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Collective cancer cell invasion in contact with fibroblasts through integrin-α5β1/fibronectin interaction in collagen matrix

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Abstract

Interaction of cancer cells with cancer-associated fibroblasts (CAFs) plays critical roles in tumor progression. Recently we proposed a new tumor invasion mechanism in which invasive cancer cells individually migrate on elongate protrusions of CAFs (CAF fibers) in 3-D collagen matrix. In this mechanism, cancer cells interact with fibronectin fibrils assembled on CAFs mainly through integrin-α5β1. Here we tested whether this mechanism is applicable to the collective invasion of cancer cells, using two E-cadherin-expressing adenocarcinoma cell lines, DLD-1 (colon) and MCF-7 (breast). When hybrid spheroids of DLD-1 cells with CAFs were embedded into collagen gel, DLD-1 cells collectively but very slowly migrated through the collagen matrix in contact with CAFs. Epidermal growth factor and tumor necrosis factor-α promoted the collective invasion, possibly by reducing the E-cadherin junction, as did the transforming growth factor-β inhibitor SB431542 by stimulating the outgrowth of CAFs. Transforming growth factor-β itself inhibited the cancer cell invasion. Efficient collective invasion of DLD-1 cells required large CAF fibers or their assembly as stable adhesion substrates. Experiments with function-blocking Abs and siRNAs confirmed that DLD-1 cells adhered to fibronectin fibrils on CAFs mainly through integrin-α5β1. Anti-E-cadherin Ab promoted the single cell invasion of DLD-1 cells by dissociating the E-cadherin junction. Although the binding affinity of MCF-7 cells to CAFs was lower than DLD-1, they also collectively invaded the collagen matrix in a similar fashion to DLD-1 cells. Our results suggest that the direct interaction with CAFs, as well as environmental cytokines, contributes to the collective invasion of cancers.

KEYWORDS

cancer-associated fibroblast, collective invasion, cytokine, fibronectin, integrin

1 | INTRODUCTION

Tumor invasion is one of the most critical steps in cancer metastasis. However, the mechanism of cancer cell invasion into the connective tissues has not sufficiently been elucidated. For understanding this mechanism, it is essential to clarify the interaction between cancer cells and their microenvironmental factors.[1,2] In addition, simple and physiologically relevant experimental models are required for these studies. Fibroblasts are the most abundant cell type in the tumor microenvironment. There are many lines of evidence that fibroblasts around cancer cells, called cancer-associated fibroblasts (CAFs), promote tumor invasion and progression by complex interaction with cancer cells.[3–6]

Cytokines such as transforming growth factor-β (TGF-β), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and tumor necrosis factor-α (TNF-α), regulate the epithelial-mesenchymal transition (EMT), motility, and proliferation of cancer cells, depending on cancer cell types.[5,7,8] Extracellular matrix-degrading enzymes such as MMPs and serine proteinases are also required for cancer cell invasion.[4,5,9] Extracellular matrices such as fibronectin (FN)[10–12] and laminins[13–15] play fundamental roles in supporting adhesion, survival, and migration of cancer cells. Fibronectin is the major ECM component produced by normal fibroblasts and CAFs.[10,11] There is accumulating evidence that FN is involved in tumor progression, and its tumor-suppressive functions have also been reported.[11,12] Recent in vitro studies have shown that the FN matrix supports cancer cell invasion.[16–18] However, some other studies suggest that CAFs support cancer cell invasion by direct interaction with cancer cells.[19–21] One of the studies indicated that fibroblasts lead collective cell invasion of epidermoid carcinoma cells in collagen matrix.[21] In this model, fibroblasts tow cancer cells into the collagen matrix by direct cell adhesion through E-cadherin/N-cadherin heterophilic interaction.

Recently we established a simple 3-D collagen gel culture model to investigate the cancer invasion mechanism. Using this system, we proposed a novel tumor invasion mechanism in which cancer cells invade the collagen matrix while binding to fibroblasts through integrin-α5β1/FN interaction.[22] In this mechanism, invasive cancer cells, such as lung carcinoma A549 and pancreatic carcinoma Panc-1 cells, individually migrate on single elongated protrusions of CAFs on which FN fibrils are densely assembled. However, it seems physically difficult for a cluster of cancer cells to migrate on a single fibroblast fiber. Pathological studies of cancer tissues showed that the migration of cancer cells in clusters, ie collective cancer invasion, occurs more often than the single cell invasion.[23–25] In the former cases, cancer cells collectively invade stromal tissues keeping tight intercellular junctions, mostly the E-cadherin-mediated junction.[25] In this study, therefore, we investigated whether our previously proposed mechanism is applicable to collective cancer invasion. We also examined the effects of some cytokines on cancer cell invasion in our model.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

Function-blocking mouse mAbs against FN (FN12-8) and E-cadherin (SHE78-7) were purchased from Takara. Other Abs used were described previously.[22] Cytokines and signaling inhibitors were purchased as follows: EGF, TNF-α, SB431542 (TGF-β type I receptor/ALK5 inhibitor), Y27632 (Rock inhibitor), LY294002 (PI3K inhibitor), and U0126 (MEK1/2 inhibitor) were from Fuji Film/Wako, and TAPI-1 (metalloproteinase inhibitor) was from Funakoshi. The RGD peptide (Gly-Arg-Gly-Asp-Ser-Pro) was purchased from Takara and bovine dermis native collagen from Koken.

2.2 | Human cell lines, colorectal cancer tissues, and culture condition

DLD-1 (colon adenocarcinoma) and MCF-7 (mammary adenocarcinoma) cells were obtained from the Japanese Collection of Research and Bioresources. Green fluorescent protein-labeled Panc-1 (pancreas adenocarcinoma), GFP-labeled A549 (lung adenocarcinoma), and WI-38 (fetal lung fibroblast) cell lines were used in our previous study.[22] DLD-1 cells were labeled with GFP by introducing the pTagGFP2-N vector (Evrogen) with the Lipofectamine 3000 reagent (Invitrogen), and the GFP-expressing cells were selected with Geneticin (Gibco). MCF-7 cells were transiently labeled with green fluorescence using Cell Explore Live Cell Tracking Kit according to the manufacturer's protocol (AAT Bioquest). Primary cultures of human lung cancer-derived fibroblasts were established in Juntendo University Hospital. The Institutional Review Board at the Juntendo University School of Medicine approved the procedures. The patient provided written, informed consent (No. 20 042). Experimental procedures were carried out according to the institutional guidelines and those of the 1995 Declaration of Helsinki. In this study, one of the fibroblast lines was used as a CAF. In some experiments, CAFs were transiently labeled with red fluorescence using the same kit as above. All these cell lines were maintained in DMEM/F12 medium (Invitrogen) supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Human cancer specimens were obtained from patients who underwent surgery at the Kanagawa Cancer Center (KCC) Hospital (Kanagawa, Japan) between 2006 and 2009 and provided by Human Cancer Tissue Bank of KCC.[22] The study protocol was approved by the Ethical Committees of KCC, and carried out as described previously.[22]

2.3 | Cancer cell invasion assay in 3-D collagen gel

Collagen gel invasion assay was undertaken as previously reported.[22] Briefly, hybrid spheroids of cancer cells and CAFs were prepared by incubating their mixture (1.5 × 10⁴ cells/well each) overnight on an EZSPHERE 96-well plate (AGC Techno Glass), which effectively

prepares homogeneous microspheroids.[26] The resulting spheroids in each well were collected into a 1.5-mL tube and suspended in 100 μL ice-cold collagen/medium solution. Each spheroid contained approximately 170 cells on average for each cell type. For the 3-D gel cultures, two layers of collagen gel, ie 70 μL lower gel and 50 μL upper spheroid-containing gel, were prepared on the 12-mm glass bottom of 35-mm culture dishes (AGC) and incubated in the standard medium for 2-14 days. Phase-contrast images with GFP fluorescence signals were obtained using an Olympus CKX41 inverted microscope. For time-lapse experiments, phase-contrast images with fluorescent signals were obtained using a Keyence BZ-9000 digital fluorescence microscope equipped with a CO_2 incubator at 60-minute intervals for one representative spheroid.

2.4 | Inhibition assays of 3-D cancer cell invasion

To assay inhibitory activity of Abs, 10 μg/mL normal mouse IgG as control, 1.3 μg/mL anti-FN mouse mAb (FN12-8), 1.3 μg/mL anti-E-cadherin SHE78-7 mouse mAb, or antiintegrin Abs at a 100-fold dilution were included in both spheroid-containing gel and culture medium. To see the effects of small molecules and cytokines, the factors were added only into culture medium. The RGD peptide was used at 0.3 mmol/L. Fluorescent images were obtained after 2 days of incubation. Relative activity of DLD-1 cell invasion was quantitated by NIH ImageJ software. One broader area enclosing invaded and noninvaded cells and a spheroid core area were separately set in each fluorescent image of 12-15 representative spheroids obtained from triplicate dishes. The tumor cell invasion was determined by subtracting the fluorescent intensity of the spheroid core from that of the broader area.

2.5 | Cell adhesion assays in 2-D cultures

Adhesion activity was assayed on 24-well culture plates. Fluorescence-labeled cancer cells were seeded at 2×10^4 cells/well on a previously prepared confluent CAF layer. To assay inhibitory activity of Abs, cancer cells were pretreated with each Ab at a concentration described above for 30 minutes and then placed on the CAF layer together with each inhibitor sample. In the case of anti-FN Ab, CAFs were pretreated with the Ab. After incubation for 5 hours, culture images were taken under a fluorescent microscope and the percentage of spread cancer cells was determined in each image with similar cell distribution.

2.6 | Immunocytochemistry

Immunofluorescent staining was undertaken as described previously.[22] Cultures or frozen tissue sections were fixed with 10% formalin, blocked with 3% (w/v) BSA/PBS. They were treated with the anti-FN mouse mAb FN12-8 or the anti-E-cadherin mouse mAb

SHE78-7 at ×100 dilution at 4°C overnight and then with the second Ab conjugated with Cy3 (red) or Alexa Fluor 488 (green) at room temperature for 2 hours. After each treatment, the cultures were extensively washed with PBS. For double immunofluorescent staining, the anti-FN mouse mAb was prelabeled with FITC and applied to the E-cadherin- or integrin-α5β1-stained samples. Fluorescent images were obtained under the Carl Zeiss 710 laser scanning microscope with ZEN software.

2.7 | Small interfering RNAs and transfection

Gene suppression (knockdown) of integrin α5 in DLD-1 cells and FN in CAFs was carried out using pools of four siRNAs and a negative control RNA (Dharmacon SMARTpool; GE Healthcare) as described previously.[22] Two or three days after the transfection, these cells were used to determine the knockdown effects. The downregulation of the targets was confirmed by immunoblotting.

2.8 | Sodium dodecyl sulfate-PAGE and immunoblotting analysis (western blotting)

Confluent cultures in 6-cm culture dishes were washed with PBS and dissolved in 0.5 mL lysis buffer containing 1% (v/v) Triton X-100. The SDS-PAGE was carried out on Bio-Rad Mini-Protean gels (4%-15%). Ten microliters of the SDS-treated cell lysates was applied on each lane. Immunoblotting was undertaken by the standard procedure, and the separated antigens were visualized by the ECL method.

2.9 | Statistical analysis

Statistical significance was evaluated with an unpaired, two-tailed Student's *t* test. A *P* value of less than .05 was considered significant. Unless otherwise noted, all statistical data shown are the means ± SD with indicated n values.

3 | RESULTS

3.1 | Single cell invasion and signal inhibitors

To compare with the collective invasion, single cell invasion was carried out using GFP-labeled A549 lung cancer cells. When the A549 cells were incubated alone on the low cell attachment microfabricated EZSPHERE plate overnight, they formed cell aggregates or loose spheroids (pure spheroids) (Figure 1A), but they produced solid spheroids when mixed with CAFs (Figure 1B). When the A549/CAF hybrid spheroids were placed into collagen gel, the cancer cells individually migrated on extremely elongated protrusions of CAFs. The fastest cancer cells migrated on the CAF protrusions at speed over 200 μm/d (approximately 250 μm/d in Figure 1C). When the loose
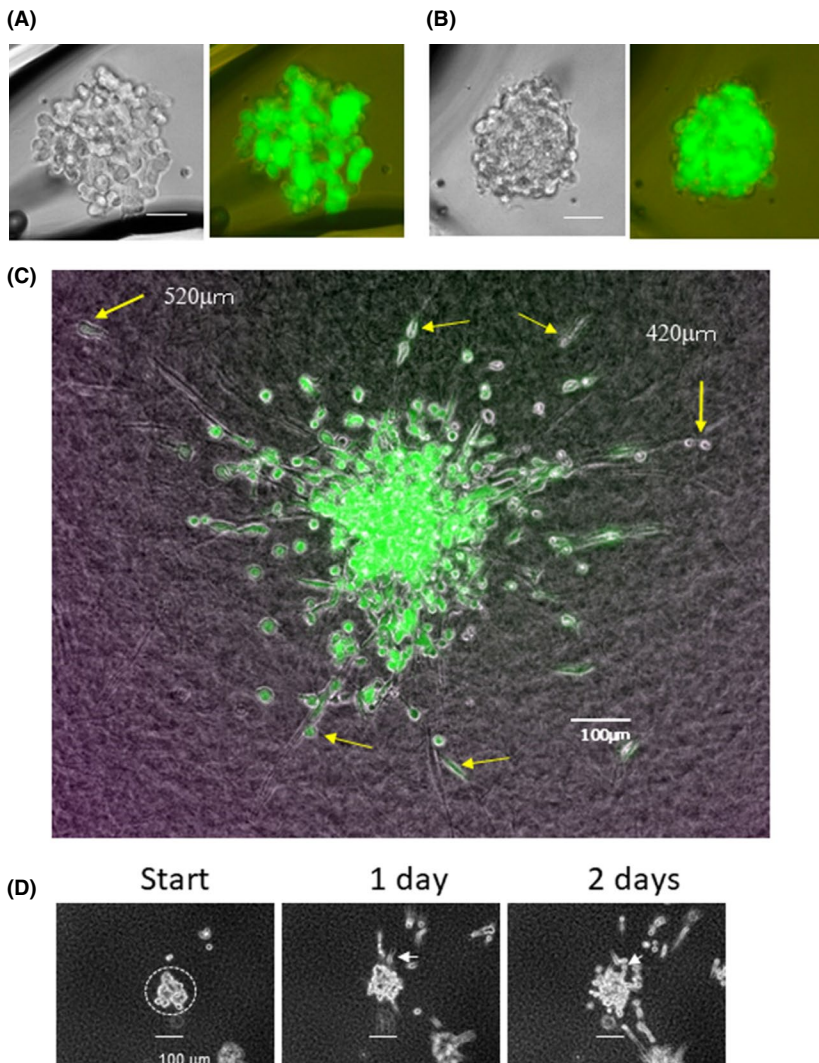
(A) (B)

(C)

(D)

FIGURE 1 Spheroid formation and single cell invasion in 3-D collagen gel of A549 cells. A, B, Phase-contrast images (left) and fluorescent images (right) of A549 spheroid (A) and A549/cancer-associated fibroblast (CAF) spheroid (B). Scale lines, 50 μm. C, A549/CAF spheroid incubated in collagen gel for 44 h. Yellow arrows indicate leading A549 cells (green) in different directions. Lengths indicate approximate distances (in μm) from the spheroid edge. Scale lines, 100 μm. D, Time course of A549 cell invasion from a pure cluster. Arrow indicates cell migrating from the cell cluster. Scale lines, 100 μm

aggregates of A549 cells were placed alone into collagen gel, they very slowly invaded the matrix (below 50 μm/d) (Figure 1D).

Using this tumor invasion model, we examined the effects of some signal inhibitors on invasion of Panc-1 pancreatic cancer cells (Figures 2 and S1). The PI3K inhibitor LY294002 inhibited the cell invasion, whereas the TGF-β signaling inhibitor SB431542 and the Rock inhibitor Y27632 promoted it. The MEK inhibitor U0126 appeared to have a weak inhibitory activity, but the activity of the metalloproteinase inhibitor TAPI-1 was unclear. The proinvasive activity of SB431542 was also found for A549 cells in our previous study with a different coculture model.[27] These data indicated that this tumor invasion model can be used for surveying various inhibitors and activators.

3.2 | Collective invasion of colon cancer cells

We next attempted to establish a collective cancer invasion model using similar hybrid spheroids. This study used two adenocarcinoma cell lines, DLD-1 (colon cancer) and MCF-7 (mammary cancer). DLD-1 cells were stably labeled with GFP, whereas MCF-7 cells were

stained with FITC dye before use. Western blotting analysis showed that both cell lines expressed high levels of E-cadherin (Figure 3A, left panels). Integrin-α5 was expressed at a lower level in MCF-7 cells than DLD-1 cells (Figure 3A, right panels). When incubated alone on the low cell attachment microplates, both cell lines formed solid spheroids as compared with A549 cells, suggesting the E-cadherin-mediated tight cell junction (Figure 3B,C, upper panels, also see Figure 1A). The two cell lines also formed solid spheroids with CAFs (Figure 3B,C, lower panels). When CAFs were labeled with a red fluorescent dye, the resultant hybrid spheroids showed that CAFs were mostly localized in the spheroid core in DLD-1/CAF and MCF7/CAF spheroids, whereas they appeared even in the spheroid surface in A549/CAF spheroids (Figure S2). Like DLD-1 and MCF-7, squamous cell carcinoma lines expressing E-cadherin, such as A431, VMRC, and CaSki, formed hard pure spheroids due to their tight intercellular junction, whereas three poorly differentiated adenocarcinoma cell lines did not form solid spheroids (Figure S3).

The pure spheroids of DLD-1 or hybrid spheroids of DLD-1 with CAFs were embedded into collagen gel (Figures 4 and S4). In the case of the hybrid spheroids, DLD-1 cells collectively invaded the matrix but very poorly and slowly (Figure 4A). Cancer-associated
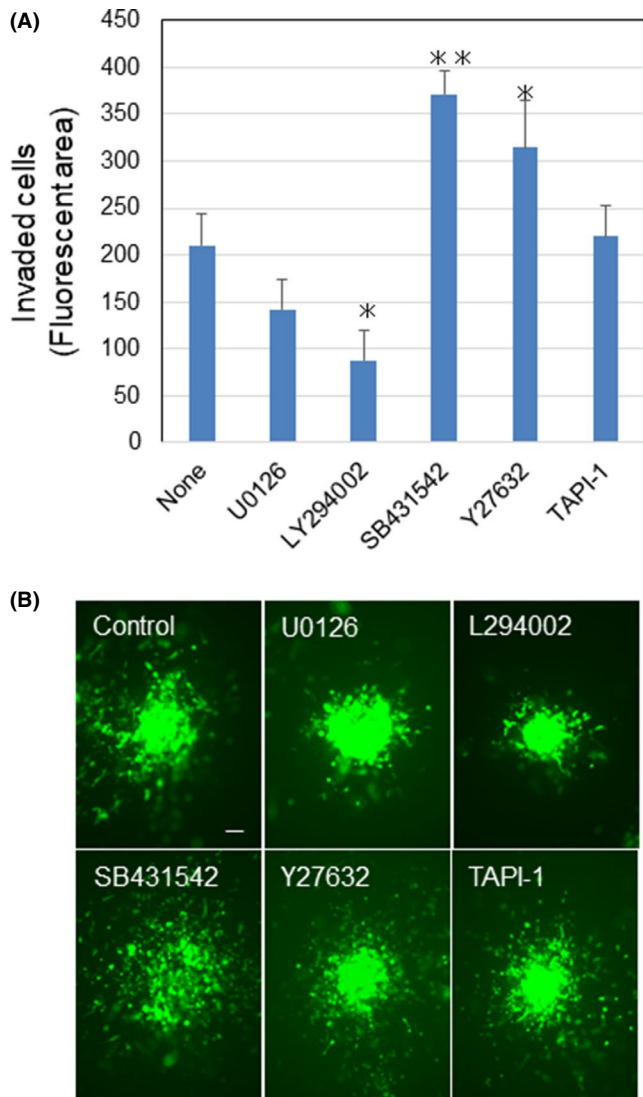
(A)

(B)

FIGURE 2 Effects of signaling inhibitors on collagen gel invasion of Panc-1 cells. Hybrid spheroids of Panc-1 and WI-38 cells were incubated for 2 d with 2 μmol/L U0126, 5 μmol/L LY294002, 10 μmol/L mmol/L SB431542, 10 μmol/L Y27632, or 2 μmol/L TAPI-1 in the culture medium. A, Quantitative data of Panc-1 cell invasion. Each column indicates the mean of fluorescent intensities ± SD in three spheroids. *$P < .05$, **$P < .01$. B, Representative images from each group. Scale line, 100 μm

(A)

(B)

FIGURE 3 Expression of E-cadherin (E-Cad) and integrin-α5 (Itg-α5) in DLD-1 and MCF-7 cells and their spheroid formation. A, Immunoblots for E-Cad (left panels) and Itg-α5 (right panels). GAPDH, internal loading control. Protein loaded per lane: 10 μg for E-Cad and 30 μg for Itg-α5. B, C, Phase-contrast images (left) and fluorescent images (right) of pure spheroids (upper panels) and hybrid spheroids (lower panels) of DLD-1 (green) (B) and MCF-7 (green) cells (C). Scale lines, 50 μm

fibroblasts also poorly migrated from the spheroids. The poor migration of CAFs was thought to result from their localization in the spheroid core (Figures 3B and S2B). This was contrasted with the case of A549/CAF spheroids (Figures 1B and S2A). We tested the effects of some cytokines and signal inhibitors. The TGF-β inhibitor SB431542 clearly promoted the invasion of both DLD-1 cells and CAFs (Figures 4B and S4B,F), whereas Y27632, which promoted single cell invasion (Figure 2A,B), did not show the proinvasive effect on DLD-1 cells (Figure S4C,G). Like SB431542, EGF and TNF-α individually stimulated the invasive activity of both cancer cells and CAFs (Figure 4C,D). SB431542 and TNF-α appeared to synergistically promote the cancer cell invasion (Figure 4E). As EGF and TNF-α
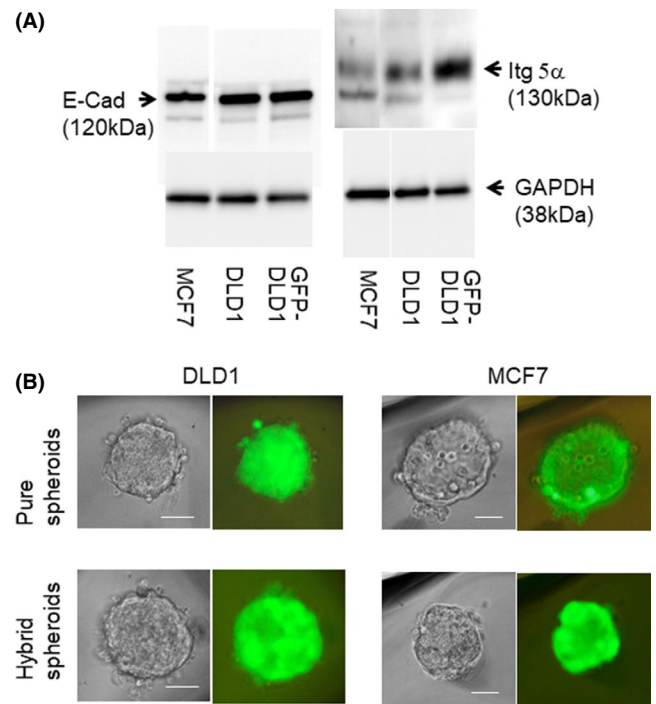
are known to induce EMT or stimulate cell motility,[8,28,29] they were supposed to loosen the cell-cell interaction of DLD-1 cells, allowing CAFs to migrate from the spheroid core. Indeed, some DLD-1 cells migrated as single cells at invasion fronts in the presence of EGF or TNF-α (Figure 4C,E, yellow arrows). In any case, the collective invasion of DLD-1 cells was associated with elongated CAF protrusions (CAF fibers) (Figure 4A,B,D,E, white arrows). Transforming growth factor-β is a well-known EMT inducer. Although TGF-β appeared to loosen the intercellular junction of DLD-1 cells, it strongly inhibited the collagen invasion of both CAFs and DLD-1 cells (Figure 4G). Judging from the spheroid outgrowth, SB431542, EGF, nor TNF-α seemed to have significant growth effect on DLD-1 cells, at least during the 7 days of incubation.

We also examined the effect of the anti-E-cadherin Ab on DLD-1 cell invasion. This Ab, which blocked the cell-cell interaction in 2-D cultures, rather promoted the CAF-dependent matrix invasion of DLD-1 cells even in the absence of SB431542 (Figures 4F and S4D). The single cell migration on fibroblast protrusions was enhanced by the Ab more evidently than EGF or TNF-α (Figure 4F, yellow arrows). This indicated that the E-cadherin/N-cadherin interaction is not important for the DLD-1 invasion in our experimental model.[21] However, DLD-1 cells could not migrate from their pure spheroids (Figure S4H).
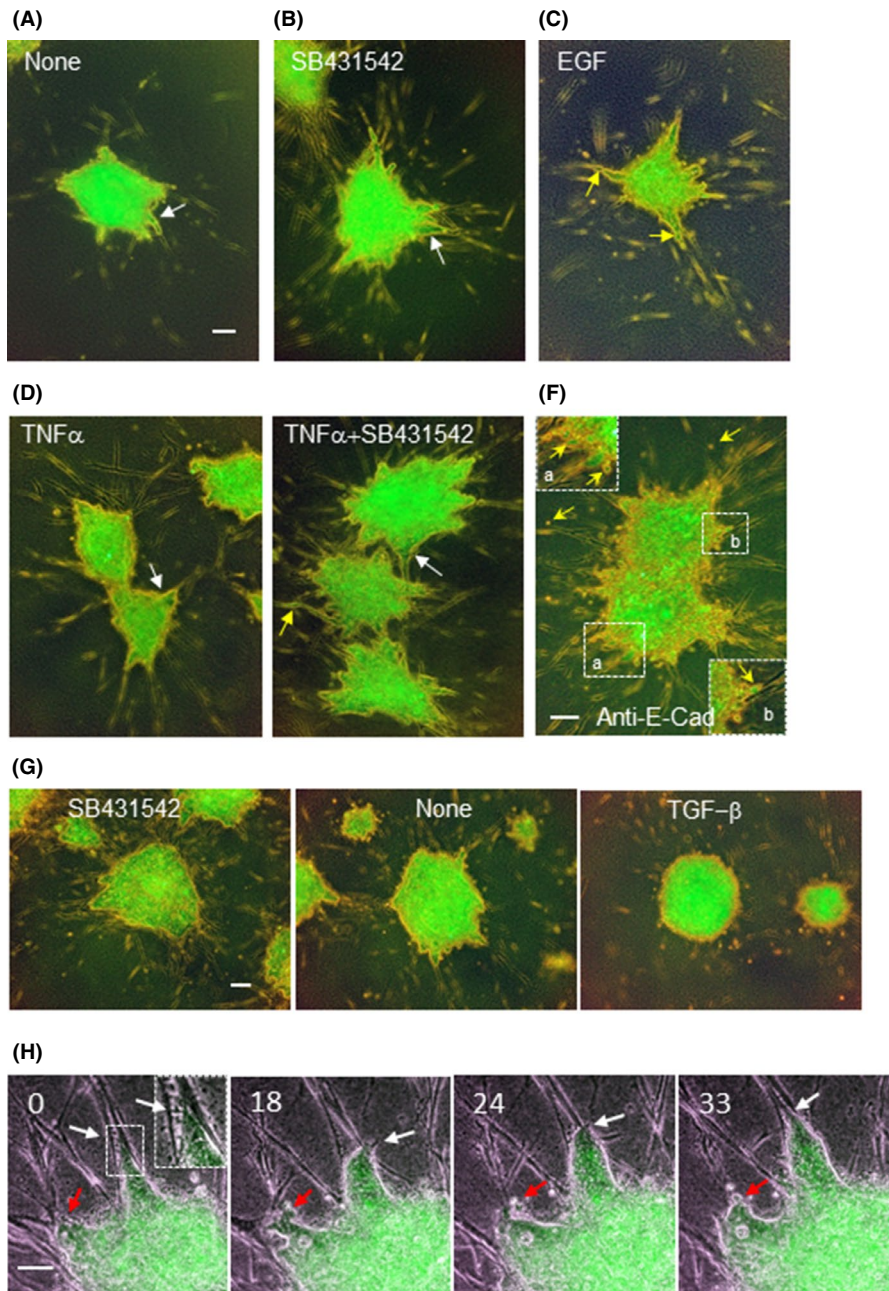
FIGURE 4 Collective invasion of DLD-1 cells (green) from hybrid spheroids with cancer-associated fibroblasts (CAFs) and its influential factors. A-E, Hybrid spheroids were incubated for 7 d in collagen gel without (A) or with 10 mmol/L SB431542 (B), 10 ng/mL epidermal growth factor (EGF) (C), 10 ng/mL tumor necrosis factor-α (TNF-α) (D), or TNF-α + SB431542 (E). (F) The spheroids were incubated with 1.3 μg/mL anti-E-cadherin mouse mAb for 9 d. White arrows, collective invasion; yellow arrows, single cell invasion. G, Spheroids were incubated in the absence (None) or presence of SB431542 or 10 ng/mL TGF-β. Scale lines, 100 μm. H, Time-lapse experiment after 5 d of preincubation. Numerical values indicate incubation lengths (h). White arrows, a DLD-1 cell cluster migrating while searching for stable CAF fibers; red arrows, a cluster being detached from CAFs. Scale line, 50 μm. Note adhesive pseudopodia in the magnified inset (left panel)

When SB431542 and Y27632 were applied to the pure spheroids of CAFs, the former supported the migration of CAFs with larger protrusions than control cells, whereas the latter promoted rapid migration of CAFs with fine and short protrusions (Figure S5). Unlike the case of the single cell invasion, the size and strength of Y27632-stimulated CAF protrusions seemed insufficient for supporting the clusters of DLD-1 cells (Figures 2, S4, and S5).

## 3.3 | Interaction between cancer cells and CAFs during collective cell invasion

As shown in Figure 4, the collective invasion of DLD-1 cells was found along with elongated CAF protrusions. The migration of multicellular clusters was rarely found on single fibroblast protrusions. In addition, we often observed the migration of single cells or small cell clusters at invasion fronts (Figure 5A, yellow arrows). This caused the separation of the leader cells from the follower cell clusters.

The requirement of stable or mechanically strong CAF fibers for collective invasion was also illustrated by a time-lapse experiment (Figure 4H, Video S1). Cancer cells collectively and slowly migrated, searching for stable CAF fibers. Detachment from CAFs terminated their collective invasion. In this experiment, the migration speed was calculated to be approximately 44 μm/d. This is far slower than the speed of A549 cell migration (250 μm/d), as shown in Figure 1C.

Immunostaining for FN more clearly showed that migrating DLD-1 cells adhered to assembled structures of multiple CAF fibers, named bundle structures, which were densely stained for FN (red) (Figure 5A,B).

FIGURE 5 Double immunofluorescent (IF) staining for fibronectin (FN) and E-cadherin (E-Cad) of collagen gel culture of DLD-1/cancer-associated fibroblast (CAF) spheroids. A, Phase-contrast fluorescent image after 14-d incubation in the presence of SB431542. Yellow arrows, leader DLD-1 cells (green) partially or completely separated from the follower cells. B, Confocal IF image of the same culture for FN (red). Note bundle structures of CAF fibers and intense staining for FN. C, Double IF staining for E-cadherin (red) and FN (green). The boxed field is magnified in the right panel. Scale lines, 100 μm
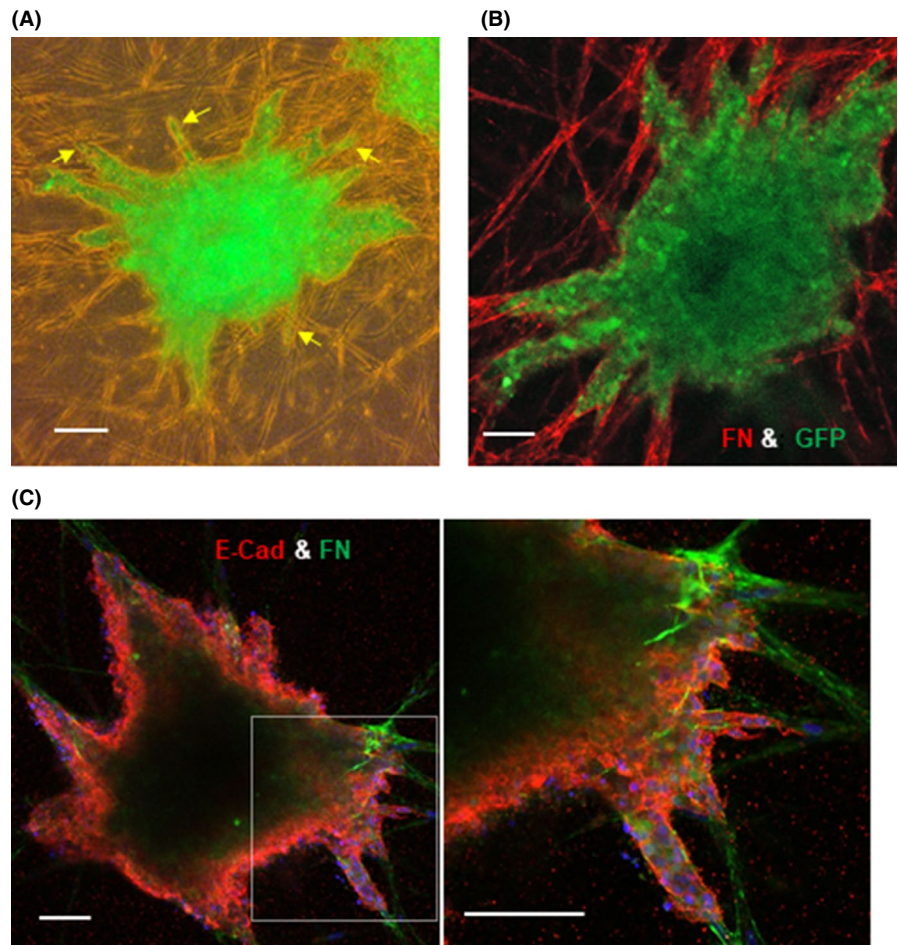
In addition, immunostaining of E-cadherin showed that the majority of cancer cells collectively migrated on the CAF fibers, keeping E-cadherin-mediated intercellular junctions (Figure 5C). These data suggested that DLD-1 cells adhered to CAF fibers through interaction with FN assembled on the surface of CAFs, as reported in the single cell invasion.[22]

Association of invading cancer cells with FN-rich CAF fibers was further examined in 2-D cocultures by double immunofluorescent staining. When DLD-1 cells were placed on a confluent CAF monolayer, they formed E-cadherin-linked cell clusters along with marked FN fibrils (Figure 6A). Peripheral cancer cells in the cell clusters and singly migrating cancer cells showed strong signals for integrin-α5β1, which were often colocalized with FN fibrils (Figure 6B,C). The possible integrin-α5β1/FN interaction was also suggested in the immunohistochemistry of human colon adenocarcinoma tissue. Consistent with the 2-D cultures, invasive E-cadherin-linked cancer cell clusters were found in stroma with abundant FN fibrils (Figure 6D). Cancer cells at invasion fronts, which contacted with FN fibrils, showed stronger signals for integrin-α5β1 than those of inner cancer cells (Figure 6E,F). Similar collective invasion was found in all of the 10 colorectal cancers examined. These data supported the possible mechanism that colon cancer cells bind to CAFs through integrin/FN interaction.

To verify the molecular mechanism, the DLD-1/CAF interaction was investigated by using function-blocking Abs in 2-D cultures.

DLD-1 cells rapidly adhered to the confluent CAF layer and spread in spindle shapes (Figure S6A). This adhesion was strongly inhibited by Abs against integrin-α5, -α5β1, -β1, and FN and by RGD peptide (Figures 7A and S7A). Anti-integrin-αv and anti-E-cadherin Abs significantly but very weakly inhibited the cell adhesion. These results indicated that DLD-1 cells bound to CAFs mainly through the interaction between integrin-α5β1 on DLD-1 cells and FN on CAFs. The integrin-α5β1/FN interaction was also verified in the 3-D invasion assay with DLD-1 cells (Figures 7B and S7B). The Abs against integrin-α5β1 and FN, as well as the RGD peptide, significantly blocked the DLD-1 invasion in the collagen matrix. Similar to the effects of the function-blocking Abs, knockdown of the integrin-α5 gene in DLD-1 cells or the FN gene in CAFs clearly inhibited the adhesion of DLD-1 cells to CAFs (Figures 7C and S8). In addition, the two kinds of gene knockdown appeared to inhibit the CAF-dependent collagen gel invasion of DLD-1 cells (Figure 7D).

3.4 | Collective invasion of MCF-7 mammary carcinoma cells

Cancer-associated fibroblast-dependent collective invasion was also examined with MCF-7 cells. When the hybrid spheroids of MCF-7 with CAFs were embedded into collagen gel, CAFs migrated from
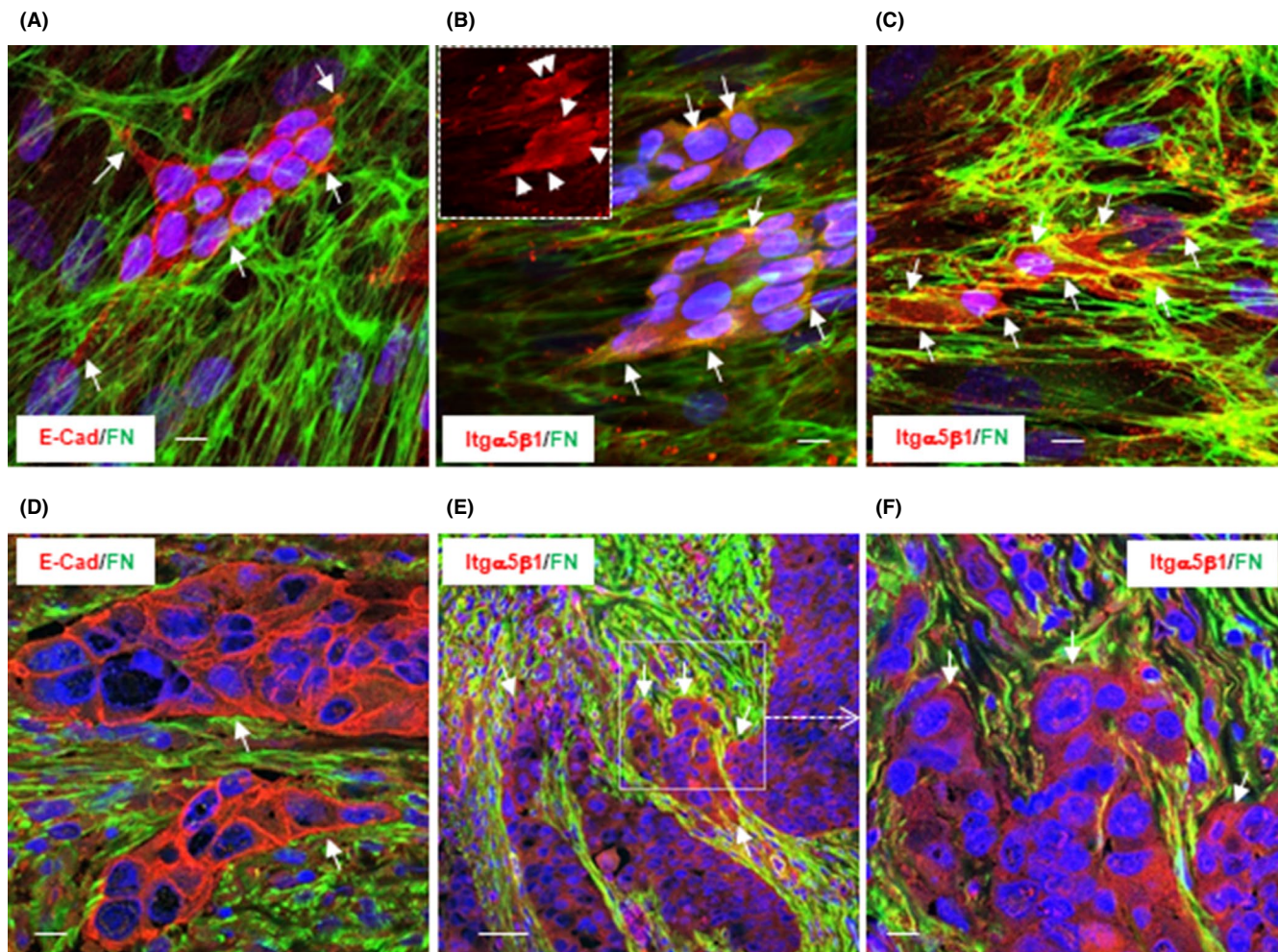
FIGURE 6 Collective invasion of colon cancer cells in contact with fibronectin (FN) fibrils in 2-D cultures (A-C) and human cancer (D-F): double immunofluorescent (IF) staining for FN (green) and E-cadherin (E-Cad, red) or integrin (Itg)-α5β1 (red). A-C, Non-labeled DLD-1 cells were seeded onto confluent cancer-associated fibroblast monolayer in an 8-well chamber slide and incubated for 3 d, followed by IF staining as indicated. In (B), the inset is a reduced image of Itgα5β1 staining. C, Two singly migrating DLD-1 cells. Arrows indicate contact sites with FN fibrils, and yellow color suggests colocalization of Itg-α5β1 and FN (B, C). D-F, Two close sections of a colon cancer specimen were subjected to double IF staining as indicated. The boxed field in (E) is magnified in (F). Arrows indicate contact sites with FN fibrils. Note that cancer cells at invasion fronts show stronger signals for Itg-α5β1 (E, F). Scale lines, 10 μm (A-D, F) and 20 μm (E)

the spheroids, supporting the collective migration of MCF-7 cells (Figures 8A and S9A). Therefore, the proinvasive effect of SB431542, if any, was not evident. Unlike DLD-1 cells, some MCF-7 cells migrated individually (Figures 8A,C and S9A, yellow arrows). This might be related to the lower expression of integrin-α5β1 and the lower adhesive activity to CAFs in this cell line as compared with DLD-1 cells (Figures 3A, right panel, and S6B). Epidermal growth factor further promoted the single cell invasion along with CAF fibers, probably inducing EMT or stimulating cell motility. The collective invasion of MCF-7 cells was always supported by bundles of multiple CAF fibers. This is shown more clearly by a time-lapse experiment (Video S2) and immunostaining for E-cadherin and FN (Figure 8E,F). The immunostaining showed that MCF-7 cells with E-cadherin junction migrated on bundles of CAF fibers, forming cell clusters. It is noted that larger bundles of CAF fibers supported the migration of larger clusters of cancer cells. Although EGF promoted single cell invasion,

the cancer cells bound to large CAF fibers maintained or reproduced tight cell-cell junctions (Figure S9E, white arrow).

4 | DISCUSSION

Collective invasion is the major mode of cancer metastasis.[23–25,30] This type of invasion is usually observed in E-cadherin-positive epithelial cancers, with some exceptions.[23,25] In this study we tested if this invasion mode can be explained by our previously proposed mechanism for single cell invasion.[22] Our results indicated again that cancer cells invaded the collagen matrix by directly binding to FN fibrils assembled on CAFs. As reported previously,[22] CAFs extended their protrusions to form fiber structures in collagen gel, and FN fibrils were densely accumulated on their surface. The FN fibrils assembled on CAF fibers seemed to be the most preferable substrate

FIGURE 7 Inhibitory effects of function-blocking Abs (A, B) and gene suppression (C, D) on 2-D adhesion to cancer-associated fibroblasts (CAFs) (A, C) and on 3-D collagen gel invasion (B, D) of DLD-1 cells. A, Ordinate, the mean of the percentage of spread cells per field $\pm$ SD in triplicate wells. E-Cad, E-cadherin; FN, fibronectin; IgG, control IgG; Itg, integrin. $**P < .01$, $***P < .001$. B, Ordinate, DLD-1 invasion for 2 d. n = 12 (PBS), 15 (IgG), 13 (RGD), 14 (FN), and 12 (Itg-α5β1). $*P < .05$, $**P < .01$. C, D, Upper panels, DLD-1 cells were treated with control (Ctr) or siRNA for integrin-α5. Lower panels, CAFs treated with Ctr or siRNA for FN. They were subjected to the cell attachment assay at day 3 (C) and to 3-D invasion assay at days 3-6 (D). C, Upper panel, n = 4. Lower panel, n = 3. $***P < .001$, $**P < .01$. D, Representative images from each group (eight spheroids)
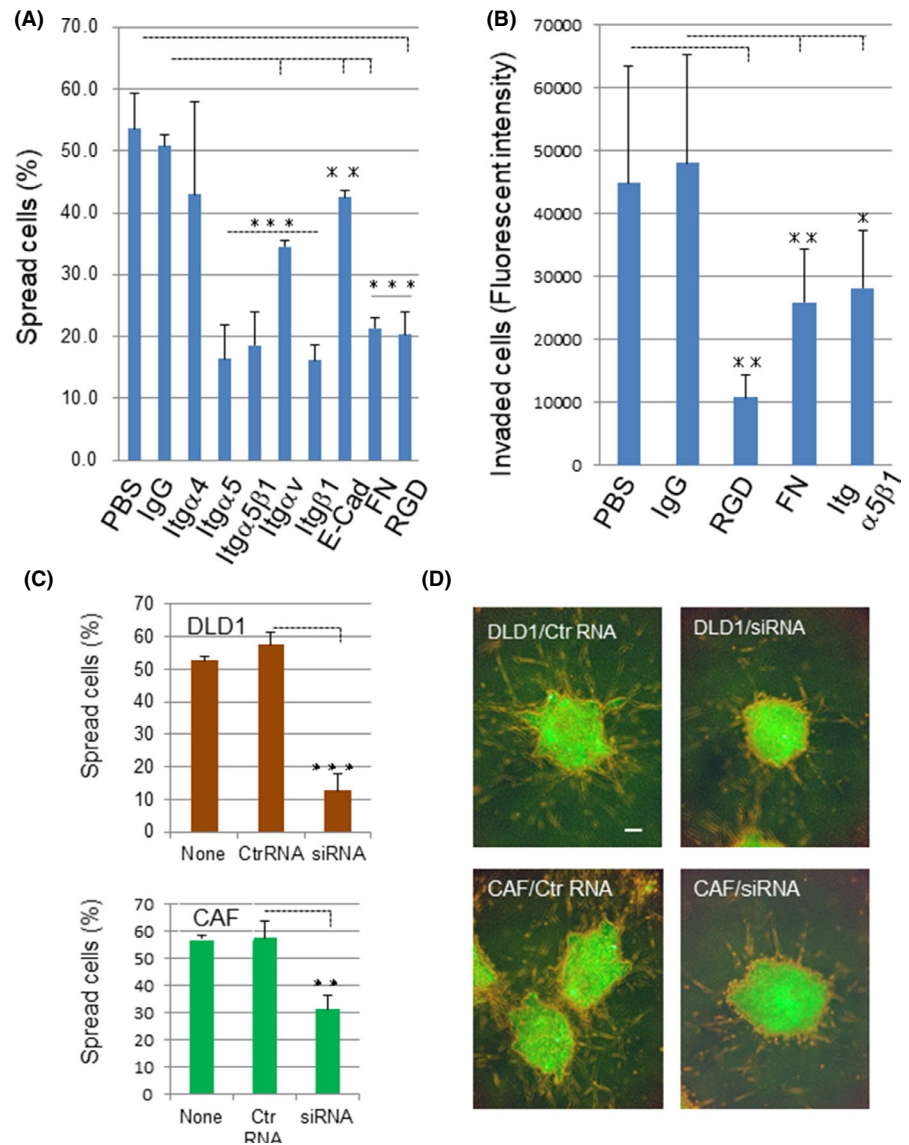
for cancer cells in the 3-D collagen matrix. This mechanism seems common to both the single-cell and the collective invasion modes. Integrin-α5β1 is known to be a major receptor for FN.[10,11] Our previous and present studies suggest that integrin-α5β1 plays a major role in the binding of cancer cells to the FN fibrils on CAFs, but other types of FN receptors, including integrin-αvβ3, might be important, depending on cell types. Important differences between the two invasion modes were the E-cadherin-based cell-cell connection and the density of CAF fibers supporting the migration of the cancer cell clusters. Both DLD-1 and MCF-7 cells formed E-cadherin-linked cell clusters, and their collective migration in collagen gel required assembly of multiple CAF fibers (bundle structure) as the stable substrate, although A549 cells individually migrated on single CAF fibers. This seems reasonable when the mechanical capacity of CAFs to hold large multicellular clusters is considered. Conversely, these cancer cells appeared to integrate multiple CAF fibers to form their bundle structures, probably by the strong cell-cell connection.

The collective invasion is thought to be controlled by the coordinated interplay between cell-cell and cell-ECM interactions.[24] The

leader cells at the invasion front acquire a mechanical traction force to pull the follower cell cluster by adhering to suitable substrates. Recent studies with 3-D collagen cultures have shown that CAFs play important roles in leading collective cancer cell migration.[19,21] One group reported that CAFs lead collective migration of A431 cells by directly binding to the cancer cells through the E-cadherin/N-cadherin junction.[21] Consistent with their and our previous findings, the present study showed the importance of the direct cancer cell-CAF interaction in collective cancer invasion. In our models, however, DLD-1 cells adhered to CAFs, at least mainly, through integrin-α5β1/FN interaction. The anti-E-cadherin Ab stimulated the CAF-dependent DLD-1 cell invasion, especially their single cell invasion. This is inconsistent with the E-cadherin/N-cadherin junction model,[21] but agreed with a recent study that reported that genetic deletion of E-cadherin enhances the invasive activity of ductal carcinoma cells in vitro but reduces tumor growth and metastasis in mice.[31] However, our results do not exclude the possibility that the E-cadherin/N-cadherin interaction might be involved in the DLD-1/CAF interaction under some specific steps.
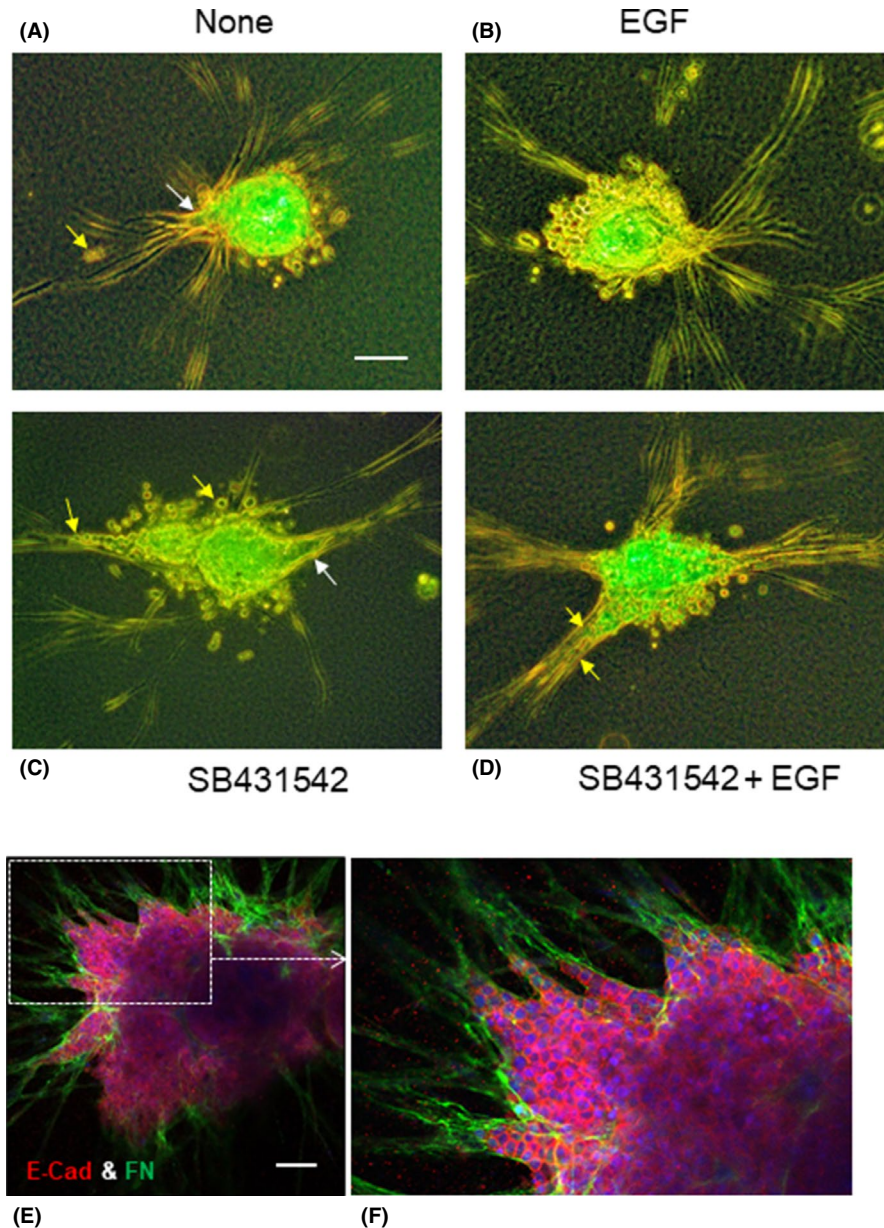
FIGURE 8 Collective invasion of MCF-7 cells (green) in collagen gel (A-D) and double immunofluorescent (IF) staining for fibronectin (FN, green) and E-cadherin (E-Cad, red) (E-F). A-D, MCF-7/cancer-associated fibroblast spheroids were incubated for 3 d without (A) or with 10 ng/mL epidermal growth factor (EGF) (B), 10 mmol/L SB431542 (C), or SB431542 + EGF (D). Scale line, 100 μm. White arrows, collective invasion; yellow arrows, single cell invasion. E, F, Confocal IF images for E-cadherin (red) and FN (green). The boxed field in (E) is magnified in (F). Scale line, 100 μm

The present study showed that the speed of collective invasion was far lower than the single cell invasion in the spheroidal invasion experiments. The E-cadherin junction is obviously an impediment for cell migration. How do such cancer cells efficiently metastasize in vivo? Many mechanisms have been proposed for this question.[24,32,33] In these mechanisms, cancer cells transiently, reversibly, or partially undergo EMT during the process of distant metastasis.[32–35] Such epithelial plasticity was also found in the present study. We found that EGF, TNF-α, and the TGF-β inhibitor SB431542 promoted the collective invasion of DLD-1 cells. Both EGF and TNF-α, which often induce EMT,[8,28,29] enhanced cancer cell migration, possibly by dissociating or loosening their E-cadherin junctions and by enhancing cell motility. It is likely that these and other cytokines in the microenvironment would transiently enhance cell migration by breaking the cell-cell junction during their collective invasion in vivo. After enhanced migration, they would reconstruct tight cell-cell junctions

as the cell number increases on the stable CAF substrate. Compared with the two cytokines, the effect of SB431542 was somewhat strange because TGF-β is an EMT inducer. However, little is known about the activity of TGF-β in 3-D conditions. Although TGF-β appeared to loosen the intercellular junction of DLD-1 cells, it strongly inhibited the collagen invasion of both CAFs and DLD-1 cells. We previously found that SB431542 stimulates HGF expression in fibroblasts and tumor cell invasion in collagen gel.[27] Hepatocyte growth factor promotes migration of normal and cancer cells in 2-D and 3-D conditions, and its expression is negatively regulated by TGF-β.[36,37] Therefore, SB431542 seemed to enhance the cancer cell invasion by stimulating the outgrowth of CAFs in our 3-D invasion model. Transforming growth factor-β is known to have both tumor-suppressive and proinvasive effects depending on tumor stages and experimental conditions.[2,3] Obviously, extensive and careful studies are needed to elucidate the clinical significance of TGF-β inhibitors.

It is also noted that in our 3-D invasion models and 2-D cocultures, cancer cells bound to CAF fibers showed elongated shapes and sometimes migrated alone (Figures 5A, 8Ac, S6A,B, and S9A,C). The integrin-α5β1-mediated adhesion to fibrillary FN facilitates cancer cell invasion by activating FAK, Erk, Akt, and other signal mediators, hence remodeling the cytoskeleton.[22,38–40] Thus, the leader cancer cells could gain a traction force to pull the follower cancer cells by FN/integrin signaling. It seems possible that small clusters of such leader cells are separated from the follower cell clusters and seeded into circulation. Such tumor cell clusters in circulation are thought to cause distant metastasis much more efficiently than singly circulating cells.[41,42] Our results are consistent with previous reports showing the importance of integrin expression in tumor prognosis.[43,44]

In conclusion, we could recapitulate the collective invasion of colon and breast cancer cell lines in a simple 3-D collagen coculture model with CAFs. The cancer cells collectively migrated through the collagen matrix while binding to bundles of CAF fibers through the integrin-α5β1 and FN interaction. These results reinforce the ideas that fibrosis, altered ECM, and local cytokines contribute to tumor progression.[32,45] Many types of 3-D experimental models for cancer studies have been reported so far.[46] Our experimental model uses two major components in the tumor microenvironment, fibroblasts and collagen, as well as their products. Our invasion assay is simple and rapid and suitable for investigating various interactions between cancer cells and CAFs. This method can be used for investigating the complex mechanisms of cancer invasion as well as for drug screening.

CONFLICT OF INTEREST

The authors do not have any financial support or relationship that may pose conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.