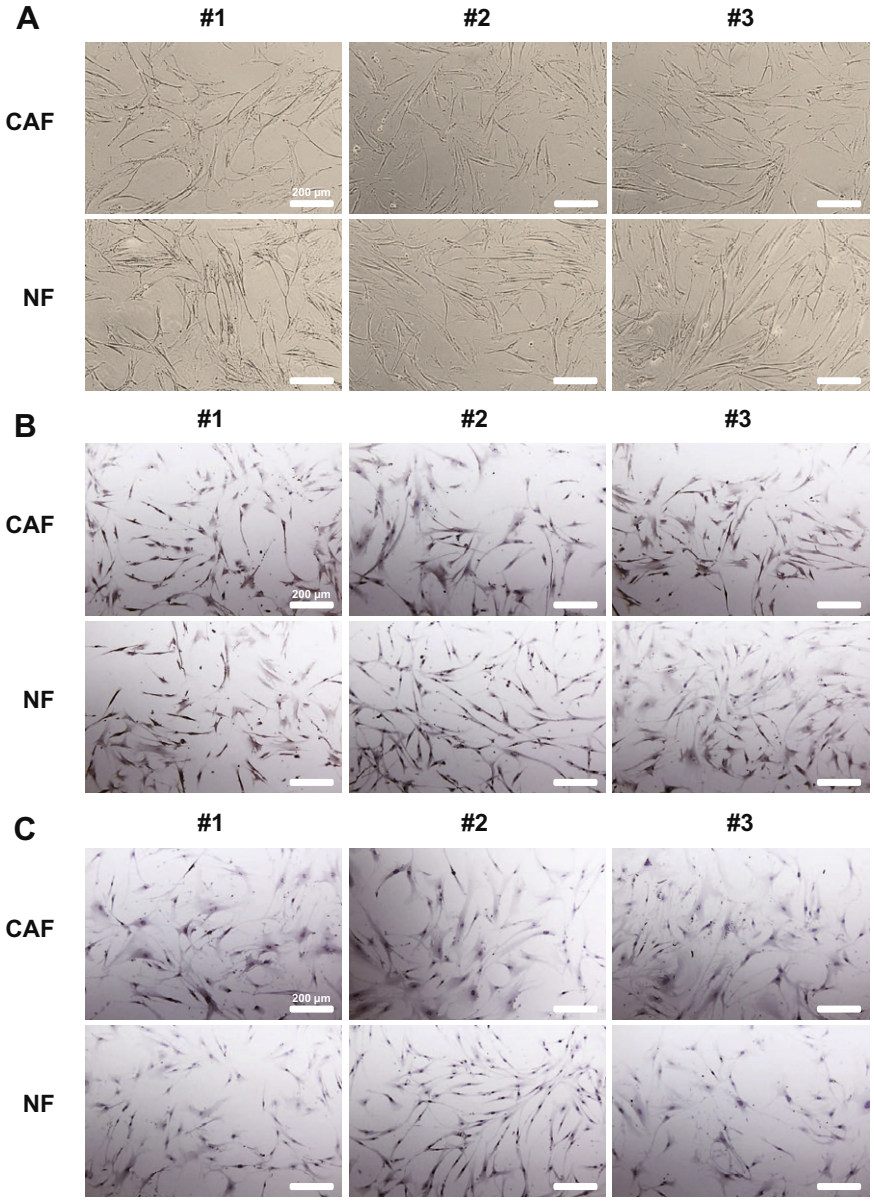


**Fig. 1.** (A) Phase-contrast micrograph of CAFs and NFs. (B) Immunostaining of CAFs and NFs for vimentin. (C) Immunostaining of CAFs and NFs for pan-keratin. Upper panel: CAFs, lower panel: NFs. Left: Case #1, middle: Case #2, right: Case #3. Scale bar, 200 μm.

NFs (Fig. 1C). These results showed that the primary cultured cells were uniformly composed of fibroblasts as confirmed by morphological and immunocytochemical studies.

### 3.2. CAFs express α-smooth muscle actin, a marker for myofibroblasts

The activated fibroblasts are characterized by expression of α-smooth muscle actin (α-SMA), which are termed as myofibroblasts [28]. Since previous reports show that CAFs display characteristics of myofibroblasts, we compared α-SMA expression levels between CAFs and NFs. Quantitative PCR and immunoblotting analysis showed that α-SMA expression levels of CAFs were higher than those of NFs, though the differences of basal expression level were also noted among the cases (Fig. 2A and B).

Next we evaluated the difference of cell proliferation between CAFs and NFs. In Case #1 and 2, cell proliferation rate of CAFs

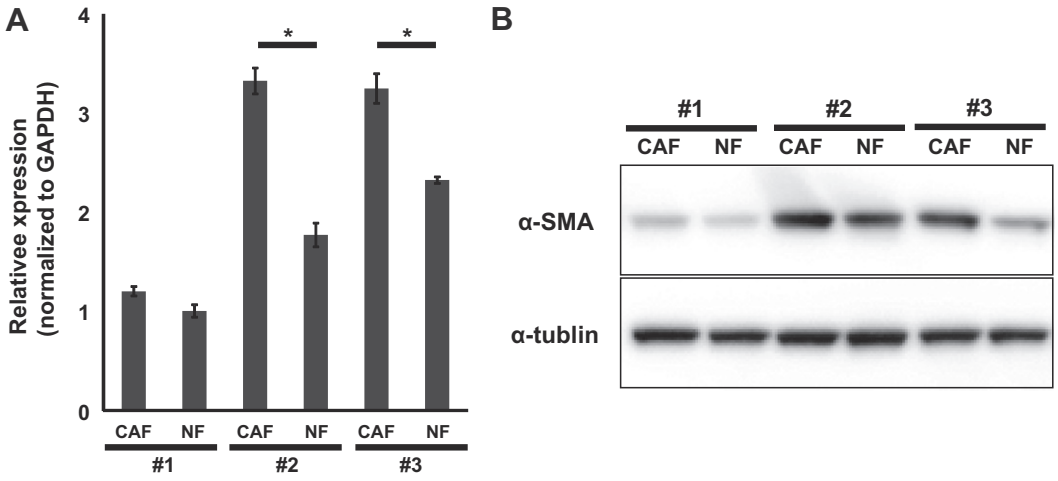


**Fig. 2.** (A) Quantitative PCR of CAFs and NFs for α-SMA. Data shown are the mean ± SEM. *$P < .05$. (B) Immunoblotting of CAFs and NFs for α-SMA. Upper panel: α-SMA, lower panel: α-tublin (loading control).

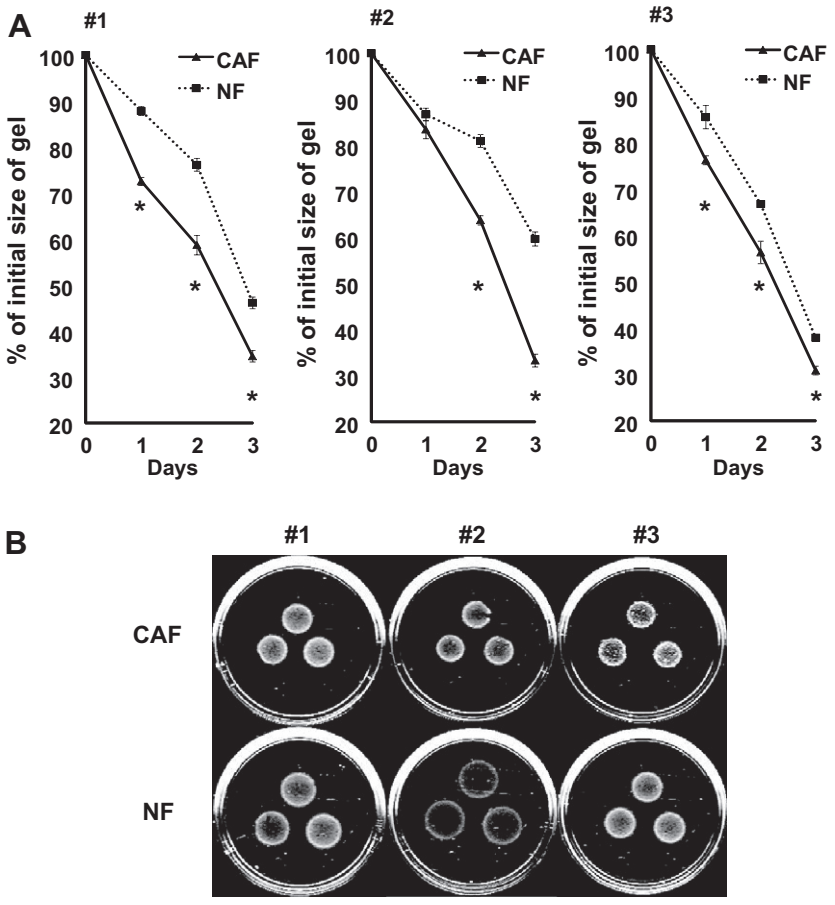


**Fig. 3.** (A) Time-course of the area of collagen gel composed of CAFs (solid line) and NFs (dot line). The relative value compared with that of the initial size was determined. The area of each gel was assessed daily for 3 days. Data shown are the mean ± SEM for triplicate cultures. Left: Case #1, middle: Case #2, right: Case #3. *$P < .05$. (B) Pictures taken on day 3 after seeding. Upper panel: CAFs, lower panel: NFs. Left: Case #1, middle: Case #2, right: Case #3.

and NFs did not show any difference, whereas that of CAFs was significantly higher than NFs in Case #3 (Fig. S1).

### 3.3. CAFs potently induce collagen gel contraction

The activated fibroblasts are characterized by enhanced contractile property, and increased expression of α-SMA has been implicated in contractile activity of fibroblasts [29]. To explore the functional differences between CAFs and NFs, we performed collagen gel contraction assay. CAFs clearly enhanced collagen gel contraction compared with NFs in all the cases (Fig. 3A and B). These results further supported that CAFs displayed the characteristics of myofibroblasts or activated fibroblasts.

### 3.4. Three-dimensional co-culture of CAFs or NFs with lung cancer cells

To examine the interaction between CAFs and lung cancer cells, we established three-dimensional (3D) co-culture model of primary cultured lung fibroblasts and A549 lung cancer cells. CAFs or NFs were embedded into the collagen gels, and A549 cells were seeded on the surface of these gels. In this co-culture model, CAFs clearly enhanced collagen gel contraction compared with NFs, in Case #2 and 3, as seen in mono-culture collagen gel contraction assay (Fig. S2).

The co-cultured collagen gels were further processed to the air–liquid interface culture for additional 5 days, followed by hematoxylin and eosin staining (Fig. 4A). In Case #2 and 3, A549 cells inside the collagen gels were observed more frequently in the samples with CAFs, as compared to those with NFs. In the specimen containing CAF #2, nodular epithelial structures were observed beneath the surface epithelial layer while these structures were not observed in collagen gels containing NF #2. These observations suggested that CAFs were more potent in enhancing A549 lung cancer cell invasion into collagen gels, compared to NFs.

In Case #2, many mucinous structures were observed inside the epithelial layer, whereas these changes were not observed in either Case #1 or 3. This result suggested that both CAFs and NFs in Case #2 might have the capacity to induce the differentiation of A549 cells into mucinous cells. These results supported the idea that fibroblasts play critical roles in regulating the phenotype and behavior of cancer cells.

To assess the effect on cell proliferation of cancer cells in 3D co-culture model, immunostaining for Ki67, which is a marker of cell proliferation and whose expression is a factor of poor prognosis for survival in NSCLC [30], was performed on cross-sections of 3D co-cultured gel (Fig. 4B). Ki-67 positive A549 cells were seen to a similar extent in 3D co-cultured gels with CAFs and NFs in all cases. These results indicated that CAFs had little effect on cell proliferation in 3D co-cultured model compared with NFs.

## 4. Discussion

Accumulating evidence suggests that cancer microenvironment plays critical roles in tumor development and progression via cross-talk between cancer and stromal cells [3,4]. Three-dimensional culture system attracts increasing attention since it recapitulates the physiological architecture, providing a useful tool for studying cell–cell communication as well as cell–ECM interaction. Here, we established 3D *in vitro* co-culture model of CAFs/NFs with A549 cells, and assessed the functional effects of CAFs/NFs on lung cancer cells. Our *in vitro* co-culture model mimicked the structure of *in vivo* cancer tissue and was useful for studying the invasive behavior of

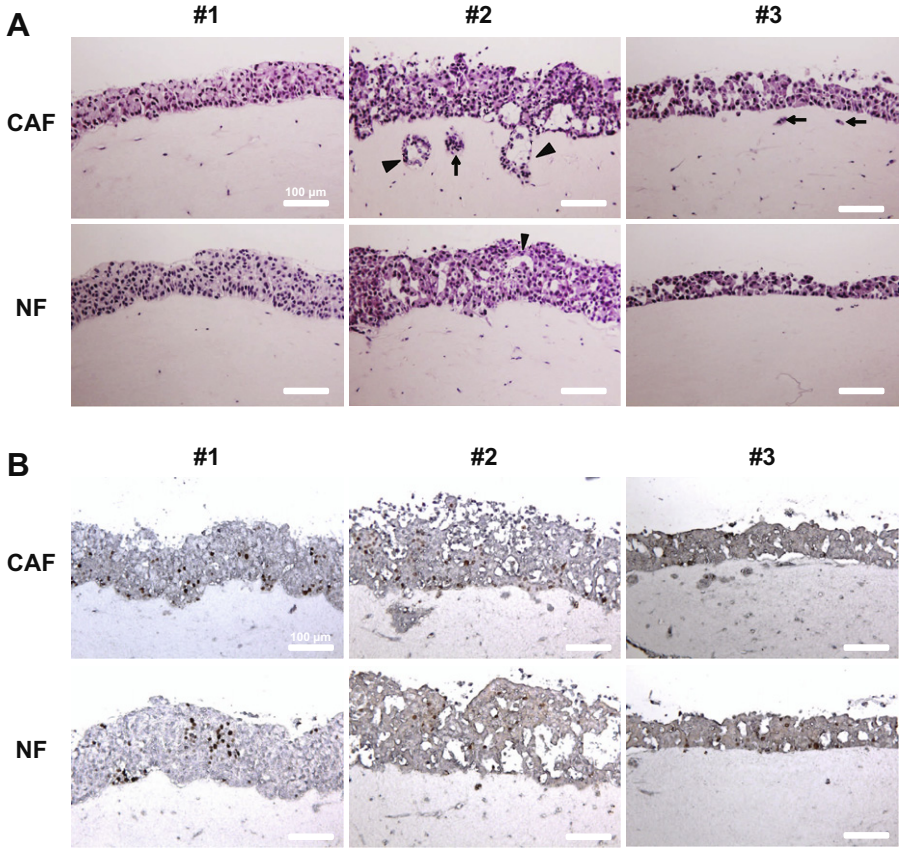


**Fig. 4.** (A) Hematoxylin and eosin stain of cross-sections of 3D cultured gel composed of A549 cells and CAFs or NFs. Arrows indicate A549 cell invasion and arrowheads indicate mucous structures. (B) Immunostaining of cross-section of 3D cultured gel for Ki-67. Upper panel: CAFs lower panel: NFs. Left: Case #1, middle: Case #2, right: Case #3. Scale bar, 100 μm.

cancer cells. In two out of three cases, CAFs had the potential to enhance A549 cell invasion into collagen gels, suggesting the tumor-promoting role of CAFs.

Stromal/mesenchymal cells play crucial roles in determining cell behaviors in cancer/epithelial cells, by producing instructive signals such as growth factors and chemokines [3,31]. Our observation that primary cultured fibroblasts had the potential to induce differentiation of cancer cells, suggests the greater roles of fibroblasts than previously recognized. Considering the heterogeneous characters of primary cultured fibroblasts among patients, future studies with larger number of samples would be necessary to obtain conclusive results.

CAFs might originally derive from several types of cells, such as pre-existing resident fibroblasts [32], nearby epithelial cells through epithelial-to-mesenchymal transition [33], bone marrow-derived cells [34], and endothelial cells [35]. Although morphological differences between CAFs and NFs were not noted in this study, CAFs had the phenotype of myofibroblasts as determined by higher expression of α-SMA and higher contractile capacity in collagen matrices. Despite the possibly different origins and heterogeneity of CAFs, they appeared to share the same characteristics.

In conclusion, CAFs from human lung cancer differ from NFs with respect to α-SMA expression and contractile property. Three-dimensional co-culture model of CAFs/NFs with lung cancer cells would help understanding the crosstalk between tumor cells and stroma.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.104.

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