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Gelatin Sponge as an Anchorage for Three-dimensional Culture of Colorectal Cancer Cells

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Abstract. *Background: Compared to two-dimensional cultures, three-dimensional (3D) cultures have many advantages in cancer studies. Nevertheless, their implementation is unsatisfactory. This study aimed to develop an anchorage-dependent 3D culture model for colorectal cancer research. Materials and Methods: Human HCT116, DLD-1 and SW620 colorectal cell lines were cultured in a gelatin sponge, and its applicability for morphological examination was studied. Results: The resulting specimens were suitable for scanning electron microscopy, transmission electron microscopy, and immunohistochemical examination. HCT116 formed smaller structures and migrated through the pores of the sponge. DLD-1 formed larger structures with tight cell-to-cell adhesion. SW620 also formed large structures but small clustered cells tended to attach to the anchorage more favorably. Immunohistochemical staining demonstrated phosphorylated yes-associated protein (YAP) localized near the attachment site in HCT116 cells. Conclusion: Because the gelatin sponge provided suitable anchorage and the cultured cells formed distinguishable 3D structures, this method may be useful for further colorectal cancer research.*

It has been more than four decades since three-dimensional (3D) cell culture became popular. Compared to ordinary two-dimensional (2D) cell cultures, 3D culture provides improved cell-to-cell contact and physiological structures similar to the *in vivo* organization. Therefore, 3D cultures have been used for studies such as of cancer proliferation, progression, migration, metastasis, drug resistance, and of stem cells. However, although 3D cultures have many advantages, the methods used in their implementation are still inconsistent and unsatisfactory. The methods are generalized as '3D culture', so different types of culture methods exist, and the results strongly depend on these methods.

At least two types of culture are categorized. One is anchorage-independent culture, without using a substrate for cellular attachment or the formation of cell aggregation. This type of culture is under nonadherent conditions, without an extracellular matrix (ECM). The other type is anchorage-dependent culture that utilizes substrates to promote cell-to-cell or cell-to-scaffold, such as those using a membrane, microfluidic channels, and ECM.

Examples of anchorage-independent cultures are cultures on low-attachment plates (1) through coating of their surfaces with compounds such as polyhydroxy ethyl methacrylate (2) and agarose (3). This can also be achieved as a result of agitation using spinner flasks or gyratory shakers (4), through the hanging drop method where a drop of medium containing a cell suspension promotes cell-to-cell interactions within the confines of the drop (5), and by culturing cells with soft agar (6). The resulting structures are commonly referred to as multicellular tumor spheroids (MCTs).

For anchorage-dependent cultures, specific substrates or materials are utilized. One of these is composed of a membrane or membranes, and the resulting structures are multilayered cell cultures composed of tumor cells and other cells on a specific membrane designed for the measurement of drug diffusion (7, 8). Microfluidic channels with micropillars are also manufactured and utilized, and ECM can be added to these chambers to allow for ECM–cell interactions (9). The basement membrane extract from an Engelbreth–Holm–Swarm murine tumor, a form of laminin-rich ECM (10-13), and collagen have also been employed as substrates or scaffolds for anchorage (14, 15). These methods have been reviewed elsewhere (16), and there are several advantages and disadvantages in anchorage-independent and

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anchorage-dependent culture. For example, there can be difficulty in retrieving cells from anchorage-dependent 3D culture formation but they can be used in large-scale production with the incorporation of growth factors (17).

The more potent advantage of anchorage-dependent culture is that it can mimic how cells move into tissues or organs in a living body and allow understanding of tumor extension dynamics as an *in vitro* culture system. In contrast, because such physiological conditions vary depending on the tissues or organs of the body, it is difficult to create a generalized model. Therefore, various culture methods are needed that correspond to various different situations.

Admittedly, further research requires the development of far more complex 3D culture models (16). This study aimed to develop an anchorage-dependent 3D culture system with existing materials. Because the numbers and choices of currently available anchorage-dependent 3D system are limited, we ho[e this method may provide an optional means of culture in colon cancer research.

Materials and Methods

Anchorage for 3D culture. As an anchorage for 3D culture, an absorbable gelatin sponge was used. This type of sponge is a sterile, water-insoluble, malleable gelatin sponge intended for hemostatic use by applying to a bleeding surface and has been in use for more than 70 years (18). The sponge is off-white and porous in appearance and was chosen because of its biocompatibility, *in vivo* stability, mesh pore size, and adequate cell attachment as anchorage for adherent cells. Because the material is mainly composed of ECM and provided in stable quality at a reasonable price, it was hypothesized that the material is useful as an alternative anchorage for 3D cultures.

Cell culture. The human colorectal adenocarcinoma cell lines DLD-1, HCT116, and SW620 (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DLD-1 and SW620) or RPMI 1640 (HCT116; Gibco, Thermo Fisher Scientific, Tokyo, Japan) supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 IU/ml and 100 μg/ml; Sigma-Aldrich, St. Louis, MO, USA). Cells (1×10^4) were inoculated and cultured in separate four-well chamber slides (Nunc Lab-Tek II; Thermo Fisher Scientific). In morphological and immuno-histochemical experiments, these 2D-cultured cells were compared to cells in 3D culture. For 3D culture, a gelatin sponge was sterilely cut into 10 mm cubes and immersed into minimum amounts of culture medium before the experiment. To allow cells to attach, dispersed colorectal cells (1×10^4 cells/100 μl medium) were injected directly into the sponge and left for 4 h at 37˚C in 5% CO_2 incubator without additional cell culture medium. After attachment of the cells, the sponges were transferred into a 10 cm dish, immersed in 10 ml culture medium and further cultivated for 3 to 20 days.

Gelatin zymography. The activities of matrix metalloproteinase 2 (MMP2) and other gelatinases expressed by these cell lines were compared using gelatin zymography. After cell culture, the medium was changed to a fetal bovine serum-free medium and cells were further cultivated for 24 h. The supernatant of the culture medium was centrifuged to eliminate dead cells and filtered with a 0.22 μm syringe filter. Using the filtered supernatant, gelatin zymography was performed with a standard protocol (19). Data were compared to that of MMP9- and proMMP2-producing SW1736 human undifferentiated thyroid carcinoma cells (from Memorial Sloan Kettering Cancer Center, NY, USA) (20).

Morphological observations. Light optical microscopy: 2D-Cultured cells were observed under phase-contrast microscopy (IX71 inverted microscope; Olympus Corporation, Tokyo, Japan). 3D- Cultured specimens were fixed with 2% paraformaldehyde in 0.1 M in phosphate buffer (pH 7.3, 400 mOsm), embedded into paraffin blocks (VIP5Jr.; Sakura Finetek, Tokyo, Japan), and sliced with a microtome (REM-700; Yamato Kohki Industrial Co., Saitama, Japan) at 5 μm thickness. The sections were then stained with hematoxylin and eosin and examined under a light microscope (BZ-9000; Keyence, Osaka, Japan). For immunohistochemical staining, 2D- and 3D-cultured cells were fixed with 1.2% glutaraldehyde in 0.1 M phosphate buffer and reacted with anti-human corticotropin-releasing hormone receptor 1 (CRHR1; GeneTex, Irvine, CA, USA) or anti-phosphorylated yes-associated protein (YAP) (Cell Signaling Technologies, Danvers, MA, USA) at 200× dilution according to the manufacturer's protocol. These antibodies were visualized with Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA).

Electron microscopy: 3D-Cultured cells were fixed with 1.2% glutaraldehyde for scanning electron microscopy (SEM) or 2% glutaraldehyde for transmission electron microscopy (TEM) in 0.1 M phosphate buffer. SEM specimens were dehydrated by a critical point dryer (HCP-2; Hitachi High-Tech Corp., Tokyo, Japan) followed by immersion in serial dilutions of ethanol/water and replacement with isoamyl acetate. These dehydrated specimens were coated by Au-Pd magnetron sputter and examined at 2.0 kV under Regulus8100 field-emission SEM (Hitachi High-Tech Corp.).

Other specimens were further fixed with 1% OsO_4 in 0.1 M phosphate buffer for 2 h, immersed in serial dilutions of ethanol/water, and replaced with methyl oxirane. After embedding in epoxy resin, the specimens were sliced using an ultra-microtome (Reichert-Nissei Ultracut; Leica, Wetzlar, Germany), stained with saturated uranium acetate aqueous solution (10 min) and Reynold's lead citrate (5 min), and examined using TEM (JEM 1400 PLUS; Japan Electron Optics Laboratory Ltd., Tokyo, Japan).

The immunohistochemically stained samples were also fixed with 1% OsO_4 for 1 h, embedded into *n*-butyl glycidyl ether (QY-1; Nisshin EM Co., Tokyo, Japan), and observed under TEM without further staining.

Results

To examine the morphology of each cell line, cells in 2D culture were observed under phase-contrast microscopy (Figure 1A). HCT116 and DLD-1 cells tended to grow in a cobblestone pattern as a single layer adhering to the bottom surface of the chamber slide. In contrast, SW620 cells less firmly attached to the chamber slide, and the boundaries of each cell were more distinct. Cells tended to grow in a columnar fashion rather than a cobblestone pattern. Lamellipodia were shorter than in the other two cell lines. Thus, these three cell lines demonstrated different morphologies in 2D culture.

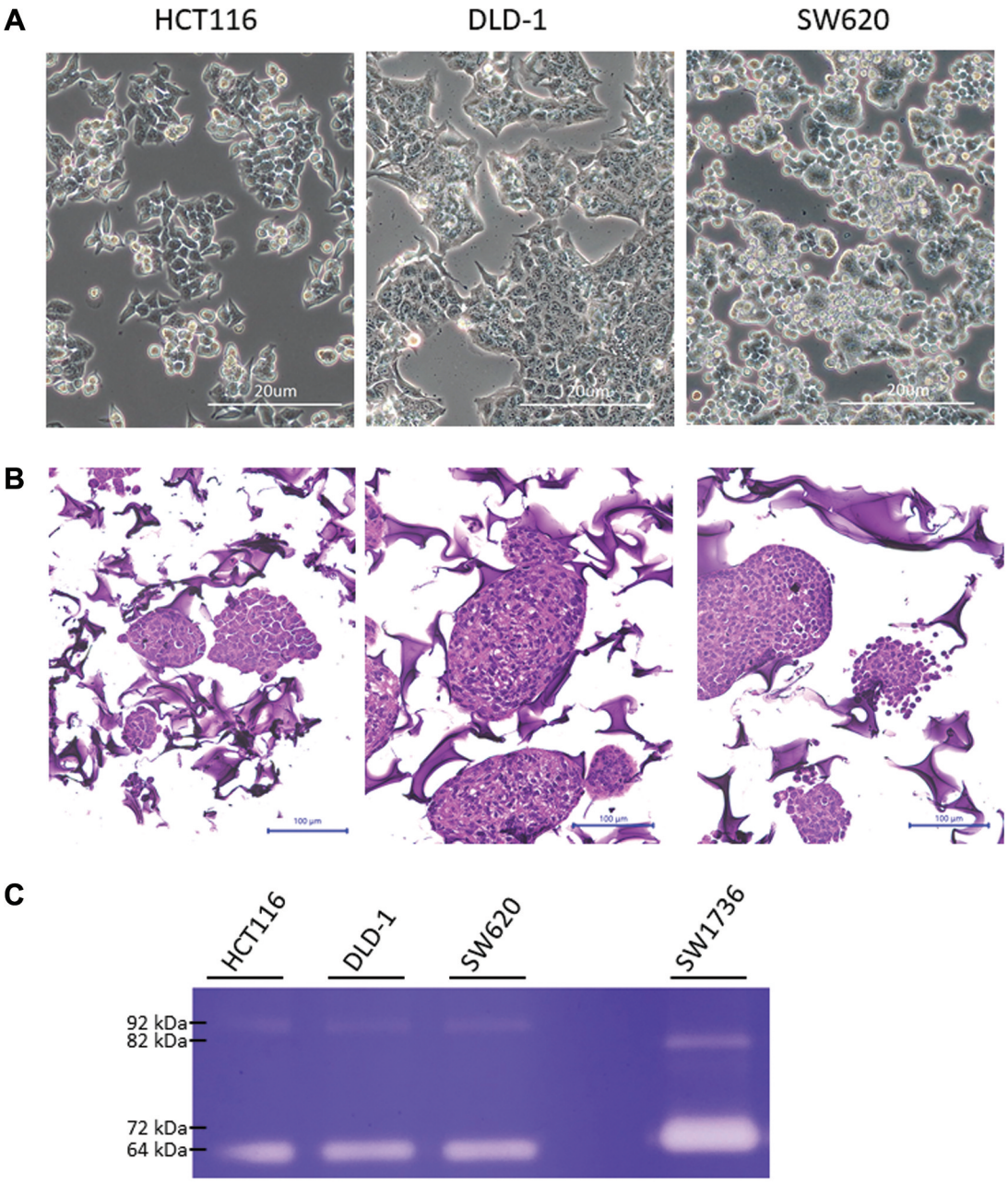

Figure 1. *Characteristics of HCT116, DLD-1, and SW620 colorectal cancer cells in 2D and 3D culture. A: Morphology of 2D-cultured cells observed under phase-contrast microscopy. B: Morphology of 3D-cultured cells. Cells were stained with hematoxylin and eosin. The gelatin sponge was also stained. C: Gelatin zymography. The activities of matrix metalloproteinase 2 (MMP2) (64 kDa) and proMMP9 (92 kDa) were demonstrated. SW1736 human undifferentiated thyroid carcinoma cells as a control, produced proMMP2 (72 kDa) and MMP9 (82 kDa).*

Next, cells were cultured in 3D in the sponge. After fixation, the cultured cells were embedded and sectioned together with the sponge for morphological examination. HCT116, DLD-1, and SW620 cells proliferated and formed a congregated 3D structure in the sponge (Figure 1B). HCT116 cells tended to form smaller structures than the other cell lines and tended to migrate through the pores of the sponge as they proliferated. HCT116 is a poorly differentiated carcinoma cell line (21) and displayed fewer lumen-like structures. DLD-1 cells formed the largest structures with tight cell-to-cell adhesion. SW620 cells also formed larger structures and additionally displayed more dispersed and

scattered cell areas where each cell was attached to the sponge independently.

Colorectal cancer is highly invasive in human tissue. Of the more than 20 MMPs that have been characterized to date, the gelatinases MMP2 and MMP9 are of particular interest for their contributions to cancer invasion and metastasis (22). To confirm if there were differences in affinity or ability of degradation of the anchorage substrate, the gelatin sponge, the activities of MMP2 and MMP9 of these cell lines were compared. Gelatin zymography was used to determine the differences in cell reactivity to gelatin. The results demonstrated that the activities of the active form of MMP2 (64 kDa) in HCT116, DLD-1 and SW620 cells were similar to those of proMMP9 (92 kDa), and no difference was observed among these cell lines (Figure 1C).

To investigate how these cells behave in the sponge, 3D-cultured cells were observed under SEM. Each HCT116 cell was recognizable as an individual even after forming an aggregated structure. Each cell was spherical and had relatively distinct boundaries. They were tightly attached to the sponge and possessed the most developed microvilli among the three cell lines. These prominent microvilli filled intercellular spaces, and some cells adhered to each other as if they had been integrated (Figure 2A).

Unlike HCT116, DLD-1 cells formed larger clusters; when aggregated, the boundaries between cells were less distinct. Cell-to-cell adhesion was the strongest among the cell lines. DLD-1 cells also demonstrated tight attachment to the sponge (Figure 2B).

SW620 formed large spherical structures similarly to HCT116 cells. However, they had the most well-marked cell boundaries (Figure 2C). This might be attributable to weak cell-to-cell adhesion, as observed in Figure 1C, and many single cells directly attached to the sponge without forming aggregates (Figure 2C, bottom right). The microvilli were shorter and fewer than in HCT116 cells.

The sponge was supplied by the manufacturer, and the detailed components and manufacturing process have not been clarified. To confirm whether the 3D culture method could be applied to various morphological studies, ultra-microtome sectioning was performed, and the images of these cells were observed under TEM. Accordingly, the morphology of the cells and cell-to-cell adhesion and cell-to-scaffold attachment were evaluated in 3D-cultured cells.

HCT116 cells adhered to neighboring cells with a round cell body (Figure 3A, left). The result was consistent with SEM observations shown in Figure 2A. Cells attached to the sponge surface using microvilli like an octopus leg (Figure 3A, right).

In contrast, DLD-1 cells did not demonstrate a round cell body when cells aggregated. Their structure was rather trabecular (Figure 3B, left). Cells were directly attached to the sponge via a cell body (Figure 3B, right).

In SW620 cells, cell-to-cell and cell-to-scaffold attachment were similar to those of HCT116. However, the adhesions were tighter and denser than in HCT116 cells (Figure 3C).

To demonstrate if it were possible to apply immunohisto-chemical studies to 3D culture, HCT116 cells were stained with an antibody to CRHR1 followed by a commercially available ABC kit. CRHR1 is a receptor for CRH or corticotropin-releasing factor and urocortin, and these molecules are associated with stress responses in the human body. The transcription of *CRHR1* in HCT116 colon cancer cells was reported previously (23). CRHR1 expression was demonstrated in both 2D and 3D cultures. The antigen localized evenly in the cells. In 2D culture, the antigen tended to be more expressed in small round cells which were less adherent to surrounding cells (Figure 4A, left). Because these round cells were less frequent in 3D culture, the antigen was underexpressed in the 3D culture (Figure 4A, right). In these figures, cells were not counterstained to clarify the localization of the antigen.

The advantage of 3D culture is that cell-to-cell adhesion and cell-to-scaffold attachment can be simultaneously observed in a single section. Because a cultured structure in this way was used for immunohistochemical examination, phosphorylated YAP expression as an example of protein relating to cell contact and density was next compared.

YAP localization is regulated by cell contact and density. At low cell densities, YAP is mainly localized in the cell nucleus. In contrast, at high densities, YAP is phosphorylated and translocated to the cytoplasm (24). In 2D culture, phosphorylated YAP was localized intracellularly to a similar extent to CRHR1 and demonstrated no significant localization in the aggregated structure (Figure 4B, left). In contrast, in 3D culture, a relatively high-density aggregated structure was formed, and phosphorylated YAP was intensely observed at the site of attachment of cells to the sponge (Figure 4B, right). This was confirmed by the TEM of these sections (Figure 4C).

Discussion

The possibility of 3D culture of colorectal cancer cells in an existing material (gelatin sponge) was explored, and the characteristics of different cell lines in the 3D culture were observed. Because the anchorage support used in this study consisted of a matrix of water-insoluble and malleable gelatin and can provide space for cell proliferation and migration, it was possible to compared the morphology of these cell lines simultaneously, such as the resulting shapes of the structure, cell-to-cell adhesion, and cell-to-scaffold attachment.

Generally, the 3D culture of colorectal cancer cells is performed for several purposes, including studies for biological behavior, immunological considerations, signal transduction and gene expression, angiogenesis, drug resistance, and cancer stem cell research.

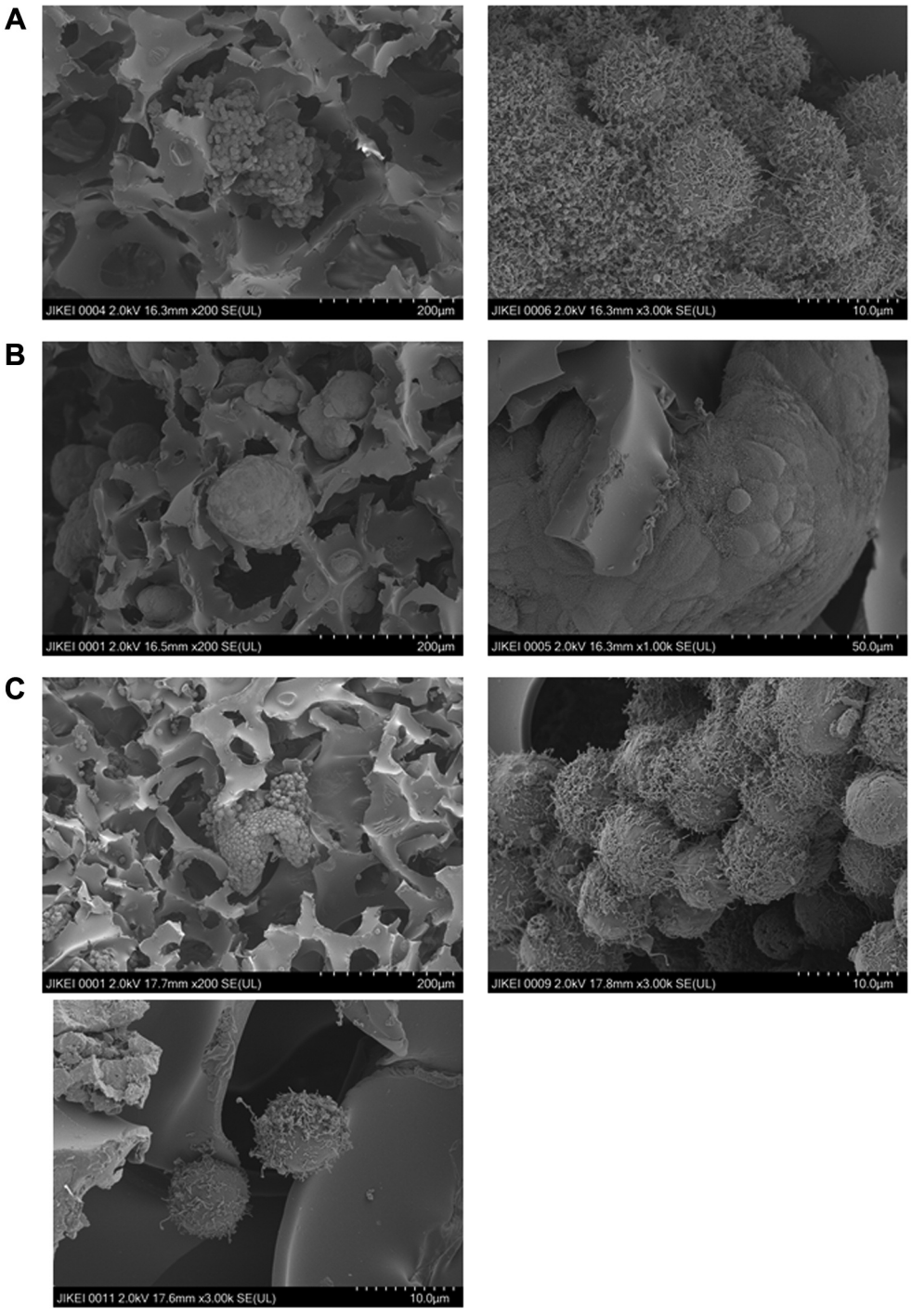

Figure 2. *Cells cultured in 3D as observed under scanning electron microscopy at low (left) and high (right) magnification. A: HCT116 cells. B: DLD-1 cells. C: SW620 cells. Many single cells were directly attached to the anchorage support without forming aggregates (bottom right).*

Biological behavior. 3D Culture provides insights to cancer structure and the understanding of homeostasis, cellular differentiation, and tissue organization in contrast to the complex host environment of an *in vivo* model (25-27). It does not require much explanation at this point. It is also useful for studying how the tumor environment regulates colon cancer (28).

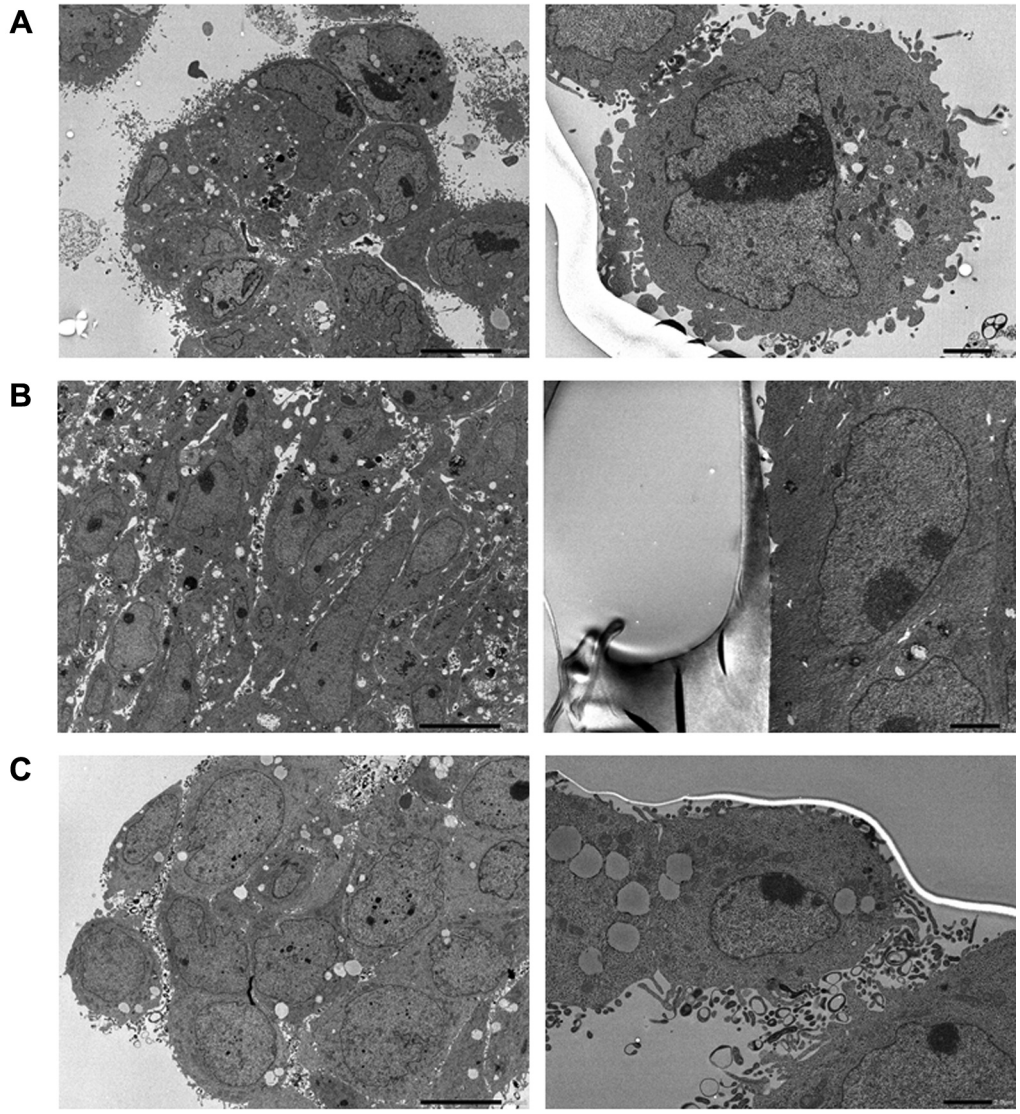

Figure 3. *Cell-to-cell adhesion (left) and cell-to-scaffold attachment (right) of colorectal cancer cells cultured in 3D as observed under transmission electron microscopy. Bars and magnifications: left 10.0 μm, ×1,000; right 2.0 μm, ×3,000. A: HCT116 cells. B: DLD-1 cells. C: SW620 cells.*

Immunological considerations. Much like in the human body, human cancer cells in 3D culture display defective immune recognition by cytotoxic T-lymphocytes, resulting in reduced cytotoxic T-lymphocyte proliferation and dendritic cell functions. Down-regulation of human leukocyte antigen and high production of lactic acid were attributable to the elicitation of these effects (29). 3D Culture revealed how chronic inflammation drives colorectal cancer development by analyzing the crosstalk between cells participating in immunity and colon cancer cells (30).

Signal transduction and gene expression. The patterns of signal transduction are known to differ significantly between cells in 2D and 3D cultures (31, 32). This is also applicable to colon cancer cells. The 3D culture of colorectal cells, including SW620, HCT116, and DLD-1, demonstrated lower activities in the AKT–mammalian target of rapamycin–S6K signaling pathway with spatial alterations. The level of phosphorylated RPS6 decreased from the cultured spheroid surface toward the center. Inhibition of the signaling pathway reduced extracellular-signal-regulated kinase (ERK) signaling, and mitogen-activated protein kinase kinase 1 (MEK1) inhibition reduced the signaling pathway in 3D but not in 2D cultures (33). MicroRNAs have also been studied in the 3D culture, and miR-101 was found to be associated with hypoxic survival and invasion of colorectal cancer

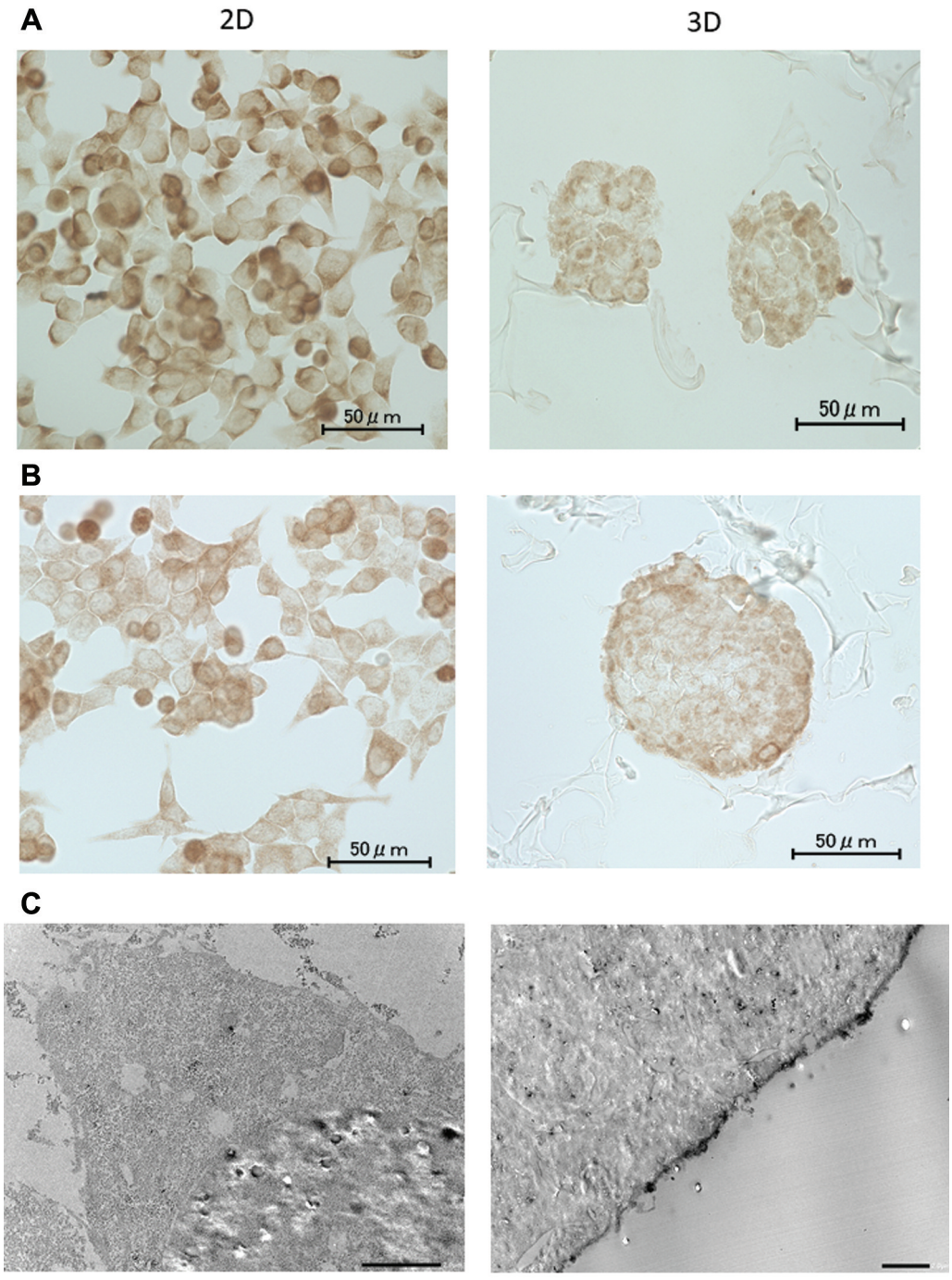

Figure 4. *Immunohistochemical staining of HCT116 cells in 2D and 3D culture. A: Expression of corticotropin-releasing hormone receptor 1. B: Expression of phosphorylated yes-associated protein. C: transmission electron microscopy observation of immunohistochemically stained sections; bars and magnification: 2.0 μm, ×3,000.*

through the WNT/β-catenin signal pathway and epithelial-to-mesenchymal transition (EMT) (34). For gene expression, when colon cancer cells were co-3D cultured with fibroblasts, they displayed a distinct gene expression profile and a subsequent pathway involved in invasion, ECM remodeling, inflammation, and angiogenesis (35).

Angiogenesis. The angiogenic characteristics of tumor cells were dramatically altered in 3D culture (36). Integrin exerts angiogenetic characteristics by enhancement of interleukin-8 (37). HCT116 cells were shown to express endothelial markers and formed tube-like structures in 3D culture with an endothelial-inducing conditioned medium. HCT116 cells secreted more endogenous vascular endothelial growth factor and expressed higher vascular endothelial growth factor receptor 2 under hypoxia (21).

Drug resistance. It has long been known that resistance to anticancer drugs differs between cells in 2D and 3D cultures. Attempts have been made to measure drug sensitivity in colon cancer cells in 3D culture (38, 39). Currently, many assays are available, and mechanisms, such as the involvement of p53 in cisplatin resistance (40), acquisition of resistance, and hypoxic stem cell characteristics have been investigated (41). 3D Cell structures generally exhibit greater resistance to anticancer drugs than 2D cultures. However, the response to drugs and differences in each experiment are dependent on cell lines and platforms used (42-44).

Cancer stem cell research. Tumorigenic cancer stem cells are present in various types of cancer. In the colon, they are present as a rare undifferentiated population of CD133+ cells and account for 2.5% of the tumor cells. The subcutaneous injection of these cells in immunodeficient mice reproduced the original tumor, and such cells can be grown exponentially for more than 1 year *in vitro* as undifferentiated tumor spheres in a serum-free medium (45-55). Methods for the isolation and culture of colon cancer stem cells have progressed (46-48) and have been applied in studies of cell death, chemoresistance, and tumor genericity (49, 50). 3D Cultures are utilized to isolate and expand various ranges of circulating cancer cells from body fluids, such as ascites, pleural fluids, and circulating blood. Tumor-derived 3D spheroids are unique because they are purposed for the enrichment of cancer stem cells or cells with stem cell-related characteristics (51).

Thus, 3D culture has become an indispensable tool for colorectal cancer research. MCTs, first described in the early 1970s cultured under nonadherent conditions to an anchorage support, are a symbolic example of 3D culture. Cell-to-cell contacts of cancer cells are maintained throughout the process of culture, and more than half of all 3D cultures reported fall in the spherical category.

Ovarian cancer cells proliferate and form multicellular spheroids within the ascites of patients. These aggregates cause further invasion or metastasis in organs, vessels, and peritoneum in the abdominal cavity. Therefore, 3D MCTs are, to a certain extent, an *in vivo* model of such cancers (52). This is also true for colorectal cancer. However, it is also known that cell subpopulations of colorectal cancer cell lines with loss of cell-to-cell adhesion (SW620 cells lack E-cadherin, DLD-1 cells have lost α-catenin, and HCT116 cells lack P-cadherin in the non-spheroid-forming state) demonstrated increased migration and invasion (53). Therefore, studies solely on anchorage-independent culture might be insufficient. Even for popular spheroid culture, anchorage is important; for this reason, materials, such as laminin-rich ECM, have been used for phenotype, gene expression, and epidermal growth factor receptor signaling pathway experiments in colorectal cancer (54).

Degradation of the basal lamina between cell-to-cell attachments is necessary not only for migrating cells, such as macrophages (55) or T-cells (56), but also for cancer cells infiltrating into surrounding tissue. *In situ* MMP2 and MMP9 activity is required to disintegrate the major basal lamina constituent gelatins, collagen types IV and V in such processes (57). The gelatin sponge used here may have a role as an anchorage support for this type of study.

One of the intrinsic limitations of anchorage-independent culture is the lack of stroma, blood vessels, and immune cells (58). In the case of invasion or metastasis by ovarian multicellular spheroids to the peritoneum in the abdominal cavity, omental mesothelial cells inhibited the early steps of ovarian cancer metastasis, whereas omental fibroblasts and the ECM enhanced the attachment and invasion of cells to the omentum (59). For colorectal cancer, when HCT116 cells were co-cultured with fibroblasts, metastatic adhesion molecules (such as β1-integrin and intercellular adhesion molecule 1), transforming growth factor-β signaling molecules (such as transforming growth factor-β3 and p-SMAD2), proliferation-associated proteins (such as cyclin D1 and Ki-67), and EMT transition factors (such as vimentin) were up-regulated, and the high-density microenvironment synergistically increased tumor-promoting factors, cancer stem cell survival, and EMT factors (60). Neighboring cells also affect the gene expression of cancer cells (61). Anchorage-dependent cultures, including the system used here, can provide such platforms for a co-culture system.

More complex systems have been developed in areas of research other than cancer, such as the 3D system for human liver function (62), and an *in vitro* small intestine model that reproduces the mechanism of drug absorption (63, 64). In colorectal cancer, the further development of 3D cultures is required to simulate and allow us to understand *in vivo* cell behavior in such complex systems. Because the gelatin sponge used here is easily applicable for the morphological studies of colorectal cancer, this system may provide a further choice of a support in anchorage-dependent 3D culture.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

Muneyuki Koyama, Erika Osada, Mayumi Nomura and Nobutake Akiyama performed 2D and 3D cell cultures. Yuki Takemura, Emi Kikuchi and Hideki Saito performed electron microscopic studies, and Go Kuwata and Kei-ichi Ikeda performed immunohistochemical studies. Morphological studies were conducted by Toshiaki Tachibana. Kazunari Sugimitsu contributed to writing and checking the manuscript and Yoshinobu Manome proposed concepts and designed as well as managed the overall study.

References

1 Vinci M, Gowan S, Boxall F, Patterson L, Zimmermann M, Court W, Lomas C, Mendiola M, Hardisson D and Eccles SA: Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. BMC Biol *10*: 29, 2012. PMID: 22439642. DOI: 10.1186/1741-7007-10-29

2 Ivascu A and Kubbies M: Rapid generation of single-tumor spheroids for high-throughput cell function and toxicity analysis. J Biomol Screen *11(8)*: 922-932, 2006. PMID: 16973921. DOI: 10.1177/1087057106292763

3 Friedrich J, Seidel C, Ebner R and Kunz-Schughart LA: Spheroid-based drug screen: considerations and practical approach. Nat Protoc *4(3)*: 309-324, 2009. PMID: 19214182. DOI: 10.1038/nprot.2008.226

4 Santini MT and Rainaldi G: Three-dimensional spheroid model in tumor biology. Pathobiology *67(3)*: 148-157, 1999. PMID: 10394136. DOI: 10.1159/000028065

5 Tung YC, Hsiao AY, Allen SG, Torisawa YS, Ho M and Takayama S: High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. Analyst *136(3)*: 473-478, 2011. PMID: 20967331. DOI: 10.1039/c0an00609b

6 Thierbach R and Steinberg P: Automated soft agar assay for the high-throughput screening of anticancer compounds. Anal Biochem *387(2)*: 318-320, 2009. PMID: 19454240. DOI: 10.1016/j.ab.2009.01.029

7 Kyle AH, Huxham LA, Chiam AS, Sim DH and Minchinton AI: Direct assessment of drug penetration into tissue using a novel application of three-dimensional cell culture. Cancer Res *64(17)*: 6304-6309, 2004. PMID: 15342419. DOI: 10.1158/0008-5472.CAN-04-1099

8 Minchinton AI and Tannock IF: Drug penetration in solid tumours. Nat Rev Cancer *6(8)*: 583-592, 2006. PMID: 16862189. DOI: 10.1038/nrc1893

9 Toh YC, Zhang C, Zhang J, Khong YM, Chang S, Samper VD, van Noort D, Hutmacher DW and Yu H: A novel 3D mammalian cell perfusion-culture system in microfluidic channels. Lab Chip *7(3)*: 302-309, 2007. PMID: 17330160. DOI: 10.1039/b614872g

10 Lovitt CJ, Shelper TB and Avery VM: Miniaturized three-dimensional cancer model for drug evaluation. Assay Drug Dev Technol *11(7)*: 435-448, 2013. PMID: 25310845. DOI: 10.1089/adt.2012.483

11 Muranen T, Selfors LM, Worster DT, Iwanicki MP, Song L, Morales FC, Gao S, Mills GB and Brugge JS: Inhibition of PI3K/mTOR leads to adaptive resistance in matrix-attached cancer cells. Cancer Cell *21(2)*: 227-239, 2012. PMID: 22340595. DOI: 10.1016/j.ccr.2011.12.024

12 Park CC, Zhang H, Pallavicini M, Gray JW, Baehner F, Park CJ and Bissell MJ: Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and *in vivo*. Cancer Res *66(3)*: 1526-1535, 2006. PMID: 16452209. DOI: 10.1158/0008-5472.CAN-05-3071

13 Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C and Bissell MJ: Reversion of the malignant phenotype of human breast cells in three-dimensional culture and *in vivo* by integrin blocking antibodies. J Cell Biol *137(1)*: 231-245, 1997. PMID: 9105051. DOI: 10.1083/jcb.137.1.231

14 Nirmalanandhan VS, Duren A, Hendricks P, Vielhauer G and Sittampalam GS: Activity of anticancer agents in a three-dimensional cell culture model. Assay Drug Dev Technol *8(5)*: 581-590, 2010. PMID: 20662735. DOI: 10.1089/adt.2010.0276

15 Manome Y, Mizuno S, Akiyama N, Fujioka K, Saito H, Hataba Y, Kobayashi T and Watanabe M: Three-dimensional cell culture of glioma and morphological comparison of four different human cell lines. Anticancer Res *30(2)*: 383-389, 2010. PMID: 20332443.

16 Lovitt CJ, Shelper TB and Avery VM: Advanced cell culture techniques for cancer drug discovery. Biology (Basel) *3(2)*: 345-367, 2014. PMID: 24887773. DOI: 10.3390/biology3020345

17 Breslin S and O'Driscoll L: Three-dimensional cell culture: the missing link in drug discovery. Drug Discov Today *18(5-6)*: 240-249, 2013. PMID: 23073387. DOI: 10.1016/j.drudis.2012.10.003

18 Ferrosan Medical Devices. Available at: http://www.Ferrosan medicaldevices.Com/ferrosan2012/products2012/spongostan.Aspx #.Ybksjnluuwc [Last accessed on July 21, 2021]

19 Abcam: MMP-9 gelatin zymograph protocol. Available at: https://wwwabcamcom/protocols/gelatin-zymography-protocol#staining [Last accessed on July 21, 2021]

20 Takeyama H, Hosoya T, Sakurai K, Mori Y, Watanabe M, Kisaki H and Ohno T: Production of a novel monoclonal antibody, JT-95, which can detect antigen of thyroid carcinoma. Cancer Res *56(8)*: 1817-1822, 1996. PMID: 8620498.

21 Liu Z, Qi L, Li Y, Zhao X and Sun B: VEGFR2 regulates endothelial differentiation of colon cancer cells. BMC Cancer *17(1)*: 593, 2017. PMID: 28854900. DOI: 10.1186/s12885-017-3578-9

22 Murnane MJ, Cai J, Shuja S, McAneny D, Klepeis V and Willett JB: Active MMP-2 effectively identifies the presence of colorectal cancer. Int J Cancer *125(12)*: 2893-2902, 2009. PMID: 19551856. DOI: 10.1002/ijc.24682

23 Kuwata GO, Ikeda K and Manome Y: Involvement of corticotropin-releasing hormone-related peptides in cellular stress caused by anticancer drugs in colorectal cancer. Anticancer Res *40(8)*: 4505-4511, 2020. PMID: 32727780. DOI: 10.21873/anticanres.14455

24 Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L, Zheng P, Ye K, Chinnaiyan A, Halder G, Lai ZC and Guan KL: Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev *21(21)*: 2747-2761, 2007. PMID: 17974916. DOI: 10.1101/gad.1602907

25 Kim JB: Three-dimensional tissue culture models in cancer biology. Semin Cancer Biol *15(5)*: 365-377, 2005. PMID: 15975824. DOI: 10.1016/j.semcancer.2005.05.002

26 Weigelt B, Ghajar CM and Bissell MJ: The need for complex 3D culture models to unravel novel pathways and identify accurate biomarkers in breast cancer. Adv Drug Deliv Rev *69-70*: 42-51, 2014. PMID: 24412474. DOI: 10.1016/j.addr.2014.01.001

27 Han J, Chang H, Giricz O, Lee GY, Baehner FL, Gray JW, Bissell MJ, Kenny PA and Parvin B: Molecular predictors of 3D morphogenesis by breast cancer cell lines in 3D culture. PLoS Comput Biol *6(2)*: e1000684, 2010. PMID: 20195492. DOI: 10.1371/journal.pcbi.1000684

28 Kawai S, Yamazaki M, Shibuya K, Yamazaki M, Fujii E, Nakano K and Suzuki M: Three-dimensional culture models mimic colon cancer heterogeneity induced by different microenvironments. Sci Rep *10(1)*: 3156, 2020. PMID: 32081957. DOI: 10.1038/s41598-020-60145-9

29 Feder-Mengus C, Ghosh S, Reschner A, Martin I and Spagnoli GC: New dimensions in tumor immunology: what does 3D culture reveal? Trends Mol Med *14(8)*: 333-340, 2008. PMID: 18614399. DOI: 10.1016/j.molmed.2008.06.001

30 Yu Y, Blokhuis B, Derks Y, Kumari S, Garssen J and Redegeld F: Human mast cells promote colon cancer growth via bidirectional crosstalk: studies in 2D and 3D coculture models. Oncoimmunology *7(11)*: e1504729, 2018. PMID: 30377568. DOI: 10.1080/2162402X.2018.1504729

31 Chitcholtan K, Asselin E, Parent S, Sykes PH and Evans JJ: Differences in growth properties of endometrial cancer in three dimensional (3D) culture and 2D cell monolayer. Exp Cell Res *319(1)*: 75-87, 2013. PMID: 23022396. DOI: 10.1016/j.yexcr.2012.09.012

32 Ekert JE, Johnson K, Strake B, Pardinas J, Jarantow S, Perkinson R and Colter DC: Three-dimensional lung tumor microenvironment modulates therapeutic compound responsiveness *in vitro* – implication for drug development. PLoS One *9(3)*: e92248, 2014. PMID: 24638075. DOI: 10.1371/journal.pone.0092248

33 Riedl A, Schlederer M, Pudelko K, Stadler M, Walter S, Unterleuthner D, Unger C, Kramer N, Hengstschläger M, Kenner L, Pfeiffer D, Krupitza G and Dolznig H: Comparison of cancer cells in 2D vs 3D culture reveals differences in AKT-mTOR-S6K signaling and drug responses. J Cell Sci *130(1)*: 203-218, 2017. PMID: 27663511. DOI: 10.1242/jcs.188102

34 Strillacci A, Valerii MC, Sansone P, Caggiano C, Sgromo A, Vittori L, Fiorentino M, Poggioli G, Rizzello F, Campieri M and Spisni E: Loss of miR-101 expression promotes Wnt/β-catenin signalling pathway activation and malignancy in colon cancer cells. J Pathol *229(3)*: 379-389, 2013. PMID: 22930392. DOI: 10.1002/path.4097

35 Dolznig H, Rupp C, Puri C, Haslinger C, Schweifer N, Wieser E, Kerjaschki D and Garin-Chesa P: Modeling colon adenocarcinomas *in vitro* a 3D co-culture system induces cancer-relevant pathways upon tumor cell and stromal fibroblast interaction. Am J Pathol *179(1)*: 487-501, 2011. PMID: 21703426. DOI: 10.1016/j.ajpath.2011.03.015

36 Fischbach C, Chen R, Matsumoto T, Schmelzle T, Brugge JS, Polverini PJ and Mooney DJ: Engineering tumors with 3D scaffolds. Nat Methods *4(10)*: 855-860, 2007. PMID: 17767164. DOI: 10.1038/nmeth1085

37 Fischbach C, Kong HJ, Hsiong SX, Evangelista MB, Yuen W and Mooney DJ: Cancer cell angiogenic capability is regulated by 3D culture and integrin engagement. Proc Natl Acad Sci USA *106(2)*: 399-404, 2009. PMID: 19126683. DOI: 10.1073/pnas.0808932106

38 Friedrich J, Eder W, Castaneda J, Doss M, Huber E, Ebner R and Kunz-Schughart LA: A reliable tool to determine cell viability in complex 3-d culture: the acid phosphatase assay. J Biomol Screen *12(7)*: 925-937, 2007. PMID: 17942785. DOI: 10.1177/1087057107306839

39 Sung JH and Shuler ML: A micro cell culture analog (microCCA) with 3-D hydrogel culture of multiple cell lines to assess metabolism-dependent cytotoxicity of anti-cancer drugs. Lab Chip *9(10)*: 1385-1394, 2009. PMID: 19417905. DOI: 10.1039/b901377f

40 He J, Liang X, Luo F, Chen X, Xu X, Wang F and Zhang Z: P53 is involved in a three-dimensional architecture-mediated decrease in chemosensitivity in colon cancer. J Cancer *7(8)*: 900-909, 2016. PMID: 27313779. DOI: 10.7150/jca.14506

41 Karlsson H, Fryknäs M, Larsson R and Nygren P: Loss of cancer drug activity in colon cancer HCT-116 cells during spheroid formation in a new 3-D spheroid cell culture system. Exp Cell Res *318(13)*: 1577-1585, 2012. PMID: 22487097. DOI: 10.1016/j.yexcr.2012.03.026

42 Chitcholtan K, Sykes PH and Evans JJ: The resistance of intracellular mediators to doxorubicin and cisplatin are distinct in 3D and 2D endometrial cancer. J Transl Med *10*: 38, 2012. PMID: 22394685. DOI: 10.1186/1479-5876-10-38

43 Hongisto V, Jernström S, Fey V, Mpindi JP, Kleivi Sahlberg K, Kallioniemi O and Perälä M: High-throughput 3D screening reveals differences in drug sensitivities between culture models of JIMT1 breast cancer cells. PLoS One *8(10)*: e77232, 2013. PMID: 24194875. DOI: 10.1371/journal.pone.0077232

44 Imamura Y, Mukohara T, Shimono Y, Funakoshi Y, Chayahara N, Toyoda M, Kiyota N, Takao S, Kono S, Nakatsura T and Minami H: Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer. Oncol Rep *33(4)*: 1837-1843, 2015. PMID: 25634491. DOI: 10.3892/or.2015.3767

45 Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C and De Maria R: Identification and expansion of human colon-cancer-initiating cells. Nature *445(7123)*: 111-115, 2007. PMID: 17122771. DOI: 10.1038/nature05384

46 Cammareri P, Lombardo Y, Francipane MG, Bonventre S, Todaro M and Stassi G: Isolation and culture of colon cancer stem cells. Methods Cell Biol *86*: 311-324, 2008. PMID: 18442654. DOI: 10.1016/S0091-679X(08)00014-9

47 Li YF, Xiao B, Tu SF, Wang YY and Zhang XL: Cultivation and identification of colon cancer stem cell-derived spheres from the Colo205 cell line. Braz J Med Biol Res *45(3)*: 197-204, 2012. PMID: 22310487. DOI: 10.1590/s0100-879x2012007500015

48 Su YJ, Lai HM, Chang YW, Chen GY and Lee JL: Direct reprogramming of stem cell properties in colon cancer cells by CD44. EMBO J *30(15)*: 3186-3199, 2011. PMID: 21701559. DOI: 10.1038/emboj.2011.211

49 Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, Russo A, Gulotta G, Medema JP and Stassi G: Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell *1(4)*: 389-402, 2007. PMID: 18371377. DOI: 10.1016/j.stem.2007.08.001

50 Todaro M, Perez Alea M, Scopelliti A, Medema JP and Stassi G: IL-4-mediated drug resistance in colon cancer stem cells. Cell Cycle *7(3)*: 309-313, 2008. PMID: 18235245. DOI: 10.4161/cc.7.3.5389

51 Ishiguro T, Ohata H, Sato A, Yamawaki K, Enomoto T and Okamoto K: Tumor-derived spheroids: Relevance to cancer stem cells and clinical applications. Cancer Sci *108(3)*: 283-289, 2017. PMID: 28064442. DOI: 10.1111/cas.13155

52 Raghavan S, Ward MR, Rowley KR, Wold RM, Takayama S, Buckanovich RJ and Mehta G: Formation of stable small cell number three-dimensional ovarian cancer spheroids using hanging drop arrays

for preclinical drug sensitivity assays. Gynecol Oncol *138(1)*: 181-189, 2015. PMID: 25913133. DOI: 10.1016/j.ygyno.2015.04.014

53 Stadler M, Scherzer M, Walter S, Holzner S, Pudelko K, Riedl A, Unger C, Kramer N, Weil B, Neesen J, Hengstschläger M and Dolznig H: Exclusion from spheroid formation identifies loss of essential cell-cell adhesion molecules in colon cancer cells. Sci Rep *8(1)*: 1151, 2018. PMID: 29348601. DOI: 10.1038/s41598-018-19384-0

54 Luca AC, Mersch S, Deenen R, Schmidt S, Messner I, Schäfer KL, Baldus SE, Huckenbeck W, Piekorz RP, Knoefel WT, Krieg A and Stoecklein NH: Impact of the 3D microenvironment on phenotype, gene expression, and EGFR inhibition of colorectal cancer cell lines. PLoS One *8(3)*: e59689, 2013. PMID: 23555746. DOI: 10.1371/journal.pone.0059689

55 Tsuji N, Matsuura T, Narama I, Yoshiki A and Ozaki K: Macrophage-associated gelatinase degrades basement membrane at the optic fissure margins during normal ocular development in mice. Invest Ophthalmol Vis Sci *59(3)*: 1368-1373, 2018. PMID: 29625460. DOI: 10.1167/iovs.17-21841

56 Leppert D, Waubant E, Galardy R, Bunnett NW and Hauser SL: T cell gelatinases mediate basement membrane transmigration *in vitro*. J Immunol *154(9)*: 4379-4389, 1995. PMID: 7722295.

57 Frederiks WM and Mook OR: Metabolic mapping of proteinase activity with emphasis on *in situ* zymography of gelatinases: review and protocols. J Histochem Cytochem *52(6)*: 711-722, 2004. PMID: 15150280. DOI: 10.1369/jhc.4R6251.2004

58 Drost J and Clevers H: Organoids in cancer research. Nat Rev Cancer *18(7)*: 407-418, 2018. PMID: 29692415. DOI: 10.1038/s41568-018-0007-6

59 Kenny HA, Krausz T, Yamada SD and Lengyel E: Use of a novel 3D culture model to elucidate the role of mesothelial cells, fibroblasts and extra-cellular matrices on adhesion and invasion of ovarian cancer cells to the omentum. Int J Cancer *121(7)*: 1463-1472, 2007. PMID: 17546601. DOI: 10.1002/ijc.22874

60 Buhrmann C, Kraehe P, Lueders C, Shayan P, Goel A and Shakibaei M: Curcumin suppresses crosstalk between colon cancer stem cells and stromal fibroblasts in the tumor microenvironment: potential role of EMT. PLoS One *9(9)*: e107514, 2014. PMID: 25238234. DOI: 10.1371/journal.pone.0107514

61 Birgersdotter A, Sandberg R and Ernberg I: Gene expression perturbation *in vitro* – a growing case for three-dimensional (3D) culture systems. Semin Cancer Biol *15(5)*: 405-412, 2005. PMID: 16055341. DOI: 10.1016/j.semcancer.2005.06.009

62 Lauschke VM, Hendriks DF, Bell CC, Andersson TB and Ingelman-Sundberg M: Novel 3D culture systems for studies of human liver function and assessments of the hepatotoxicity of drugs and drug candidates. Chem Res Toxicol *29(12)*: 1936-1955, 2016. PMID: 27661221. DOI: 10.1021/acs.chemrestox.6b00150

63 Youhanna S and Lauschke VM: The past, present and future of intestinal *in vitro* cell systems for drug absorption studies. J Pharm Sci *110(1)*: 50-65, 2021. PMID: 32628951. DOI: 10.1016/j.xphs.2020.07.001

64 Macedo MH, Martínez E, Barrias CC and Sarmento B: Development of an improved 3D *in vitro* intestinal model to perform permeability studies of paracellular compounds. Front Bioeng Biotechnol *8*: 524018, 2020. PMID: 33042961. DOI: 10.3389/fbioe.2020.524018

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