Novel 3-D cell culture system for in vitro evaluation of anticancer drugs under anchorage-independent conditions

Ayako Aihara,¹ Natsuki Abe,¹ Koichiro Saruhashi,² Tatsuro Kanaki¹ and Taito Nishino¹

¹Biological Research Laboratories, Nissan Chemical Industries Ltd, Saitama; ²Material Research Laboratories, Nissan Chemical Industries Ltd, Chiba, Japan

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Correspondence
Taito Nishino and Tatsuro Kanaki, Biological Research Laboratories, Nissan Chemical Industries Ltd, 1470, Shiraoka, Shiraoka-shi, Saitama 349-0218, Japan. Tel: +81-480-92-2513; Fax: +81-480-92-2516; E-mails: nishino@nissanchem.co.jp; kanaki@nissanchem.co.jp

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Anticancer drug discovery efforts have used 2-D cell-based assay models, which fail to forecast in vivo efficacy and result in a lower success rate of clinical approval. Recent 3-D cell culture models are expected to bridge the gap between 2-D and in vivo models. However, 3-D cell culture methods that are available for practical anticancer drug screening have not yet been fully attained. In this study, we screened several polymers for their ability to suspend cells or cell spheroids homogeneously in a liquid medium without changing the viscosity behavior, and identified gellan gum (FP001), as the most potent polymer. FP001 promoted cell dispersion in the medium and improved the proliferation of a wide range of cancer cell lines under low attachment conditions by inhibiting the formation of large-sized spheroids. In addition, cancer cells cultured with FP001-containing medium were more susceptible to inhibitors of epidermal growth factor (EGF) signaling than those cultured under attachment conditions. We also showed that ligands of the EGF receptor family clearly enhance proliferation of SKOV3 ovarian carcinoma cells under anchorage-independent conditions with FP001. Consistent with this result, the cells grown with FP001 showed higher EGF receptor content compared with cells cultured under attachment conditions. In conclusion, we developed a novel 3-D cell culture system that is available for high throughput screening of anticancer agents, and is suitable for evaluation of molecular-targeted anticancer drugs. Three-dimensional cell culture using FP001 will be of value in the development of useful technologies for anticancer drug discovery.
and additionally mediated low attachment to multiwell plates. A large-scale sphere system for culture of human pluripotent stem cells by applying FP001 as a sedimentation-suppressive agent has recently been reported. In that system, FP001 fulfills an important role by resolving major problems within suspension culture for mass cell production. Here, we report a novel 3-D cancer cell culture system utilizing FP001 that is available for anticancer drug assays under anchorage-independent conditions.

Materials and Methods

Compounds and reagents. Gellan gum was purchased from Sansho (Osaka, Japan). In order to prepare gellan gum (FP001) containing media, gellan gum was suspended in pure water to 0.3% (w/v) and dissolved by stirring at 90°C. The aqueous solution was sterilized at 121°C for 20 min in an autoclave. The solution was then added to each medium at the given concentration with stirring at room temperature.

Human growth factors, epidermal growth factor (EGF), hepatocyte growth factor (HGF)-like growth factor (HB-EGF), transforming growth factor-β (TGF-β), basic fibroblast growth factor, insulin-like growth factor 1, and platelet-derived growth factor-BB, were purchased from PeproTech (Rocky Hill, NJ, USA). Gefitinib, erlotinib, trametinib, and MK2206 were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Adriamycin, paclitaxel, and mitomycin C were purchased from Wako Pure Chemical Industries (Osaka, Japan). Adriamycin, paclitaxel, and mitomycin C were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cancer cell lines. Human cancer cell lines were obtained from DS Pharma Biomedical (Osaka, Japan; A549, SKOV3, and HepG2), A549 and HepG2 cells were cultured in DMEM with 10% FBS. SKOV3 cells were cultured in McCoy’s 5A medium with 15% FBS. These media were supplemented with a 1% penicillin–streptomycin mixture (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured at 37°C in a humidified atmosphere flushed with 5% CO₂ in air.

HepG2 cell spheroids were made for the dispersion test by using EZSPHERE (AGC Techno Glass, Tokyo, Japan). After 6–10 days of culture on the plate (initial cell number, 250 000 cells/100-mm dish), spheroids of 100–200 μm in diameter were collected by centrifugation at 420 g for 10 min.

Measurement of viscosity. The viscosity of the medium was measured by using the MCR 302 rheometer (Anton Paar, Graz, Austria), a 25-mm cone plate, and a gap of 0.107 mm at 25°C with a shear rate of 10 s⁻¹.

Quantitative analysis of spheroid size distribution. A549 cells were grown in 96-well low attachment plates (Corning, New York, NY, USA) for 5 or 10 days with 0.015% (v/v) FP001 (initial cell number, 2000 cells/100 μL/well). Spheroids were stained by adding 100 μL phenol red-free DMEM supplemented with 40 μg/mL Hoechst 33342 (Life Technologies, Carlsbad, CA, USA) per well. After incubation for 45 min at 37°C, the culture plates were centrifuged at 420 g for 10 min and supernatants (150 μL) were changed to fresh media. The culture plates were centrifuged at 420 g for 10 min, and then spheroid size was analyzed using Cellomics Arrayscan VTI (Thermo Fisher Scientific, Waltham, MA, USA).

Cell culture using medium with FP001. Each human cancer cell line was seeded at a density of 10 000–50 000 cells/mL into the indicated medium composition containing 0.01–0.03% (v/v) FP001, and dispensed into the wells of a 96-well flat-bottom low attachment plate using 100–200 μL/well. For the monolayer culture method (2-D), each cancer cell line was inoculated at a density of 2000–15 000 cells/mL in medium without FP001, and dispensed into the wells of a 96-well flat-bottom plate (Corning) using 100–200 μL/well. These plates were cultured unstirred in a CO₂ incubator (37°C, 5% CO₂) for 5–12 days.

For the anticancer drug test and the growth factor test, medium containing a 10-fold concentration of various anticancer drugs and/or growth factors and FP001 at a final concentration of 0.015% (w/v) was added to cell culture media at a ratio of 9:1, and the cells were cultured using the method stated above.

Cell proliferation assay. Cultured cells were counted as follows. For the WST-8 assay, WST-8 solution (1:10 volume; Dojindo Laboratories, Kumamoto, Japan) was added to the cell culture and incubated at 37°C for 100 min. Viable cells were counted by measurement of the absorbance at 450 nm using the absorbance spectrometer, SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA). For the ATP assay, an equal volume of the ATP reagent (CellTiter-Glo Luminescent Cell Viability Assay; Promega, Madison, WI, USA) was added to the cell culture, and the luminescence intensity (relative light unit value) was measured using FlexStation3 (Molecular Devices). For the Trypan blue exclusion assay, spheroids were degraded into single cells by trypsin–EDTA treatment for 10 min and then cells were stained with an equal volume of 0.015% (w/v) Trypan blue solution (Life Technologies). Viable cells were counted using an automated cell counter (TC20; Bio-Rad Laboratories, Hercules, CA, USA).

Flow cytometric analysis of apoptosis. Apoptosis was measured using the Annexin V–FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manual and all samples were analyzed by flow cytometry using a FACSAria III (BD Biosciences).

Apoptosis assay. Caspase 3/7 reagent (Caspase 3/7-Glo; Promega) was added to the culture medium after 5 days of cell culture as indicated. Following incubation for 1 h at room temperature, luminescence intensity (relative light unit value) was measured using FlexStation3. The values were normalized to the ATP value.

Immunoblotting. Western blot analysis was carried out as described previously. SKOV3 cells were incubated with human HB-EGF for 15 min or 1 h, washed with ice-cold PBS after removing the medium, and lysed. The blot was incubated with anti-phosphorylated EGF receptor (p-EGFR; Tyr1092), anti-EGFR (Santa Cruz Biotechnology), anti-Akt, anti-p-Akt (Cell Signaling Technology, Danvers, MA, USA) and anti-GAPDH (Cell Signaling Technology) antibodies. Sites of antibody binding were visualized using the ECL Western blotting detection system (Wako Pure Chemical Industries) after incubation with peroxidase-conjugated anti-Ig (SouthernBiotech, Birmingham, AL, USA).

Ethics statement. All experiments using mice were carried out in accordance with our institutional guidelines for the use of laboratory animals and approved by the review board for animal experiments of Nissan Chemical Industries.

Statistical analysis. All results are presented as the mean ± SD. Statistical significance was analyzed with Student’s t-test, Dunnett’s test, or the Kruskal–Wallis test followed by the Steel–Dwass test by using EXSAS version 7.1.6.1 (Arm Systex, Osaka, Japan). The level of significance was set at 0.05.

Results

Gellan gum suspends cancer cell spheroids homogeneously. In order to build 3-D cell culture systems suitable for compound
screening in anticancer drug discovery, we assumed that specific polymers will have the ability to suspend cells or cell spheroids uniformly in liquid medium without increasing its viscosity. It was also anticipated that these polymers would suppress the formation of large-sized spheroids (>500 µm in diameter) in low attachment vessels. We therefore screened various concentrations of seven polysaccharide polymers (Table S1) for their ability to suspend HepG2 cell spheroids uniformly in liquid medium, and ultimately identified gellan gum (FP001) as the most active polymer (Fig. 1a). We next confirmed that FP001 could reach a concentration of over 0.010% (w/v) when added to cell culture medium (data not shown). Figure 1(b) shows that addition of FP001 at a concentration of 0.015% (w/v) to DMEM elicited a homogeneous suspension of HepG2 cell spheroids with suppression of sedimentation of the spheroids. The degree of viscosity of the FP001-containing DMEM at FP001 concentrations of 0.010% and 0.020% (w/v) was 1.31 and 1.92 mPa·s, respectively, which is the same fluidity as DMEM without FP001 (0.98 mPa·s). In contrast, the degree of viscosity of methyl cellulose-containing DMEM at methyl cellulose concentrations of 0.6% and 1.0% (w/v) was 13.0 and 48.2 mPa·s, respectively, and HepG2 cell spheroids in this medium sank to the bottom of a vessel (Table S1).

We next cultured the A549 human lung cancer cell line and the SKOV3 human ovary cancer cell line on low attachment 96-well plates in 0.015% (w/v) FP001-containing medium for 5 days. These cells formed spheroids of huge size around the edge of the wells in medium without FP001. However, in the medium with FP001, no large-sized spheroids were formed and the spheroids were suspended homogeneously (Fig. 1c).

Next, we measured the distribution of spheroid size by using an Arrayscan imaging analyzer (Fig. 1d). The average diameter of A549 cell spheroids that were formed in FP001-containing medium was 40 and 50 µm after 5 and 10 days of culture, respectively. Scanning electron microscopy also showed that the average diameter of 5-day cultured spheroids was approximately 40 µm (Fig. S1). These data indicated that FP001 dispersed the cells uniformly in the medium at the beginning of culture and that each single cell formed a spheroid of approximately 40-µm diameter in size during 5 days of culture. FP001 also inhibited the fusion of spheroids.

**FP001 improves cell proliferation on low attachment culture plates.** To evaluate the effect of FP001 on cell proliferation under low attachment conditions, we cultured A549 cells in FP001-containing medium on low attachment plates for 5 days and counted the cell number. FP001 facilitated the proliferation of A549 cells compared with blank control, based on three different cell counting methods (Fig. 2a). The positive effect of FP001 on cell growth on low attachment plates was
observed for the representative cancer cell lines A375, HCT116, HeLa, MCF7, HepG2, MNNG/HOS, and SKOV3 cells (Table S2). In contrast, the growth of A549 cells on normal plates remained unaffected by addition of FP001 to the culture medium (Fig. 2b). Furthermore, A549 cells could not adhere to the surface of FP001-coated cell culture plates and instead formed spheroids (Fig. S2). These data suggested that FP001 improved the proliferation of cancer cells on low attachment plates, but had no direct effect on cell proliferation. FP001 might promote cell proliferation under anchorage-independent conditions by dispersing the cell spheroids uniformly and inhibiting the formation of huge spheroids.

We next examined global transcription levels of genes in cancer cells cultured with FP001 by using a DNA microarray. There was no significant difference in the protein-coding gene expression profile between A549 cells cultured with and without FP001 for 5 days under 2-D or 3-D conditions (Fig. S3a). In contrast, 3-D culture of A549 cells with FP001 led to the upregulation of 141 protein-coding genes and the downregulation of 98 protein-coding genes versus 2-D culture (Fig. S3a, Table S3). Of these genes, we reconfirmed the expression change of carcinoembryonic antigen-related cell adhesion molecule genes by using real-time quantitative PCR (Fig. S3b). Additionally, we confirmed that there was little difference in gene expression between 2-D versus 3-D and 2-D versus 3-D with FP001 (data not shown). These data showed that FP001 had little effect on the gene expression of cells cultured under 2-D or 3-D conditions. In contrast, there was a huge difference in gene expression between cells cultured under 2-D or 3-D conditions with FP001.

Next, we investigated the global gene expression profiling in A549 cells transplanted s.c. into athymic nude mice to form tumors, and compared the profiling with that in cells cultured under 3-D conditions with FP001. Tumor formation of A549 cells in nude mice led to the upregulation (>2.0-fold) of 489 protein-coding genes and the downregulation (<0.5-fold) of 2129 protein-coding genes versus 2-D culture (data not shown). Table S4 shows upregulated and downregulated genes in tumors versus 2-D culture that are overlapping in cells cultured under 3-D with FP001. Among them, we confirmed higher expression levels of carcinoembryonic antigen-related cell adhesion molecule proteins in tumors and cells cultured under 3-D with FP001 compared to those under 2-D conditions (Fig. S4a). These results indicated that the expression profile of some of the proteins related to cell adhesion was similar between xenografted tumors and cells cultured under 3-D conditions with FP001. As for apoptosis-related genes, a further 23 genes were upregulated and 95 genes were downregulated in tumors compared to cells cultured under 2-D; expression of only four more genes (RNF144B, UNC13B, CHAC1, and GADD45B) was changed in cells cultured under 3-D conditions compared to 2-D.

Fig. 2. Gellan gum (FP001) improves the growth of A549 cells in 3-D cultures. (a) A549 cells were cultured on low attachment plates with FP001. The numbers of cultured cells were counted with WST-8, ATP, and Trypan blue exclusion assays. (b) A549 cells were inoculated into 96-well normal plates for 1 day; 10% FBS-containing DMEM with FP001 was added to the culture after removal of the initial medium. The cells were subsequently cultured for 5 days and the numbers of cultured cells were counted using the ATP assay. Data represent means ± SD for three independent experiments.
Drug screening using 384-well low attachment plates and cells grown under attachment conditions. Under low attachment conditions with FP001 compared with agents was more pronounced when the cells were cultured in 3-D culture with FP001 in 3-D culture but not to cells in 2-D culture (Fig. 4b). Consistent with these observations, the apoptosis-inducing effect of paclitaxel was more striking for cells cultured in the presence of FP001 in 3-D culture compared with those in 3-D culture without FP001 (Fig. 3b).

We next investigated the effect of molecular targeted anticancer agents on A549 cells cultured under 3-D conditions with FP001. Trametinib, an inhibitor of MEK and MK2206, an inhibitor of protein kinase B (PKB/AKT) showed the strongest effect on the cells cultured with FP001. Trametinib, an inhibitor of MEK and MK2206, an inhibitor of protein kinase B (PKB/AKT) showed the strongest effect on the cells cultured with FP001. Consistent with these observations, the apoptosis-inducing effect of paclitaxel was more striking for cells cultured in the presence of FP001 in 3-D culture compared with those in 3-D culture without FP001 (Fig. 3b).

Next, we constructed a cell proliferation assay for anticancer drug screening using 384-well low attachment plates and FP001-containing medium. The coefficient of variation and the Z-factor for the plate when counting A549 cell number using WST-8 after 5 days of culture were 7.7 ± 0.8 and 0.71 ± 0.03, respectively. We also detected the growth-inhibitory effect of the anticancer drugs mitomycin C, paclitaxel, and trametinib by using the 384-well low attachment plates with FP001-containing medium (data not shown). These results indicated that FP001-containing medium is available for HTS using 384-well plates.

We also evaluated the efficacy of anticancer drugs to cancer cells cultured in medium with or without serum under 2-D or 3-D conditions. In these experiments, we used the MCF7 human breast adenocarcinoma cell line because MCF7 cells showed higher sphere-forming ability in medium without serum than A549 cells, SKOV3 cells, or HeLa cells (data not shown). In medium without serum, FP001 suppressed the formation of large-sized spheres on low attachment plates (Fig. S5a), and cells cultured under 3-D conditions with FP001 showed higher levels of expression of POU5F1 (also known as OCT4), SOX4, and ITGA6 (also known as CD49F gene) compared with cells cultured under 2-D or 3-D without FP001 (Fig. S5b). Consistently, the population of CD44+CD24- breast cancer stem cells was significantly greater in 3-D cultures with FP001 than in those under 2-D or 3-D without FP001 (Fig. S5c). Table S7 shows the sensitivity of MCF7 cells cultured under 2-D or under 3-D with or without FP001 to paclitaxel, trametinib, and MK2206. As with the case of A549 cells, MCF7 cells cultured in serum-containing medium under 3-D conditions with FP001 showed highest sensitivity to trametinib and MK2206. In contrast, cells cultured in serum-free medium under 3-D with FP001 lost the strong sensitivity. These results suggested that serum-free 3-D culture conditions with FP001 may induce the stemness of cancer cells more efficiently than 2-D or 3-D without FP001.

**FP001 provides cancer cells with increased susceptibility to EGF family stimulation.** In the course of examining the efficacy of FP001 for typical cancer cell lines (Table S2), we found that the SKOV3 cell line required HB-EGF to grow on low attachment plates and that FP001 enhanced its growth under this condition (Table S5). Correspondingly, we observed higher levels of apoptosis in tumors (Fig. S4b). Absence of the blood vascular system inside the tumors might induce apoptosis.

Cancer cells cultured with FP001 are more sensitive to inhibitors of cell signaling. To evaluate the availability of medium with FP001 for use in the search for anticancer drugs, we carried out an anticancer assay to estimate the anticancer drug sensitivity of A549 cells cultured with FP001 in low attachment plates. Figure 3(a) and Table S6 show the sensitivity of A549 cells cultured under attachment conditions (2-D) or under low attachment conditions (3-D) with or without FP001 to the conventional anticancer drugs Adriamycin, paclitaxel, and mitomycin C. Although there was no big difference in sensitivity between cells cultured under 2-D and 3-D conditions with FP001, cells cultured under 3-D conditions without FP001 showed somewhat less sensitivity to the anticancer drugs than those cultured under 3-D conditions with FP001. These results suggested that serum-free 3-D culture conditions with FP001 may induce the stemness of cancer cells more efficiently than 2-D or 3-D without FP001.

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Fig. 3. Conventional anticancer agents inhibit the proliferation of A549 cells under both 2-D and 3-D conditions. (a) A549 cells were inoculated at a density of 2000 cells/well into 96-well normal plates in 10% FBS-containing DMEM (2-D) and into 96-well low attachment plates in 10% FBS-containing DMEM (3-D) with or without 0.015% (w/v) gellan gum (FP001). After 1 day of culture, anticancer drugs or DMSO (Control) were added to each cell culture. The cells were subsequently cultured for 3 days and the cell numbers were counted using the ATP assay. (b) A549 cells were inoculated without (3-D) or with 0.015% (v/v) FP001 (3D + FP001) as in (a). Paclitaxel or DMSO (vehicle) was added to each cell culture for 24 h and the populations of annexin V+/propidium iodide (PI) cells were measured. Statistical significance was analyzed with the Kruskal-Wallis test followed by the Steel-Dwass test. Data represent means ± SD for three independent experiments. n.s., not significant.
condition. We next evaluated the effect of various growth factors on SKOV3 cells cultured under 3-D conditions with FP001. As shown in Figure 5(a), HB-EGF, EGF, and TGF-β, which are members of the EGF family, had a growth-stimulating effect on SKOV3 cells only under anchorage-independent conditions. FP001 enhanced the effect of these growth-stimulating factors on SKOV3 cells under this condition. Apart from the EGF family, TGF-β1 exerted a similar effect on growth only under 3-D conditions with FP001, although its effect was not particularly strong. In contrast, fibroblast growth factor, platelet-derived growth factor-BB, and insulin-like growth factor 1 had no effect on the proliferation of SKOV3 cells under any condition.

We next evaluated the inhibitory effect of gefitinib and erlotinib, EGFR inhibitors, on the growth of SKOV3 cells in the presence of HB-EGF. These inhibitors showed a growth-inhibitory effect only in the presence of HB-EGF under anchorage-independent conditions (Fig. 5b). Consistent with these data, Gefitinib dose-dependently induced caspase activity under anchorage-independent conditions in the presence of HB-EGF (Fig. 5c). These data indicated that members of the EGF family are effective against SKOV3 cells only under anchorage-independent conditions and that their effect was elicited more clearly by the addition of FP001.

FP001 increased the protein amount of EGFR in cancer cells. To elucidate the molecular mechanisms by which FP001 enhances cellular sensitivity to the EGF family, we used Western blot analysis to examine the protein and phosphorylation levels of EGFR and AKT in SKOV3 cells cultured with FP001. Figure 6 shows that the protein level of EGFR was increased and the phosphorylation level of AKT was decreased in cells cultured on low attachment plates with FP001. However, the transcription levels of the EGFR gene were decreased in the cells cultured in 3-D with FP001; this finding was confirmed by quantitative PCR (data not shown). These results indicated that the EGFR was stabilized at the protein level but that phosphorylation of AKT, which reflects downstream signaling, was inhibited under anchorage-independent conditions.

**Discussion**

A quest for novel 3-D cell culture substrata identified gellan gum (FP001). F001 can inhibit cell–cell assembly and suppress the formation of large-sized spheroids in low attachment vessels without increasing the viscosity of the medium. FP001 has been reported to form a microstructure in an aqueous solvent in the presence of divalent cations. The microstructure formed by FP001 in medium might play an important role in inhibiting cell–cell assembly. Three-dimensional culture methods often use low attachment vessels to promote the formation of multicellular spheroids. However, the conventional 3-D method frequently results in the formation of large-sized spheroids. Nutrients and oxygen cannot reach the cells on the inside of these large spheroids, and the cells inside become necrotic. Consistent with these data, we showed increased cell death in 3-D cultures, that is, high
annexin V-positive cell numbers in cells cultured in low attachment plates. In contrast, FP001 prevented the formation of huge spheroids and distinctly improved cell proliferation in low attachment plates. Addition of FP001 led to the generation of relatively small and uniformly sized spheroids. Spheroids of this size have the advantage that they can absorb nourishment and oxygen from the medium. FP001 could not promote A549 cell growth and did not have an effect on the extensive gene expression of the cells when cultured under 2-D conditions. Further examination indicated that FP001-coated plates promoted the formation of spheroids. These data imply that cells are unlikely to attach to the surface of the microstructure formed by FP001 and that FP001 does not function as a cell scaffold. Thus, FP001 may not directly affect cell proliferation and may not communicate a growth signal to cells but instead induces the formation of spheroids of a size that is advantageous for growth under low attachment conditions.

Cells grown in the 3-D culture system with FP001 showed higher sensitivity and reproducibility in the growth assay for detection of anticancer drugs as compared with cells grown in 3-D systems without FP001. FP001 probably improves drug permeability into cell spheroids by suppressing the formation of gigantic spheroids. The growth inhibitory effect of inhibitors of cell signal transduction was greater against cells grown in

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**Fig. 5.** Cells cultured with gellan gum (FP001) are sensitive to epidermal growth factor (EGF) family stimulation. (a) SKOV3 cells were inoculated at a density of 300 cells/well into 96-well normal plates (2-D) and at a density of 5000 cells/well into 96-well low attachment plates (3-D) with or without 0.015% (w/v) FP001. After 1 day of culture, growth factors and medium (Control) were added to each cell culture. The cells were subsequently cultured for 4 days and the numbers of cultured cells were counted using the ATP assay. (b) SKOV3 cells were inoculated as in (a). After 1 day of culture, 100 ng/mL heparin-binding EGF-like growth factor (HB-EGF) or medium (Vehicle), and anticancer drugs and DMSO (Control) were added to each cell culture. The cells were subsequently cultured for 3 days and the numbers of cultured cells were counted. (c) SKOV3 cells were treated with anticancer drugs for 5 days as described for (b). The numbers of cultured cells were counted using the ATP assay and apoptosis activity was evaluated with the Caspase 3/7-Glo assay. Statistical significance was analyzed with Dunnett’s test (vs. Control). Data represent means ± SD for three independent experiments.
3-D than in 2-D culture systems and FP001 also enhanced their activity, especially the activity of the AKT inhibitor, MK2206. We also confirmed that FP001 is available to ordinary 3-D culture systems that use serum-free medium containing EGF, basic fibroblast growth factor, and supplements like B27, while the conditions weaken the activity of some anti-cancer drugs. Anchoragel-independent growth is a characteristic of many tumor cells that distinguishes them from their normal counterparts, and results in several cellular changes including loss of contact inhibition, alteration in motility, and adhesiveness. The ability of tumor cells to grow in the anchoragel-independent manner has been linked to elevation of the phosphatidylinositol 3-kinase/AKT cell survival pathway in the cells. In contrast with those observations, we found that phosphorylated AKT protein levels were decreased in SKOV3 cells cultured under 3-D conditions with FP001 compared with 2-D culture. It has been reported that spheroid formation results in decreased phosphorylation levels of AKT in A549 cells. Hirai et al. reported that MK2206 caused growth inhibition at high concentrations in 2-D cell culture; the IC$_{50}$ ranges were between 3.4 and 28.6 $\mu$mol/L. Similarly, our result indicated that A549 cells are resistant to MK2206 when grown under 2-D conditions, but are sensitive when grown under 3-D conditions. These results suggest that AKT activation is suppressed under the 3-D anchorage-independent condition with FP001 and that the observed decrease in AKT activation may be responsible for the increased sensitivity of the cancer cells to AKT inhibitors.

Growth factor HB-EGF, which belongs to the EGF family, has a critical role in ovarian cancer progression. We observed that members of the EGF family showed growth-promoting activity for SKOV3 cells in the 3-D culture system with FP001, but not in the 2-D system. The EGF inhibitors gefitinib and erlotinib specifically reduced HB-EGF-induced cell growth in 3-D culture with FP001. Remarkably, the protein level of EGFR was increased in SKOV3 cells when cultured under 3-D conditions with FP001. These results suggested that 3-D culture conditions led to stabilization of EGFR, which then contributed to higher sensitivity to EGFR family stimulation. Although the mechanism that is responsible for EGFR stability in 3-D cultures remains obscure, a number of factors have been previously directly or indirectly associated with EGFR stabilization. Whatever the mechanism, the culture system using FP001 would contribute to the development of anti-ovarian cancer drugs, which include EGFR inhibitors, HB-EGF inhibitors, Akt inhibitors, and combination therapy with EGFR inhibitors. This notion is consistent with a previous report that utilized 3-D culture conditions to efficiently obtain EGFR inhibitors.

In summary, we have developed a novel 3-D culture method that improves the growth of spheroid-forming cancer cells under anchorage-independent conditions by exploiting a feature of FP001. The method is robust, reproducible, and available for HTS, and is particularly beneficial for the in vitro evaluation of molecularly targeted drugs such as EGFR and AKT inhibitors regardless of the presence or absence of serum. We expect that this culture method will accelerate the development of new anticancer drugs as well as an anticancer sensitivity test that will help in accurately choosing the best drug or drugs for the cancer being treated.

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**Disclosure Statement**

The authors have no conflict of interest.
Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 Scanning electron microscopy image of a cell spheroid grown in medium with gellan gum (FP001).

Fig. S2 Effect of gellan gum (FP001) coating on cell attachment.

Fig. S3 Gene expression changes in gellan gum (FP001)-treated A549 cells.

Fig. S4 Features of A549 cells transplanted in immunosuppressed mice.

Fig. S5 Features of MCF7 cells cultured in serum-free medium.

Table S1 Screening of substrates for 3-D cell culture.

Table S2 Growth of cancer cell lines cultured in medium with gellan gum (FP001).

Table S3 Gene expression changes in gellan gum (FP001)-treated A549 cells.

Table S4 Gene expression changes in transplanted A549 cells.

Table S5 Expression of apoptosis-related genes in transplanted A549 cells.

Table S6 IC_{50} value of compounds for A549 cells cultured in 2-D, 3-D, and 3-D with gellan gum (FP001) conditions.

Table S7 IC_{50} value of compounds for MCF7 cells cultured in 2-D, 3-D, and 3-D with gellan gum (FP001) conditions.