JSHC
Japan Society of Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 57 (5): 149–155, 2024
doi: 10.1267/ahc.24-00043

Regular Article

Three-Dimensional Culture of Glioblastoma Cells Using a Tissueoid Cell Culture System

Natsume Okamoto[1], Naoko Taniura[1,2], Takahisa Nakayama[1], Eri Tanaka[1], Yusuke Kageyama[1], Mai Noujima[1], Ryoji Kushima[1] and Ken-ichi Mukaisho[2]

[1]Division of Human Pathology, Department of Pathology, Shiga University of Medical Science, Otsu, Japan and [2]Education Center for Medicine and Nursing, Shiga University of Medical Science, Otsu, Japan

Received August 6, 2024; accepted August 12, 2024; published online October 12, 2024

In classical cell culture techniques, cancer cells typically proliferate in a single layer by adhering to the undersurface of laboratory vessels. Consequently, concerns have been raised regarding the fidelity of the morphological and functional characteristics of these cultured cancer cells compared to those of their *in vivo* counterparts. Our previous studies have investigated various epithelial malignant tumors utilizing the Tissueoid cell culture system, a three-dimensional (3D) cultivation method employing Cellbed—a nonwoven sheet composed of high-purity silica fibers as a scaffold. In this investigation, we have achieved successful 3D culturing of glioblastoma cells (A172 and T98G), which are non-epithelial in nature. As such our focus is to juxtapose their morphological features against that of those cultivated via conventional two-dimensional (2D) methods. Our findings will be elucidated using immunostaining, immunofluorescence staining, and scanning electron microscopy, substantiated with accompanying imaging. Notably, cells cultured in the 3D environment exhibited distinct morphological attributes compared to those of their 2D counterparts, notably featuring pronounced cellular protrusions. We envisage the continued utilization of the 3D culture platform to facilitate diverse avenues of research, encompassing the exploration of novel therapeutic modalities for glioblastoma cells and beyond.

Key words: glioblastoma, three dimensional culture, cellular protrusion

I. Introduction

The occurrence rate of primary malignant brain tumors stands at approximately 7 per 100,000 individuals, with glioblastoma constituting roughly 49% of these cases. Most patients die of progressive illness [24]. This subtype represents the most formidable manifestation of glioma, contributing to 16% of all primary neoplasms affecting the brain and central nervous system [26]. Glioblastoma epitomizes the pinnacle of malignancy among central nervous system neoplasms, necessitating a multidisciplinary therapeutic approach encompassing surgical intervention, radiotherapy, and chemotherapy. Glioblastoma and other high-grade astrocytomas are among the most common and lethal brain tumors, with 5-year survival rates hovering below 5% despite decades of genomic analysis [12]. Glioblastoma, has an extremely poor prognosis, usually resulting in death within 2 years [13]. Consequently, there persists an imperative to advance novel treatment modalities, given the stagnant progress in this domain. While recent strides, such as the advent of temozolomide and bevacizumab, have marginally enhanced patient survival rates, the overall prognosis remains dismal [5, 14, 31].

It has gradually become clear that mammalian cells grown as conventional two-dimensional (2D) monolayer cultures on rigid and treated surfaces could show different behavior and functional properties from those in the native microenvironment [11, 23, 27, 28]. The predominant focus of cancer research employing *in vitro* experimental models has traditionally centered on 2D culture systems. However, the disparity between the 2D culture milieu—typically characterized by rigid tissue culture plates composed of

Correspondence to: Ken-ichi Mukaisho, Education Center for Medicine and Nursing, Shiga University of Medical Science, Seta-tsukinowa-cho, Otsu, Shiga 520–2192, Japan. E-mail: mukaisho@belle.shiga-med.ac.jp

© 2024 The Japan Society of Histochemistry and Cytochemistry

polystyrene or glass—and the complex *in vivo* microenvironment in humans is substantial [3, 7, 9, 32]. Consequently, the translational fidelity of experimental findings derived from 2D cultures to physiological outcomes *in vivo* may be compromised [2]. When cells procured from tissues are subjected to 2D culture conditions, a gradual phenotypic transition is observed, characterized by cell flattening, aberrant proliferation, and loss of cellular differentiation [20, 30]. In this context, three-dimensional (3D) culture methodologies, distinguished by their capacity to integrate heterogeneous cell populations and emulate the intricate tumor microenvironment *in vivo*, have garnered escalating interest.

We have implemented the Tissueoid cell culture system, a novel 3D cultivation approach employing high-purity nonwoven silica fiber sheets (Cellbed®). Through this methodology, we have effectively demonstrated 3D culturing of diverse epithelial malignant tumors, encompassing tongue, bladder, and gastrointestinal cancer cells [8, 15, 16]. Notably, Cellbed, serving as the foundational scaffold within this system, closely mimics the architecture of loose connective tissue found *in vivo* [18]. This unique framework facilitates the unimpeded movement of epithelial malignant tumor cells within a highly porous Cellbed, allowing for the manifestation of distinct 3D structures characteristic of each cell type. In the present investigation, we present our findings utilizing glioblastoma cells, which are reputed to exhibit numerous cellular protrusions in their native *in vivo* environment, in contrast to that by epithelial malignant tumor cells.

II. Materials and Methods

Cell lines

Two glioblastoma cell lines, A172 (JCRB0228 lot:12192018) and T98G (JCRB9041 lot:10112011), were purchased from JCRB Cell Bank (Tokyo, Japan). The medium used for A172 was Dulbecco's Modified Eagle Medium (Low Glucose) (Nacalai Tesque Inc., Kyoto, Japan), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, USA) and ABAM (Sigma-Aldrich, St Louis, USA). For T98G, MEM (Nacalai Tesque Inc, Kyoto, Japan) supplemented with ABAM (same as the above-mentioned), 1 mmol/L Sodium pyruvate (Nacalai Tesque Inc, Kyoto, Japan), and 10% FBS (CORNING, NY, USA) was used as a culture medium.

Cell culture

Both 2D and 3D cultures were conducted at 37°C under a 5% CO_2 atmosphere. In our experimentation, Cellbed served as the scaffold for the 3D culture system. For 2D cell culture, cells were seeded onto 18-mm coverslips and cultured for a duration of 1 week. In the 3D cell culture methodology, a circular Cellbed with a diameter of 19 mm was utilized, with 1 mL of cell suspension added onto a 12-well plate containing an additional 1 mL of medium. The culture medium was replenished every 3 days, and the size of the culture vessel was sequentially increased to a 35-mm dish after the initial week and a 60-mm dish after 2 weeks. Notably, commencing from the first medium change post-seeding, the culture vessel underwent gentle rotation at a low speed of 20 revolutions per minute using a cell shaker (High Humidity-resistant Shaker CS-LR, Taitec Corporation, Koshigaya-city, Japan) (see Fig. 1). For more information, T98G started rotation on day 3 (the first medium change). A172 was cultivated without moving for 4 days, and rotation started. This rotational motion served to prevent the accumulation of medium within the Cellbed, a dense scaffold, thereby mitigating the risk of nutrient deprivation and consequent cell death. By ensuring continuous medium circulation, this approach safeguards against medium stagnation, which is detrimental to cell viability, while maintaining optimal conditions for 3D culture, which necessitates a substantial volume of medium consumption.

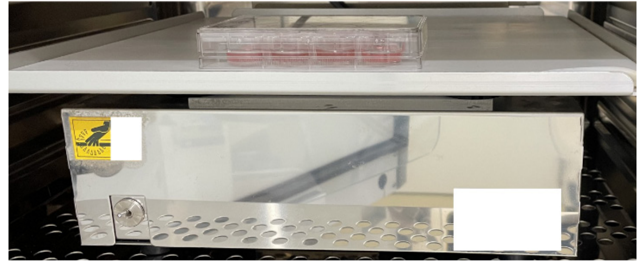

Fig. 1. Cell shaker. Despite the modest rotational speed (20 revolutions per min), employing a bioshaker to rotate the culture container serves the purpose of averting stagnation of the culture medium both within and surrounding the Cellbed, thereby mitigating cellular death.

Hematoxylin and eosin (HE) staining

Cells (T98G: 1×10^5 cells per mL; A172: 1×10^5 cells per mL) were seeded into a 12-well plate. Following a cultivation period of 21 days, cells underwent washing with phosphate-buffered saline (PBS) and subsequent fixation with 10% formalin in PBS for a duration of 30 min. Following two additional washes with PBS, the Cellbed was affixed onto a specialized holder and subjected to staining with HE (refer to Fig. 2). Subsequently, histological examination was performed utilizing a light microscope, and images were captured for analysis.

For 2D cell culture, cells were seeded on 18-mm cover glasses, cultured for 1 week, and then washed with PBS. After fixing with 10% formalin containing PBS for 30 min, HE staining was performed, and the samples were observed and imaged using a light microscope.

Immunostaining

A172 and T98G cells, cultured in 3D within Cellbed for a duration of 2 weeks, underwent embedding in paraffin. Subsequently, serial sections with a thickness of 6 micrometers were horizontally sliced and subjected to his-

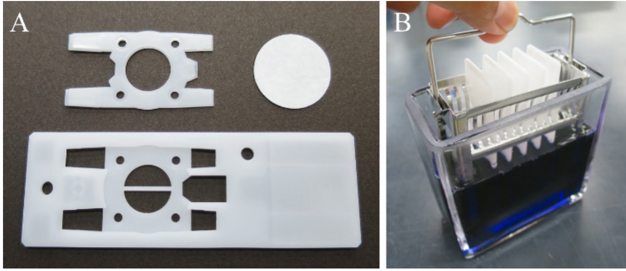

Fig. 2. Staining holder exclusively for Cellbed. A specialized staining holder, designed akin to a microscope slide in shape (**A**), facilitates the fixation of the Cellbed, rendering it more manageable. This configuration enables simultaneous staining of multiple Cellbeds, streamlining the process (**B**).

tological examination via immunostaining. The primary antibodies employed included an anti-GFAP mouse monoclonal antibody sourced from Proteintech Japan (Tokyo, Japan) and an anti-Ki67 rabbit monoclonal antibody procured from Roche Diagnostics GmbH (Mannheim, Germany). Immunostaining procedures were conducted utilizing the automated immunostaining apparatus, VENTANA (Discovery-ULTRA) by Roche (Tokyo, Japan).

Fluorescent staining

Cells (A172: 2×10^4/ml, T98G: 1×10^3 cells/ml) were seeded into 12-well plates and subjected to 3D culture for a duration of 3 weeks. Following culture, cells were fixed utilizing 4% paraformaldehyde phosphate buffer solution (Nacalai Tezue Inc. Kyoto, Japan) for 30 min. Subsequent steps included membrane permeabilization and blocking, conducted for 1 hr using a solution comprising PBS containing 0.1% Tween 20 (Nacalai Teszue Inc. Kyoto, Japan) and 10% normal goat serum (Nichirei Biosciences, Tokyo, Japan). The primary antibody utilized was an anti-GFAP mouse monoclonal antibody sourced from Proteintech Japan, with incubation performed overnight at 4°C. Following primary antibody incubation, cells were subjected to an 1-hr incubation period at room temperature with secondary antibodies. Two secondary antibodies were employed: Alexa Fluor 488 goat anti-mouse IgG (Thermo Fisher Scientific, Massachusetts, USA) and Rhodamine phalloidin (Invitrogen, Oregon, USA). Additionally, cell nuclei were counterstained with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride), sourced from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The mounting medium used for microscopy was ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific, Massachusetts, USA). Visualization of cells was achieved using a confocal laser scanning microscope (FV-1000D IX-81; Olympus, Tokyo, JAPAN).

Scanning electron microscopy

A172 cells, cultured as previously described for 3 weeks within a 19-mm diameter circular Cellbed, were utilized for subsequent analysis. Upon completion of the culture period, the cells were transferred to a 10-cm dish for further experimentation. Samples were prepared for imaging by Tokai Electron Microscope Co., Ltd. (Nagoya, JAPAN) following the detailed experimental protocol below. Cells were initially fixed using a solution containing 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer (PB; pH 7.4). Following initial fixation, cells underwent additional fixation with 2% glutaraldehyde in 0.1 mol/L PB at 4°C overnight. Cells were further treated with 2% osmium tetroxide in 0.1 mol/L PB at 4°C for 90 min. The cells were then subjected to propylene oxide (PO) infiltration twice for 20 min each. Subsequently, the cells were immersed in a 70:30 mixture of PO and resin (Quetol-812; Nissin EM, Tokyo, Japan) for 1 hr followed by overnight volatilization of PO with the tube cap open. Polymerized resin was sectioned into 70-nm ultrathin sections with diamond knife using an ultramicrotome (Ultracut UCT; Leica, Vienna, Austria), and the sections were mounted onto copper grids. The resin-embedded sections were stained with 2% uranyl acetate for 15 min at room temperature, followed by secondary staining with lead staining solution (Sigma-Aldrich, Tokyo, JAPAN). Grids were observed utilizing a transmission electron microscope (JEM-1400Plus; JERO Ltd., Tokyo, Japan) operating at an accelerating voltage of 80 kV.

III. Results

HE staining

Figure 3 illustrates HE staining images comparing 2D and 3D cultured cells of A172 and T98G cell lines. In the 2D culture condition, A172 and T98G cells exhibited a rounded morphology or short cellular protrusions. Conversely, in the 3D culture environment, both A172 and T98G cells displayed numerous elongated cellular protrusions, indicative of morphological changes induced by the 3D culture setting (Fig. 3).

Immunohistochemical staining

Both A172 and T98G were positive for GFAP, specifically expressed in astroglial cells, and Ki67 was positive in the nucleus of cells with proliferation potential. Figure 4 shows the results of immunohistochemical staining using an anti-GFAP antibody for a 3D culture of A172 (Fig. 4A) and an anti-Ki67 antibody for T98G cells (Fig. 4B).

Immunofluorescence staining

Figure 5 illustrates the outcomes of immunofluorescent staining conducted on A172 and T98G cells following 3D culture. In both instances, GFAP staining demonstrated cytoplasmic distribution across all cells, confirming its presence (Fig. 5B, F). Furthermore, phalloidin staining revealed robust cytoplasmic and marginal localization, indicating abundant actin content within the cells' protrusions (Fig. 5C, G).

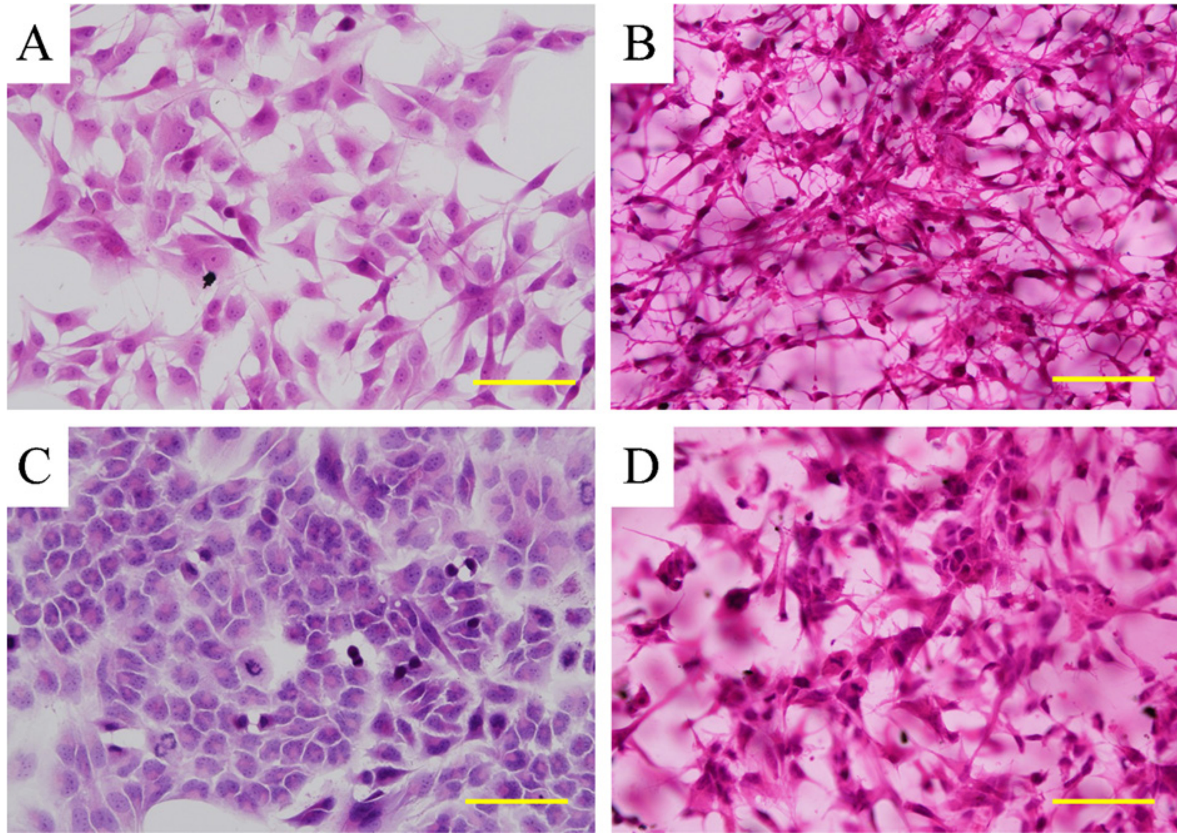

Fig. 3. Morphology of glioblastoma cells. **A**: 2D culture of A172 cells. **B**: 3D culture of A172 cells. **C**: 2D culture of T98G cells. **D**: 3D culture of T98G cells. Cells cultured on cover glasses in a 2D configuration for a duration of 7 days predominantly exhibited rounded morphology or only displayed short cellular protrusions. In contrast, cells cultured on Cellbed in a 3D setting consistently demonstrated elongated cellular protrusions. This observation was confirmed through hematoxylin and eosin (HE) staining, with a scale bar provided for reference (Bars = 100 μm).

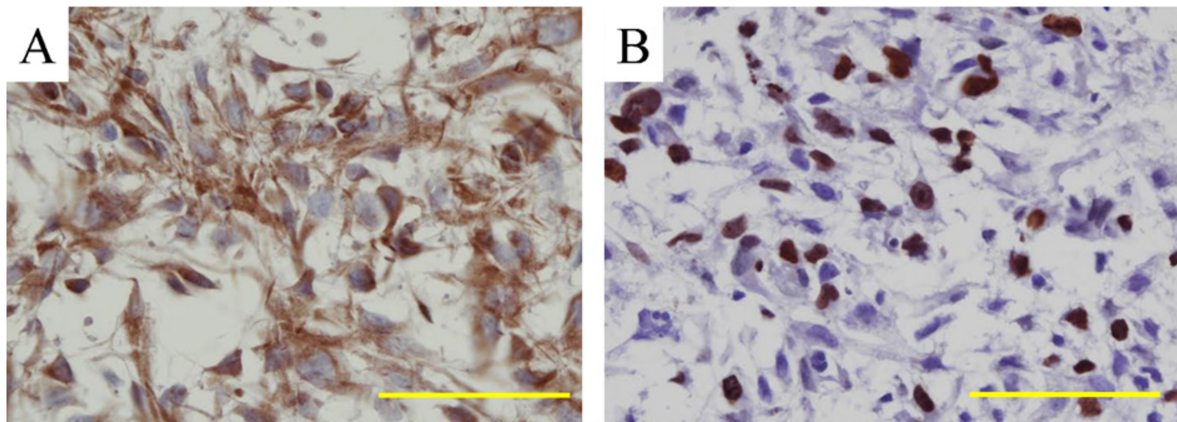

Fig. 4. Immunostaining of glioblastoma cells. **A**: GFAP staining of A172. **B**: Ki67 staining of T98G. Given that the color development involves DAB, cells expressing positivity are discernibly stained brown. Notably, T98G and A172 cell lines exhibited positivity in the protruding regions of cells, as evidenced by GFAP staining, signifying the presence of intermediate filaments. Additionally, strong positivity for Ki67 was observed in numerous nuclei. These findings were confirmed through immunohistochemical analysis, with a scale bar provided for measurement (Bars = 100 μm).

Scanning electron microscopy

A172 cells cultured in 3D for approximately 3 weeks were observed using a scanning electron microscope. It was confirmed that glioblastoma cells extended protrusions along the fibers of the Cellbed (Fig. 6).

IV. Discussion

Employing a tissueoid cell culture system, we expanded our investigations beyond culture of epithelial cancer cells, which have been previously reported, to

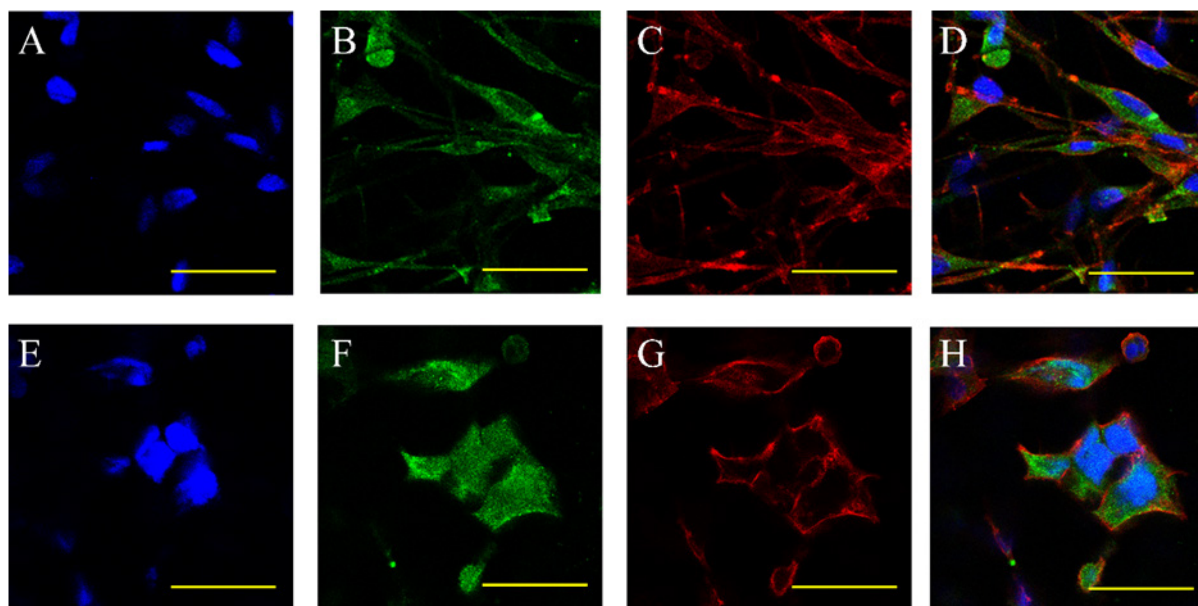

Fig. 5. Immunofluorescent staining of glioblastoma cells. **A**–**D**: A172. **E**–**H**: T98G. A scale bar provided for measurement (Bars = 50 μm). **A** and **E**: DAPI, **B** and **F**: GFAP, **C** and **G**: phalloidin, **D** and **H**: Merge. DAPI staining appears in blue, GFAP in green, and phalloidin in red. Across all cells, cellular protrusions were evident, with phalloidin concentrated at the boundary between the cytoplasm and these protrusions.

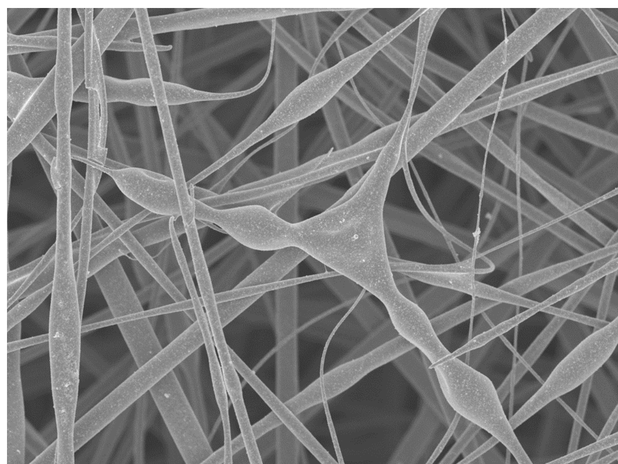

Fig. 6. Scanning electron microscopy image of A172 cells. A172 cells are seen extending cellular protrusions along the silica fibers.

include glioblastoma cells using a methodology akin to 2D culture. Consequently, we observed glioblastoma cells manifesting elongated cellular protrusions reminiscent of those observed in living organisms, a feature not discernible under 2D culture conditions.

The conventional approach in cancer research has long relied on 2D culture systems for *in vitro* cell studies. However, evidence suggests that such systems inadequately recapitulate the intricate architecture and dynamic interactions observed within 3D tissues *in vivo*, including the complex interplay between tumor cells and the tumor microenvironment [1, 10, 33]. Conversely, 3D culture systems are purported to offer a more faithful representation of *in vivo* tumors, replicating their structural, metabolic, histopathological, genetic, and molecular attributes, as well as their responsiveness to therapeutic agents [6]. Notably, the 3D culture environment facilitates the formation of complex cellular structures, with microfiber scaffolds recognized for their capacity to induce cellular differentiation, proliferation, tissue organization, and *in vivo*-like metabolic activities in neural stem cells and various tumor models [4, 21, 25]. In our experiment, we observed notable differences in the morphology of glioblastoma cells between 2D and 3D cultures. Consequently, it is conceivable that 3D culture systems may bridge the gap between conventional 2D cultures and *in vivo* tumor models, rendering the experimental model more representative of actual tumor tissues [29]. Moving forward, there is anticipation that the establishment of experimental systems utilizing 3D culture methodologies will yield enhanced predictive capabilities for drug responses in patients, thereby offering valuable insights for therapeutic development.

In our prior investigations, we have documented that the morphological and metabolic characteristics of tongue squamous cell carcinoma cells exhibit disparities when cultured in both 2D and 3D settings [17]. The establishment of *in vivo* animal models poses considerable challenges due to factors such as cost, ethical considerations, and the logistical complexities associated with monitoring tumor growth and conducting drug screening [22]. Furthermore, the process of drug development, spanning from clinical trials to market availability, demands substantial investments of time and resources, with statistics indicating that at least 92% of new drug candidates fail to progress beyond clinical trials [19]. Notably, cancer metabolism displays vari-

ances between 2D and 3D cultures, and should 3D cultured cells demonstrate metabolic profiles akin to those observed in living organisms, the adoption of 3D culture methodologies could serve as an invaluable preliminary step prior to embarking on animal experiments. Additionally, conventional 3D culture models necessitate the incorporation of specific cellular constituents, such as fibroblasts and immune cells, to accurately model the intricate architecture of the tumor microenvironment [1]. Our Tissueoid cell culture system relies on a Cellbed scaffold, yet it can be seamlessly executed utilizing the same culture medium as that of 2D systems. Moreover, by supplementing the Cellbed with appropriate extracellular matrix (ECM) components such as collagen and conducting culture, it becomes feasible to evaluate the efficacy of the supplemented ECM [16]. Furthermore, a diverse array of staining techniques can be applied post-culture, and since no additional substrate beyond Cellbed is necessitated, proteins can be extracted solely from cells, facilitating downstream experiments such as Western blotting. Leveraging this 3D culture system, we previously demonstrated the suppression of tongue squamous cell carcinoma invasion by an ERK activity inhibitor [18]. By extending similar investigations to glioblastoma utilizing the Tissueoid cell culture system, which offers operational simplicity akin to 2D cultures, we anticipate advancing research endeavors toward the development of novel therapeutics.

To summarize, although glioblastoma represents a formidable challenge in oncology with limited treatment options, the development of a novel experimental system for 3D culture of glioblastoma cells utilizing the Tissueoid cell culture system presents a promising avenue for conducting diverse cancer research endeavors. Specifically, this system offers opportunities to delve into cancer metabolism, investigate drug responses, and facilitate screening for effective therapeutic agents. Such advancements hold potential for enhancing our understanding of the glioblastoma biology and ultimately improving treatment outcomes for this devastating disease.

V. Conflicts of Interest

This research was done in collaboration with Japan Vilene Company, Ltd. The company provided Cellbed and information on Cellbed materials.

VI. Acknowledgments

We thank Japan Viline Co., Ltd., which supplied Cellbed material. We also thank Mr. Takuya Iwasa and Mr Masaaki Kawabe (Japan Vilene Co., Ltd.), who provided information regarding the Cellbed characteristics. We thank Mr. Michiharu Nasu, who prepared paraffin sections and performed immunohistochemistry.

VII. References

1. Atat, O. E., Farzaneh, Z., Pourhamzeh, M., Taki, F., Abi-Habib, R., Vosough, M., *et al.* (2022) 3D modeling in cancer studies. *Hum. Cell* 35; 23–36.
2. Baker, B. M. and Chen, C. S. (2012) Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. *J. Cell Sci.* 125; 3015–3024.
3. Branco da Cunha, C., Koshy, S. T., Weaver, J. C., Chaudhuri, O., Seruca, R., *et al.* (2016) CD44 alternative splicing in gastric cancer cells is regulated by culture dimensionality and matrix stiffness. *Biomaterials* 98; 152–162.
4. Christopherson, G. T., Song, H. and Mao, H. Q. (2009) The influence of fiber diameter of electrospun substrates on neural stem cell differentiation and proliferation. *Biomaterials* 30; 556–564.
5. Cohen, M. H., Johnson, J. R. and Pazdur, R. (2005) Food and Drug Administration Drug approval summary: temozolomide plus radiation therapy for the treatment of newly diagnosed glioblastoma multiforme. *Clin. Cancer Res.* 11; 6767–6771.
6. Dutta, D., Heo, I. and Clevers, H. (2017) Disease Modeling in Stem Cell-Derived 3D Organoid Systems. *Trends Mol. Med.* 23; 393–410.
7. Friedl, P., Sahai, E., Weiss, S. and Yamada, K. M. (2012) New dimensions in cell migration. *Nat. Rev. Mol. Cell Biol.* 13; 743–747.
8. Ikari, R., Mukaisho, K. I., Kageyama, S., Nagasawa, M., Kubota, S., Nakayama, T., *et al.* (2021) Differences in the central energy metabolism of cancer cells between conventional 2d and novel 3d culture systems. *Int. J. Mol. Sci.* 22; 1805.
9. Jaalouk, D. E. and Lammerding, J. (2009) Mechanotransduction gone awry. *Nat. Rev. Mol. Cell Biol.* 10; 63–73.
10. Kyriakopoulou, K., Koutsakis, C., Piperigkou, Z. and Karamanos, N. K. (2023) Recreating the extracellular matrix: novel 3D cell culture platforms in cancer research. *FEBS J.* 290; 5238–5247.
11. Lane, A. N., Higashi, R. M. and Fan, T. W.-M. (2016) Preclinical models for interro-gating drug action in human cancers using Stable Isotope Resolved Metabolomics (SIRM). *Metabolomics* 12; 118.
12. Liu, J., Cao, S., Imbach, K. J., Gritsenko, M. A., Lih, T. M., Kyle, J. E., *et al.* (2024) Multi-scale signaling and tumor evolution in high-grade gliomas. *Cancer Cell.* 42; 1217–1238. e19.
13. Michiba, A., Shiogama, K., Tsukamoto, T., Hirayama, M., Yamada, S. and Abe, M. (2022) Morphologic Analysis of M2 Macrophage in Glioblastoma: Involvement of Macrophage Extracellular Traps (METs). *Acta Histochem. Cytochem.* 55; 111–118.
14. Mrugala, M. M. (2013) Advances and challenges in the treatment of glioblastoma: a clinician's perspective. *Discov. Med.* 15; 221–230.
15. Mukaisho, K., Yoshida, S., Noi, M. and Nakayama, T. (2017) Morphology of various cancer cells using the silicate fiber scaffold Cellbed in a novel three-dimensional cell culture system. *Gastroenterology.* 152; S352–S353.
16. Murakami, S., Mukaisho, K. I., Iwasa, T., Kawabe, M., Yoshida, S., Taniura, N., *et al.* (2020) Application of "Tissueoid Cell Culture System" Using a Silicate Fiber Scaffold for Cancer Research. *Pathobiology.* 87; 291–301.
17. Murakami, S., Tanaka, H., Nakayama, T., Taniura, N., Miyake, T., Tani, M., *et al.* (2021) Similarities and differences in metabolites of tongue cancer cells among two- and three-dimensional cultures and xenografts. *Cancer Sci.* 112; 918–931.
18. Noi, M., Mukaisho, K. I., Yoshida, S., Murakami, S.,

Koshinuma, S., Adachi, T., *et al.* (2018) ERK phosphorylation functions in invadopodia formation in tongue cancer cells in a novel silicate fibre-based 3D cell culture system. *Int. J. Oral Sci.* 10; 30.
19. Pampaloni, F., Stelzer, E. H. and Masotti, A. (2009) Three-dimensional tissue models for drug discovery and toxicology. *Recent Pat. Biotechnol.* 3; 103–117.
20. Petersen, O. W., Rønnov-Jessen, L., Howlett, A. R. and Bissell, M. J. (1992) Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proc. Natl. Acad. Sci. U S A* 89; 9064–9068.
21. Ranjan, V. D., Qiu, L., Lee, J. W., Chen, X., Jang, S. E., Chai, C., *et al.* (2020) A microfiber scaffold-based 3D: in vitro human neuronal culture model of Alzheimer's disease. *Biomater. Sci.* 8; 4861–4874.
22. Robinson, N. B., Krieger, K., Khan, F. M., Huffman, W., Chang, M., Naik, A., *et al.* (2019) The current state of animal models in research: A review. *Int. J. Surg.* 72; 9–13.
23. Sant, S. and Johnston, P. A. (2017) The production of 3D tumor spheroids forcancer drug discovery. *Drug Discov Today Technol.* 23; 27–36.
24. Schaff, L. R. and Mellinghoff, I. K. (2023) Glioblastoma and Other Primary Brain Malignancies in Adults: A Review. *JAMA* 329; 574–587.
25. Terada, E., Bamba, Y., Takagaki, M., Kawabata, S., Tachi, T., Nakamura, H., *et al.* (2023) Silicate Microfiber Scaffolds Support the Formation and Expansion of the Cortical Neuronal Layer of Cerebral Organoids With a Sheet-Like Configuration. *Stem Cells Transl. Med.* 12; 825–837.
26. Thakkar, J. P., Dolecek, T. A., Horbinski, C., Ostrom, Q. T., Lightner, D. D., Barnholtz-Sloan, J. S., *et al.* (2014) Epidemiologic and molecular prognostic review of glioblastoma. *Cancer Epidemiol. Biomarkers Prev.* 23; 1985–1996.
27. Tseng, H., Gage, J. A., Shen, T., Haisler, W. L., Neeley, S. K., Shiao, S., *et al.* (2015) A spheroid toxicity assay using magnetic 3D bioprinting and real-time mobile device-based imaging. *Sci Rep.* 5; 13987.
28. Tsukada, T., Kouki, T., Fujiwara, K., Ramadhani, D., Horiguchi, K., Kikuchi, M., *et al.* (2013) Reassembly of anterior pituitary organization by hanging drop three-dimensional cell culture. *Acta Histochem. Cytochem.* 46; 121–127.
29. Unnikrishnan, K., Thomas, L. V. and Ram Kumar, R. M. (2021) Advancement of Scaffold-Based 3D Cellular Models in Cancer Tissue Engineering: An Update. *Front. Oncol.* 11; 733652.
30. von der Mark, K., Gauss, V., von der Mark, H. and Müller, P. (1977) Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* 267; 531–532.
31. Wick, W., Gorlia, T., Bendszus, M., Taphoorn, M., Sahm, F., Harting, I., *et al.* (2017) Lomustine and Bevacizumab in Progressive Glioblastoma. *N. Engl. J. Med.* 377; 1954–1963.
32. Wirtz, D., Konstantopoulos, K. and Searson, P. C. (2011) The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nat. Rev. Cancer* 11; 512–522.
33. Zhang, L., Liao, W., Chen, S., Chen, Y., Cheng, P., Lu, X., *et al.* (2023) Towards a New 3Rs Era in the construction of 3D cell culture models simulating tumor microenvironment. *Front. Oncol.* 13; 1146477.

This is an open access article distributed under the Creative Commons Attribution-NonCommercial 4.0 International License (CC-BY-NC), which permits use, distribution and reproduction of the articles in any medium provided that the original work is properly cited and is not used for commercial purposes.