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Scaffold-Free Coculture Spheroids of Human Colonic Adenocarcinoma Cells and Normal Colonic Fibroblasts Promote Tumorigenicity in Nude Mice[1,2,3]

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Abstract

The aim of this study was to form a scaffold-free coculture spheroid model of colonic adenocarcinoma cells (CACs) and normal colonic fibroblasts (NCFs) and to use the spheroids to investigate the role of NCFs in the tumorigenicity of CACs in nude mice. We analysed three-dimensional (3D) scaffold-free coculture spheroids of CACs and NCFs. CAC Matrigel invasion assays and tumorigenicity assays in nude mice were performed to examine the effect of NCFs on CAC invasive behaviour and tumorigenicity in 3D spheroids. We investigated the expression pattern of fibroblast activation protein-α (FAP-α) by immunohistochemical staining. CAC monocultures did not form densely-packed 3D spheroids, whereas cocultured CACs and NCFs formed 3D spheroids. The 3D coculture spheroids seeded on a Matrigel extracellular matrix showed higher CAC invasiveness compared to CACs alone or CACs and NCFs in suspension. 3D spheroids injected into nude mice generated more and faster-growing tumors compared to CACs alone or mixed suspensions consisting of CACs and NCFs. FAP-α was expressed in NCFs-CACs cocultures and xenograft tumors, whereas monocultures of NCFs or CACs were negative for FAP-α expression. Our findings provide evidence that the interaction between CACs and NCFs is essential for the tumorigenicity of cancer cells as well as for tumor propagation.

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Introduction

The local tumor microenvironment plays an essential role in tumor growth and propagation. The surrounding stromal environment of a tumor not only provides nutrition, waste removal, and structure but also affects tumor progression via the development of specific surroundings that enable tumor transformation [1,2]. Tumor

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progression requires changes in both tumor cells and the surrounding stroma, including the release of cytokines and proteolytic enzymes, the production of a specific extracellular matrix, and angiogenesis [3]. Fibroblasts have been reported to be the predominant cell type within the stromal components of carcinomas. In many carcinomas, such as those of the breast, stomach and pancreas, the stromal compartment surrounding the cancer cells has been reported to account for 80% of the carcinoma mass, which is in contrast to other cancers such as sarcoma, lymphoma, and mammary medullary carcinoma [2]. Normal fibroblasts appear to be recruited into tumors by tumor cells, and then resting fibroblasts in the tumor microenvironment become activated fibroblasts in response to tumor cell signalling [4,5]. Activated fibroblasts in tumors are often called cancer-associated fibroblasts (CAFs) or peritumor fibroblasts. CAFs produce growth factors and chemokines and are important for promoting extracellular matrix remodelling and angiogenesis because they deposit many proteins in the surrounding tumor stroma. Paracrine signalling between tumor cells and fibroblasts surrounding the tumor cells are important determinants of tumor progression [1,6,4]. CAFs have been reported to be abundant in colorectal carcinoma and have been observed to express fibroblast activation protein-α (FAP-α). FAP-α is expressed in CAFs and transiently expressed in healing wounds, in contrast to other ubiquitous tumor-associated products [7–9].

Three-dimensional (3D) coculture models that mimic tumor-stromal cell interactions have been established to verify cell-cell and cell-matrix interactions during tumor progression [10–12]. The 3D coculture models of tumor and stromal cells enable the use of a 3D environment to reproducibly and systematically study a tumor cell's response to therapy *in vitro*. Multicellular 3D culture promotes the formation of well-developed spheroids that resemble avascular tumor sites or micrometastatic regions *in vivo* [10,13,14]. During spheroid formation, resting fibroblasts undergo activation and acquire morphological or physiological features that resemble those of CAFs in tumor stroma [15].

We aimed to introduce a scaffold-free coculture spheroid model of colonic adenocarcinoma cells (CACs) and normal colonic fibroblasts (NCFs) by a forced aggregation technique using a rotary orbital shaker that mimics avascular tumor sites in a microgravity environment and to verify that our scaffold-free coculture spheroid model increases tumor invasion into a basement membrane matrix (BD Matrigel) and promotes tumorigenicity in nude mice. Additionally, we examined the expression of FAP-α in monocultured NCFs, NCFs cocultured with CACs, 3D coculture spheroids and xenograft tumors in nude mice by immunofluorescent or immunohistochemical staining.

Materials and Methods

Cell lines

Human colonic adenocarcinoma cell lines COLO320HSR (KCLB 10020.1) and SNU-C1 (KCLB 0000C1) and the human normal colonic fibroblast cell line CCD-18Co (KCLB 21459) (those from Korean Cell Line Bank, Seoul, South Korea) were cultured in RPMI 1640 Medium (Gibco, Grand Island, NY, cat. 11875-093) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO_2. COLO320HSR cells grow in a loosely adherent manner, and SNU-C1 cells grow in suspension without attaching to a surface.

Coculture Spheroid Formation Assay

The scaffold-free spheroids formed naturally from 1,000 cancer cells (COLO320HSR or SNU-C1) and 500 fibroblasts (CCD-18Co) per well in an ultra-low attachment 24-well plate (Corning Costar, Corning, NY, USA; cat. CLS3473-24EA) in RPMI 1640 Medium (Gibco, Grand Island, NY; cat. 11875-093) with 10 % FBS and 1% penicillin-streptomycin. In cultures subjected to rotating conditions, ultra-low attachment 24-well plates containing the cells were placed on an orbital shaker (FINEPCR twist shaker; TW3, Seoul, South Korea) rotating at 70 rpm in an environmentally controlled incubator at 37°C in 5% CO_2 for the entire 3 days [16].

Electron Microscopy

The spheroids were fixed in 2.5% glutaraldehyde and 0.1 M sodium phosphate butter at pH 7.2 and postfixed in 1% osmium tetroxide. Semithin sections (1 mm) were stained with toluidine blue. These sections were embedded in Epon and further sectioned using an ultramicrotome. After uranyl acetate and lead citrate staining, electron microscopy was performed using a transmission electron microscope (H-7650; Hitachi, Japan) equipped with a soft imaging system (Morada, Japan).

Immunofluorescence

COLO320HSR-CCD-18Co coculture cells or CCD-18Co monoculture cells cultured on Lab-Tek II Chamber Slide 8 Chambers (Nalgene; Nunc, Rochester, NY; cat. 154941) for 3 days were fixed with 100% cold methanol at room temperature for 15 minutes and then washed in wash buffer (cold phosphate-buffered saline (PBS) and 0.05% Tween 20). The cells were then incubated in 0.25% Triton X-100 for 10 minutes and washed in wash buffer. Image-It Fx Signal Enhancer (Invitrogen, Carlsbad, CA; cat. I-36933) was used to block the samples for 30 minutes at room temperature. After three rinses, the cells were incubated overnight at 4 °C with rabbit polyclonal anti-FAP-α antibody (1:50; Abcam, England; Ab53066). After three rinses, the cells were stained with Alexa Fluor® 488 goat anti-rabbit IgG (H + L) (1:500; Invitrogen, Carlsbad, CA, cat. A-11008). The primary antibody was omitted for the negative controls.

Cell Invasion Assay

The cell invasion assays were performed using Lab-Tek II Chamber Slide™ 8 Chambers (Nalgene, Rochester, NY; cat. 154941). Each chamber was filled with matrix (one volume of FBS, two volumes of culture medium, and seven volumes of Matrigel; BD Matrigel Basement Membrane Matrix, Cat. No. 354234) and overlaid with

Table 1. Effect of Exogenous Scaffold-Free Spheroids Derived from Cocultured CACs and NCFs on Tumorigenicity in Nude Mice

Cancer cell line	Cell condition	No. of cells inoculated subcutaneously	No. of mice.	Tumor-bearing mice [1]
COLO320	Suspension	2000×10^3 COLO320	5	0
		1280×10^3 COLO320 + 160×10^3 CCD-18Co	5	2
		1280×10^3 COLO320	5	0
	Spheroid	10 spheroids (1280×10^3 COLO320 + 160×10^3 CCD-18Co)	5	4
		One spheroid (128×10^3 COLO320 + 16×10^3 CCD-18Co)	5	1
SNU-C1	Suspension	1000×10^3 SNU-C1	5	0
		30×10^3 SNU-C1 + 160×10^3 CCD-18Co	5	1
		30×10^3 SNU-C1	5	0
	Spheroid	10 spheroids (30×10^3 SNU-C1 + 160×10^3 CCD-18Co)	5	3
		One spheroid (3×10^3 SNU-C1 + 16×10^3 CCD-18Co)	5	0

[1] 50 days after injection.

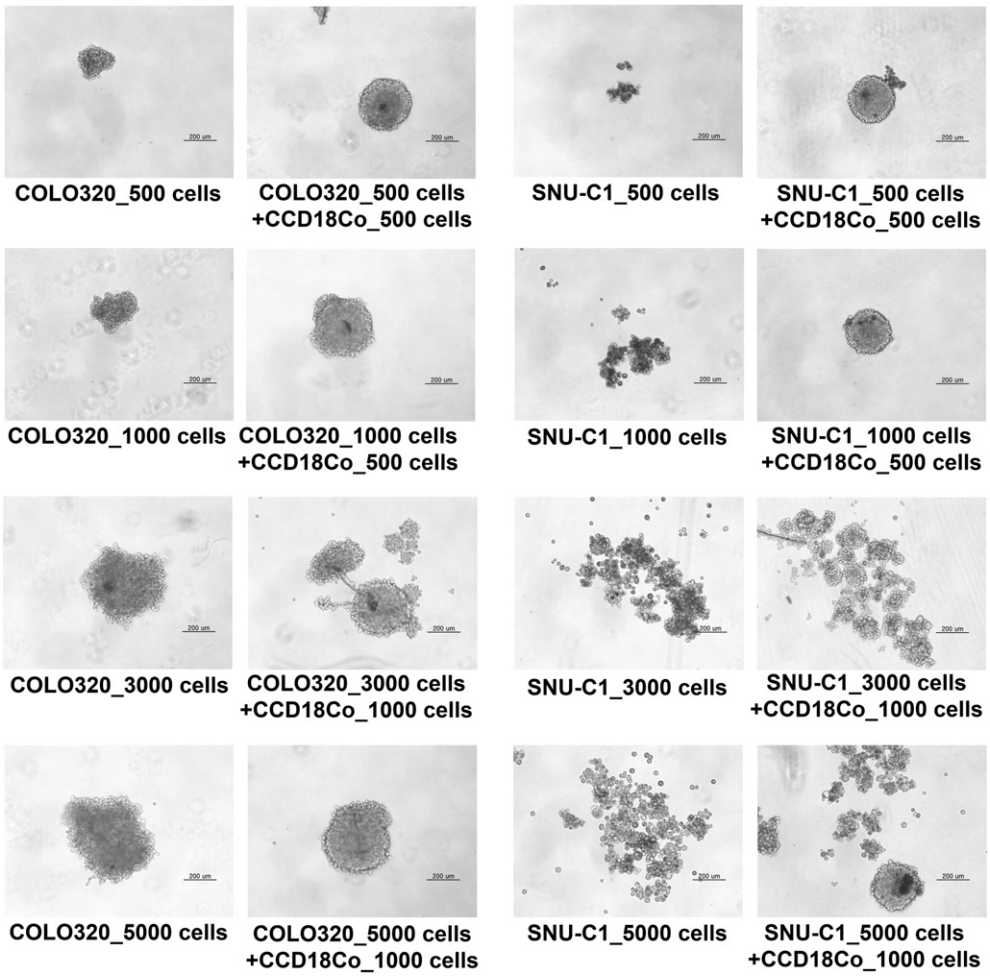

Figure 1. Representative images of CACs grown alone or mixed with CACs-NCFs in scaffold-free rotating conditions after 3 days in culture. Rounded spheroids were formed by cocultured cancer cells and fibroblasts. The CACs grown without fibroblasts formed irregular and loose aggregates. Rounded spheroids with no scattered irregular aggregates were formed by cancer cells grown with fibroblasts at ratios of 500:500 or 1000:500 in the seeding mixture (100×; scale bars = 200 μm).

RPMI 1640 Medium with COLO320HSR or SNU-C1 cancer cells alone, CCD-18Co fibroblasts and colon cancer cells (COLO320HSR or SNU-C1), or coculture 3D spheroids after 3 days of cultivation [7]. After 4 days, the cells in the invasion assays were fixed in 4% formaldehyde and 0.25% glutaraldehyde, paraffin-embedded, and sectioned for hematoxylin and eosin staining. The depth of CAC invasion into the matrix was measured under 400× magnification using the image analysis software Olympus cellSens Standard 1.7.

Tumorigenicity Assay in Nude Mice

All the animal studies were approved by the Institutional Review Board of Chungnam National University Hospital (CNUH-A0012). Male BALB/c nu/nu nude mice aged 4 to 5 weeks (Central Lab. Animal Inc, South Korea) were housed in a specific pathogen-free environment in the Animal Laboratory Unit, Chungnam National University Hospital, Korea. COLO320HSR or SNU-C1 cells with or without CCD-18Co fibroblasts were suspended in 0.1 ml of phosphate-buffered saline and injected subcutaneously into the flanks of the mice (one injection/mouse). Coculture 3D spheroids of colonic cancer cells (COLO320HSR or SNU-C1) and CCD-18Co fibroblasts for the entire 3 days were suspended in 0.1 ml of phosphate-buffered saline and injected subcutaneously into the flanks of the mice (one injection/mouse) (Table 1). CCD-18Co fibroblasts alone were used as controls. Tumor growth was monitored weekly, and tumor volume was assessed by measuring tumor size. These specimens from the mice were provided by the National Biobank of Korea, Chungnam National University Hospital, Daejeon, Korea.

Immunohistochemistry

The sections of the paraffin embedded 3D coculture spheroids in the Matrigel or xerograft tumors on microslides were deparaffinized with xylene, dehydrated using serial dilutions of alcohol, and immersed in peroxidase-blocking solution (Dako, Glostrup, Denmark) to block endogenous peroxidase activity. Heat-mediated antigen retrieval was performed with 10 M sodium citrate (pH 6.0) (Dako, Glostrup, Denmark) for 15 minutes followed by 3 minutes in a pressure cooker at full power. The sections were incubated overnight at 4°C with rabbit polyclonal anti-FAP-α antibody (1:50, Abcam, England; Ab53066), rabbit polyclonal anti-wide spectrum cytokeratin antibody (1:50; Abcam Inc, Cambridge, MA, USA; cat. Ab9377) and mouse monoclonal anti-human cytokeratin antibody (1:100; Dako, Glostrup, Denmark; code No. M0821). The sections were then incubated in DakoREAL EnVision/HRP rabbit/mouse detection reagent (Dako, Glostrup, Denmark) for 20 minutes at

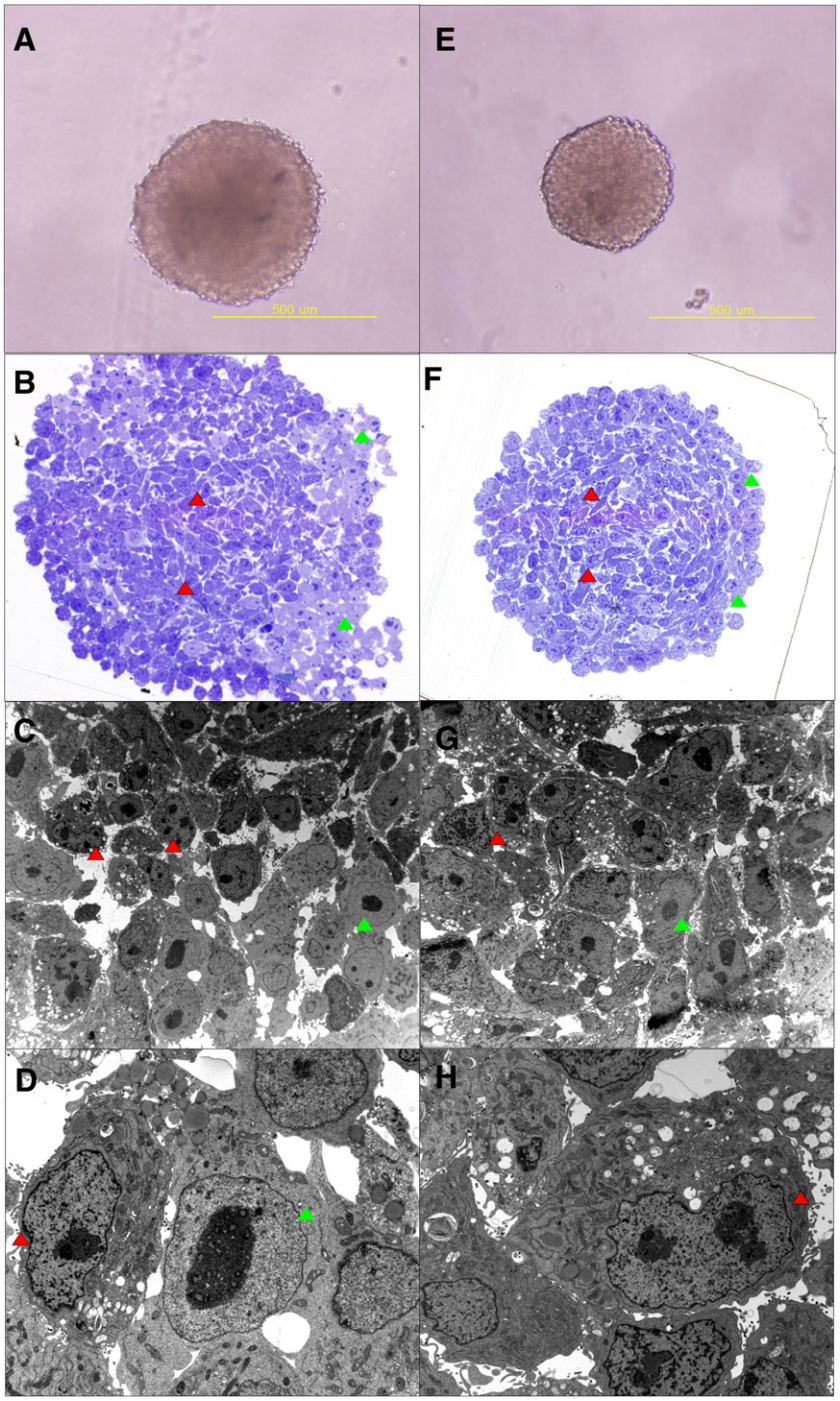

Figure 2. The scaffold-free coculture spheroids of 1,000 human CACs and 500 NCFs after 3 days on a rotary orbital shaker exhibited a ball shape. (A-D) COLO320HSR and CCD-18Co spheroid. (E-H) SNU-C1 and CCD-18Co spheroid (scale bar = 500 μm; 100×). Fibroblasts (green triangle) are predominantly positioned in the periphery of the spheroid, with less basophilic staining than cancer cells (red triangle) after toluidine blue stain (B, F; 400×). The peripheral fibroblast layer of a COLO320HSR-containing spheroid was thicker than that of a SNU-C1-containing spheroid, and COLO320 spheroids were larger than SNU-C1 spheroids. By electron microscopy, fibroblasts are more transparent than adenocarcinoma cells, and they typically have a large nucleolus, relatively dispersed chromatin and no microvilli. Adenocarcinoma cells appear darker than fibroblasts, and they have slightly irregular nuclei with nucleoli and numerous microvilli. Adenocarcinoma cells partially adhered close to fibroblasts (C and G, 2000×; D and F, 7000×).

room temperature. The chromogen was developed for 2 minutes, and the slides were counterstained with Meyer hematoxylin, dehydrated and coverslipped. The primary antibody was omitted in the negative controls.

Statistical Analysis

The xenograft tumorigenicity assay results were examined by nonparametric Kruskal-Wallis analyses. Statistical significance was set at $P < .05$ (SPSS 21; SPSS Inc., Chicago, IL).

Results

Spheroid Formation of COLO320HSR and SNU-C1 CACs is Induced by Cocultivation With CCD-18Co Fibroblasts

Monocultures of COLO320HSR or SNU-C1 CACs in scaffold-free rotating conditions were organized in irregular and loose aggregates after 3 days in culture. However, cocultivated CACs and NCFs in scaffold-free rotating conditions formed densely packed rounded spheroids. A low density of seed cells, with a ratio of CACs to NCFs of 500:500 or 1000:500 in the cocultures, resulted in the formation of rounded spheroids with few scattered cells or loose aggregates for the entire 3-day period. Unexpectedly, a high density of seed cells, with a ratio of CACs to NCFs of 3000:1000 or 5000:1000 in the cocultures, resulted in the formation of many scattered cells and loose aggregates (Figure 1). After 3 days, apoptosis was apparent in the centre of the coculture spheroids. Thus, 3 day-cultivated spheroids from a coculture seeding mixture of 1000:500 of CACs to NCFs were used for morphologic examinations, tumor invasion assays and tumor xenografts.

Scaffold-Free Coculture Spheroids of Human CACs and NCFs Exhibit In Vivo-Like Features

The three-day coculture spheroids of 1000 COLO320HSR CACs and 500 CCD-18Co NCFs were larger (355.1 ± 34.18 μm average diameter) than spheroids of 1000 SNU-C1 CACs and 500 CCD-18Co NCFs (284.3 ± 21.77 μm average diameter). The doubling times were 10.81 hours for COLO320HSR cells and 51.58 hours for SNU-C1 cells [17,18]. Transmission electron microscopy revealed CACs with characteristic intestinal cell microvilli and nuclei with irregular contour and mildly coarse chromatin and nucleoli compared to colonic fibroblasts with no microvilli, nuclei with a smooth nuclear membrane and dispersed chromatin, and a typically large nucleolus. The fibroblasts surrounded the cancer cells; they were primarily located on the periphery of the spheroids, similar to the location of peritumor fibroblasts in cancer tissue. The COLO320HSR and CCD-18Co coculture spheroids had a larger diameter and a greater population of fibroblasts and cancer cells than SNU-C1 and CCD-18Co coculture spheroids (Figure 2).

Fibroblasts Influence Tumor Cell Invasion into the Extracellular Matrix

COLO320HSR or SNU-C1 cancer cells were seeded on a Matrigel extracellular matrix and cultured for 4 days. When a mixture of COLO320HSR cancer cells and CCD-18Co colonic fibroblasts were seeded on a Matrigel extracellular matrix, COLO320HSR cancer cell invasion was observed. The COLO320HSR cancer cells alone exhibited only a faint invasion into the extracellular matrix. When

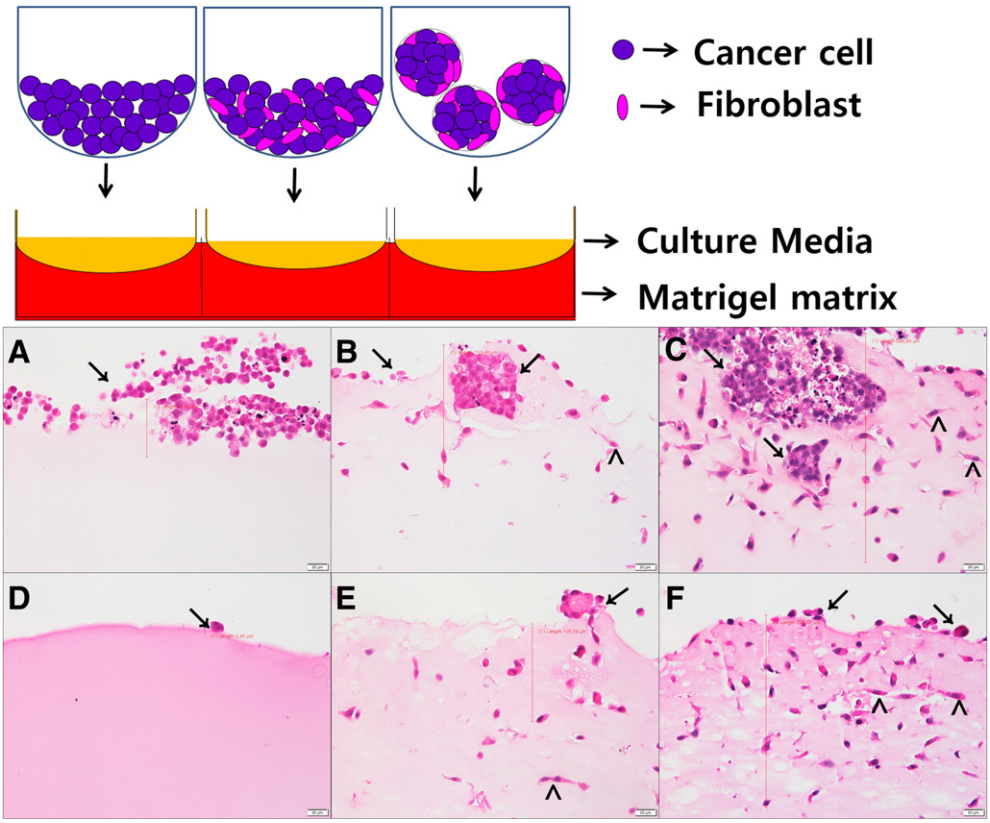

Figure 3. Tumor invasion assays using a 3D organotypic cell culture model with a basement membrane matrix (BD Matrigel™) after a 4-day culture. The following cells were seeded on the basement membrane mixture: (A) COLO320HSR cells alone (depth of tumor cell invasion: 61.89 μm), (B) a mixture of COLO320HSR and CCD-18Co cells in suspension (depth of tumor cell invasion: 153.40 μm), (C) coculture spheroids of COLO320HSR and CCD-18Co cells after 3 days in culture (depth of tumor cell invasion: 245.93 μm), (D) SNU-C1 cells alone (depth of tumor cell invasion: 4.40 μm), (E) a mixture of SNU-C1 and CCD-18Co cells in suspension (depth of tumor cell invasion: 105.29 μm) and (F) coculture spheroids of SNU-C1 and CCD-18Co cells after 3 days in culture (depth of tumor cell invasion: 207.07 μm). CCD-18Co fibroblasts induced colonic adenocarcinoma cell invasion into the basement membrane matrix. The seeded spheroids exhibited greater invasiveness. The arrows indicate cancer cells, and the arrowheads designate fibroblasts (hematoxylin and eosin (H&E), scale bar = 20 μm; 400×).

COLO320HSR and CCD-18Co coculture spheroids were tested, the COLO320HSR cells demonstrated marked invasiveness. CCD-18Co colonic fibroblasts induced SNU-C1 suspension cancer cells to attach to the surface of the extracellular matrix (Figure 3).

Spheroids Enhance Tumorigenicity in a Tumor Xenograft Model

The injection of COLO320HSR or SNU-C1 CACs alone in suspension did not generate any tumors. The co-injection of CCD-18Co fibroblasts and COLO320HSR cancer cells in suspension generated tumors in two of five mice. The injection of CCD-18Co and COLO320HSR spheroids generated tumors in four of five mice. The injection of a single CCD-18Co and COLO320HSR spheroid generated a tumor in one of five mice. The tumors from CCD-18Co and COLO320HSR spheroids were larger than those generated by co-injecting CCD-18Co and COLO320HSR cells in suspension (P = .022). The co-injection of CCD-18Co fibroblasts and SNU-C1 cancer cells in suspension generated a tumor in one of five mice. Injected CCD-18Co and SNU-C1 spheroids generated tumors in three of five mice. The injection of a single SNU-C1 and CCD-18Co spheroid did not generate a tumor in any of the five mice. The tumors from CCD-18Co and SNU-C1 spheroids tended to be larger than those generated by co-injecting CCD-18Co and SNU-C1 cells in suspension (P = .077). The tumors derived from both COLO320HSR and SNU-C1 cancer cells expressed cytokeratin. The interaction between fibroblasts and cancer cells indeed plays an essential role in enhancing tumorigenesis in nude mice (Table 1; Figure 4; see also Supplemental Figure S1).

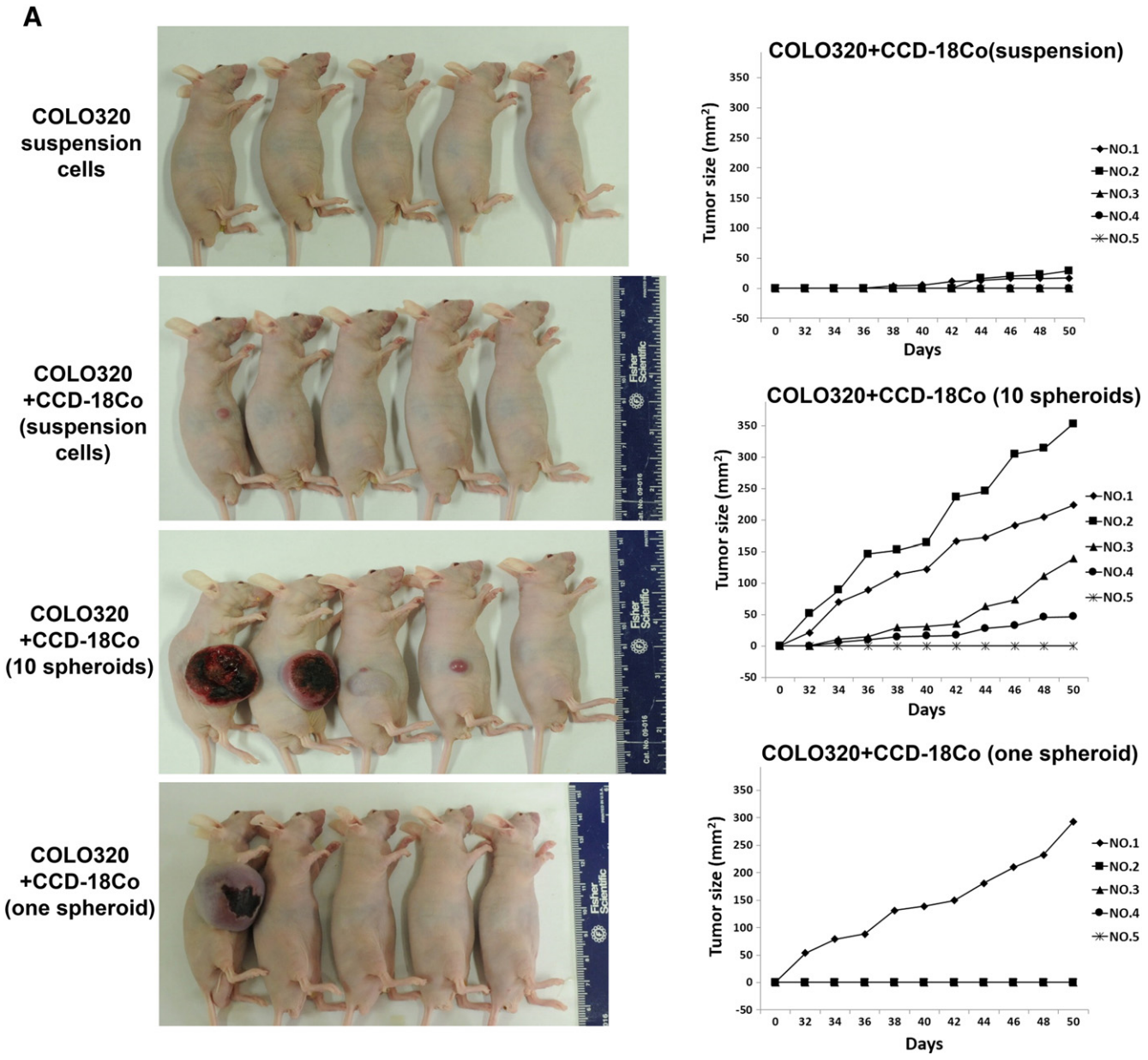

Figure 4. Cancer cell spheroids exhibited increased tumorigenicity *in vivo*. (A) COLO320HSR cancer cells with CCD-18Co fibroblasts generated xenograft tumors in nude mice. The growth curves implicate the spheroid form of cancer cells in tumorigenicity and tumor growth. (B) SNU-C1 cancer cells with CCD-18Co fibroblasts generated xenograft tumors in nude mice. The growth curves implicate the spheroid form of cancer cells in tumorigenicity and tumor growth.

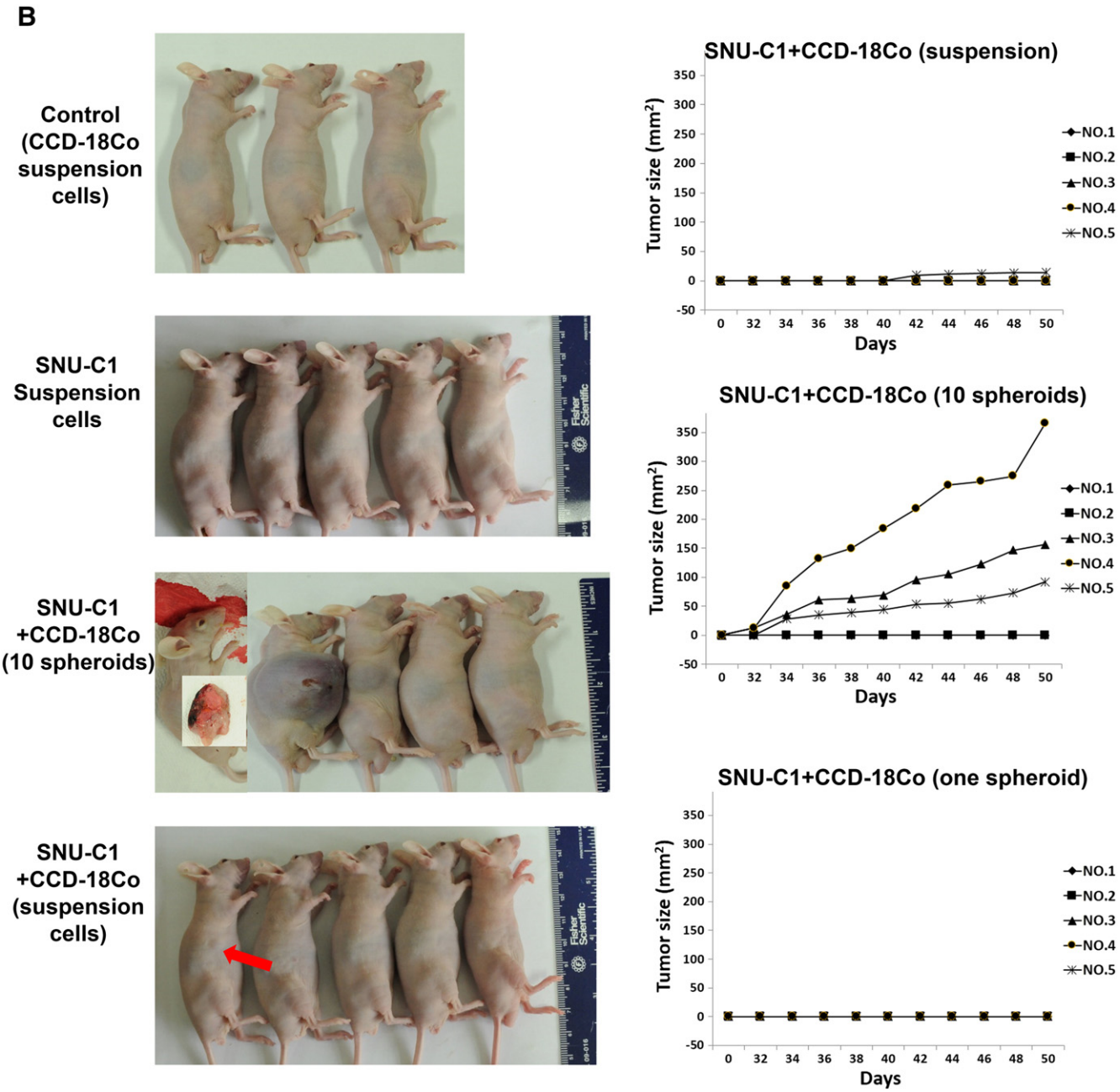

Figure 4 (*continued*)

Differential Expression of FAP-α in NCFs-CACs Cocultures and Monocultures of NCFs or CACs

The CCD-18Co fibroblasts cocultured with the COLO320HSR cancer cells expressed FAP-α as determined by immunofluorescence, whereas monocultured CCD-18Co fibroblasts did not (Figure 5, *A* and *B*). The scaffold-free spheroids of CCD-18Co and COLO430HSR or SNU-C1 exhibited FAP-α expression at the periphery of the spheroid (Figure 5, *C* and *D*). The xenograft tumors in nude mice also showed FAP-α expression at the periphery of the tumors (Figure 5, *E* and *F*). The fibroblasts or other stromal cells were predominantly positioned at the periphery of the spheroid or xenograft tumors (Figures 2 and 5, *C*–F).

Discussion

Fibroblasts are capable of matrix remodelling and can promote tumor progression. Squamous cell carcinoma cells reportedly do not invade into the extracellular matrix when cultured alone; cancer cell stromal invasion is induced by the presence of CAFs, indicating that squamous cell carcinoma cells follow fibroblasts that remodel the extracellular matrix [19]. Previous studies have not focused on the tumorigenicity of pure cancer cells and fibroblasts without molecular or genetic modifications [20–22]. In the present study, we evaluated that NCFs increased cancer cell stromal invasion into the basement membrane-like extracellular matrix, which consists primarily of laminin and collagen type 4, and enhanced xenograft tumor

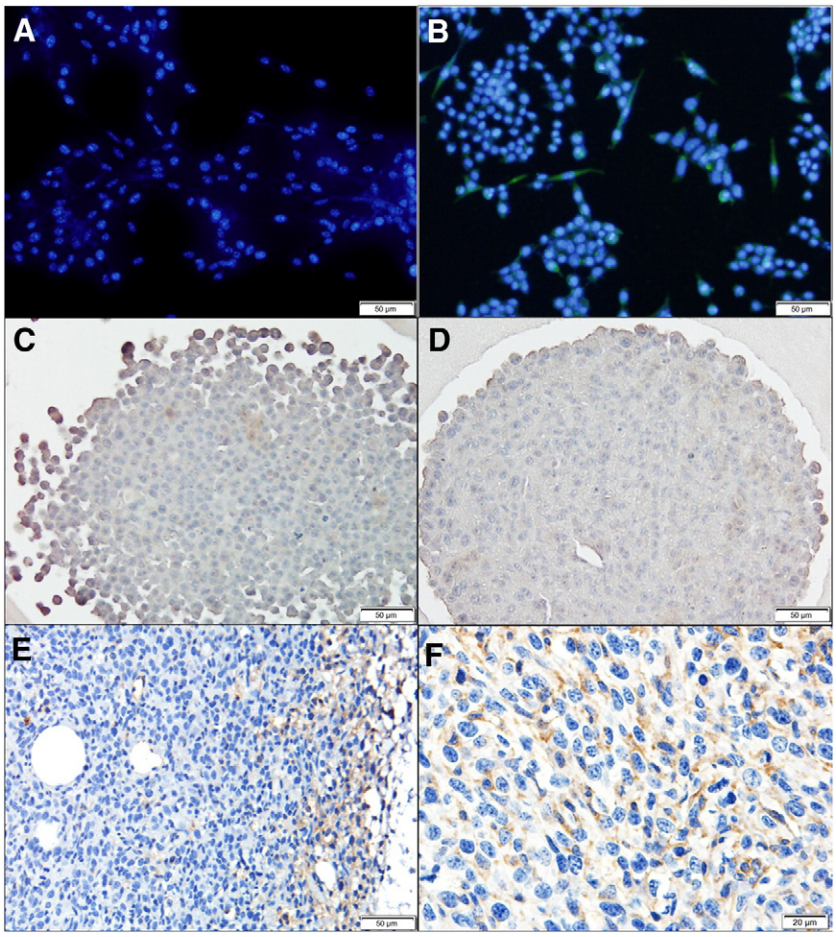

Figure 5. FAP-α expression in cocultures of CCD-18Co fibroblasts and colonic adenocarcinoma cells and in the xenograft tumors in nude mice. Immunofluorescent staining of FAP-α in monocultured CCD-18Co fibroblasts (A) and cocultures of COLO320HSR cancer cells and CCD-18Co fibroblasts (B). The cocultured CCD-18Co fibroblasts showed FAP-α expression (B, the green colour corresponds to FAP-α expression). Immunohistochemical staining of FAP-α in the CCD-18Co and COLO320HSR spheroid (C), the CCD-18Co and SNU-C1 spheroid (D) and the xenograft tumors in nude mice (E, F) revealed FAP-α expression at the periphery of the spheroids and the tumors (A-E: scale bar = 50 μm, 200 ×; F: scale bar = 20 μm, 400 ×).

induction. We also provide a model in which the subcutaneous injection of organotypic spheroids consisting of CACs and NCFs without exogenous matrix into nude mice more effectively promoted the tumorigenicity of COLO320HSR and SNU-C1 CACs than the injection of CACs and NCFs in suspension.

Carcinogenesis is greatly influenced by the surrounding stroma [23]. Normal fibroblasts can become CAFs through genetic and epigenetic alterations [24], and CAFs in the peritumoral stroma have been implicated in tumor progression [25]. CAFs found in different cancers are highly heterogeneous [26]; they up-regulate the expression of serine proteases and matrix metalloproteinases that degrade and remodel the extracellular matrix [27,28] and can regulate growth factor availability and presentation [29]. Although cancer cells also generate proteolytic enzymes to remodel the extracellular matrix, tumor progression and cancer cell stromal invasion were shown to be limited in the absence of fibroblasts in our study and others. Cross-talk between cancer cells and fibroblasts induces invasive behaviour and promotes the phenotypic transformation of cancer cells [1].

This study demonstrates that the cocultivation of CACs and NCFs results in synergistic effects on CAC and NCF spheroid formation. CAFs differ from resting NCFs with regard to myofibroblastic characteristics such as the production of growth factors and proteases [4,7,30]. Activated fibrobalsts called CAFs have been reported to produce the protease FAP-α [7,31]. We provide evidence that FAP-α is expressed in CCD-18Co fibroblasts during coculture with CACs; in contrast we show that there is no FAP-α expression in monocultured CCD-18Co fibroblasts (Figure 5, *A* and *B*). FAP-α expression is present at the periphery of our 3D scaffold-free spheroids and xenograft tumors in nude mice (Figure 5, *C*–F). During CAC and NCF spheroid formation, NCFs probably acquire characteristics that resemble those of CAFs in the peritumoral stroma [15], resulting in CAFs that surround the tumor cells [32,33].

Solid carcinomas with proliferating tumor cells can grow near the blood vessels, whereas malignant cells surrounded by fibroblasts remain separated from the blood vessels in the early stage of tumor development or in micrometastatic sites *in vivo* [6,32,33]. The coculture 3D spheroid model is not a new system for the investigation

of intercellular interaction. In general, spheroid cocultures have been established in extracellular scaffolds including agarose [10], methylcellulose [34,35], a mixture of collagen I gel and Matrigel (BD Biosciences) [36], a hanging drop (cells in hanging drops of culture media descend under the pull of gravity to assemble as a miniature tissue) [6] or an Algimatrix 3D culture system (Invitrogen) that uses a lyophilized alginate sponge [12]. Scaffold-free 3D aggregation monocultures of mesenchymal stem cells or single cell types maintained in suspension on a rotary orbital shaker have been used in cancer research [37–39], but to our knowledge, the scaffold-free coculture of cancer cells and fibroblasts in a microgravity environment using a rotary orbital shaker has not been reported. Our scaffold-free spheroids were formed naturally in RPMI 1640 medium (Gibco, Grand Island, NY; cat. 11875-093) with 10 % FBS and 1% Penicillin-Streptomycin for only 3 days with no periodic medium change. A medium change could lead to fluctuations in the culture environment including variations in the pH level [40,41]. Thus, our scaffold-free spheroid model mimicked the *in vivo* characteristics of CACs in their microenvironment and may represent an avascular early stage of tumorigenicity and the state of tumor metastasis.

Activated fibroblasts, or CAFs, affect the migration and proliferation of tumor cells [6]. CAFs have been reported to be abundant in colorectal carcinomas and have been shown to express FAP-α. FAP-α is expressed in CAFs in the peritumoral stroma of human colorectal adenocarcinomas and represents an attractive target protein for preclinical therapeutic research [8,42]. FAP-transfected HEK293 cells were more tumorigenic and elicited greater tumor growth in mice compared to vector-transfected HEK293 controls. Additionally, inhibiting FAP enzymatic activity by site-directed mutagenesis of FAP attenuated tumor growth *in vivo* [20].

This *in vitro* scaffold-free spheroid model addresses the importance of obtaining a model that better represents cancer cells *in vivo*. This spheroid model is a promising tool for the generation of tumor spheroids. Furthermore, these spheroids enable the investigation of tumor-stroma interactions and offer a better representation of the *in vivo* conditions of cancer cells in their microenvironment.

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