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METHODS ARTICLE

Generation of Homogenous Three-Dimensional Pancreatic Cancer Cell Spheroids Using an Improved Hanging Drop Technique

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In vitro characterization of tumor cell biology or of potential anticancer drugs is usually performed using tumor cell lines cultured as a monolayer. However, it has been previously shown that three-dimensional (3D) organization of the tumor cells is important to provide insights on tumor biology and transport of therapeutics. Several methods to create 3D tumors *in vitro* have been proposed, with hanging drop technique being the most simple and, thus, most frequently used. However, in many cell lines this method has failed to form the desired 3D tumor structures. The aim of this study was to design and test an easy-to-use and highly reproducible modification of the hanging drop method for tumor sphere formation by adding methylcellulose polymer. Most pancreatic cancer cells do not form cohesive and manageable spheres when the original hanging drop method is used, thus we investigated these cell lines for our modified hanging drop method. The spheroids produced by this improved technique were analyzed by histology, light microscopy, immunohistochemistry, and scanning electron microscopy. Results show that using the proposed simple method; we were able to produce uniform spheroids for all five of the tested human pancreatic cancer cell lines; Panc-1, BxPC-3, Capan-1, MiaPaCa-2, and AsPC-1. We believe that this method can be used as a reliable and reproducible technique to make 3D cancer spheroids for use in tumor biology research and evaluation of therapeutic responses, and for the development of bio-artificial tissues.

Introduction

Currently, most of the research in the field of cancer cell biology is performed on cells grown in monolayers. While studies performed on monolayers formed the basis of our understanding of cell biology, accumulating evidence in the literature has expounded the merits of utilizing three-dimensional (3D) rather than two-dimensional (2D) *in vitro* models, to more precisely replicate the biology and physicochemical features of the tumor microenvironment.[1–3] Compared to 2D-monolayers, 3D-cultures more completely recapitulate *in vivo* cancer cell–cell interactions, including the distribution of nutrients and exogenous therapeutics within the tumor stroma. Thus, the importance of 3D tumor cell cultures in the study of cancer pathogenesis, evaluation of drug efficacy, and metastatic behavior is increasingly recognized by the biomedical community.[1,4–9]

To accurately predict the efficacy of new drugs for cancer therapy it is essential to develop cellular models that better mimic physiologic conditions within the tumor microenvironment. Malignant cells in neoplastic tissue, like healthy cells in normal tissue, are organized in 3D networks displaying nutrient and signal gradients, and complex cell–cell and cell–extracellular matrix (ECM) contact interactions.[10–12] Thus, cellular functions and responses that occur in tissues are often lost in conventional 2D cell cultures, limiting the predictive capability of screening assays. Cells in a 3D conformation, on the other hand, exhibit numerous biological differences compared with 2D-monolayers that greatly influence how cells respond to therapeutics.[6,13–15] Second, monolayers do not pose the barrier to drug penetration or provide many of the microenvironmental influences found in solid tumors and 3D cultures.[16]

Spheroid formation is one of the best-characterized models for 3D cell culture due to its similarity to physiological tissues.[6,17] Spheroids are self-assembled clusters of cell colonies cultured in microenvironments where cell–cell interactions dominate over cell–substrate interactions. The concentric arrangement and growth pattern of heterogeneous cell populations in spheroids mimic initial, avascular stages of solid tumors

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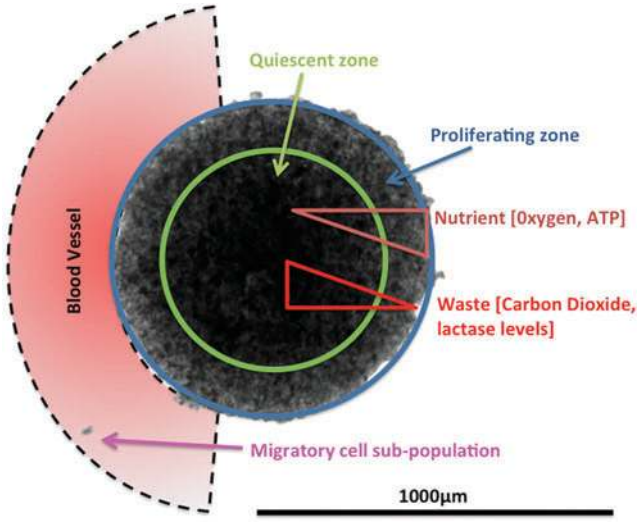

FIG. 1. A schematic presentation of 3D spheroid micro-regions and nutrient and waste gradients. 3D, three dimensional. Color images available online at www.liebertpub.com/tec

in vivo, not-yet vascularized micro-metastatic foci, and inter-capillary tumor micro-regions in which there is highly proliferative activity close to the capillaries, hypoxic and necrotic regions far from the capillaries, and quiescent cells in between[12,18] (Fig. 1). Differentiation in spheroid culture includes not only conservation of morphogenic capacities and histotypic reorganization but also maintenance of functional activities and gene expression patterns.[19–24] Traditional methods for tumor spheroid generation include hanging drop,[25] liquid overlaying, spinner flasks,[26] rotary cell culture systems,[27] poly-2-hydroxyethyl methacrylate, low binding plates,[28] gel/matrix based culture,[29,30] microencapsulation,[31] polymeric scaffolds,[30,32] and micropatterned plates.[8,33–35] However, these methods are frequently associated with poor reproducibility, scalability, adaptability, and lack high-throughput formats amendable to automation and drug screening. Furthermore, undesirable effects on the cells due to physical forces (spinner flasks, rotary cell culture systems), chemicals (coating substrates such as agarose or agar), or the multi-constituent nature of commonly used scaffolds, such as Matrigel™, cannot be excluded.[36] For instance, Matrigel is animal derived and may contain endogenous growth factors and signals that are poorly characterized and do not represent the human tumor environment. Different techniques for spheroid culturing have been extensively described in studies, including their advantages and disadvantages.[5,6,37] Therefore, 3D spheroid assays are not routinely incorporated in cancer drug development studies.

The growth of spheroids utilizing the hanging drop method is well established[38] and described in detail in the literature. The characterization and properties of methylcelluloses are well understood and have been recently reviewed by Nasatto *et al.*[39] and used in several biomedical applications.[40–43] In general, methylcellulose is a nonionic polymer, which can be mixed with other polymers, salts, and different ingredients. These polymers are enzyme resistant, nontoxic for humans, and are not cell permeable. Aqueous methylcellulose solutions are good thickening agents of which the gel matrix-forming behavior may be controlled by the polymer concentration and molecular weight.[39] Hence, a methylcellulose constituent added to cell media provides cells with a semi solid matrix, which better mimics the extracellular geometry *in vivo* and allows 3D growth support. Methylcellulose has also previously been shown to protect suspended cells from shear stresses,[44] which is valuable when processing spheroids for various microscopic analyses.

The aim of this study was to design and test a modified, highly reproducible and easy to perform hanging drop method for tumor sphere formation based on the addition of methylcellulose. We based this study on pancreatic adenocarcinoma cell lines (PDAC), since, in ours and others experience, PDAC cells are difficult to culture into cohesive and manageable spheres when any of the traditional spheroid forming methods are used.[45] The proposed technique enables simultaneous, cost- and time-effective generation of numerous spheroids. The tumor spheroids are extremely uniform in nature and do not easily dissemble when handled, allowing increased reproducibility and scalability.

Methodology

Materials

To prepare media for spheroid formation, cell media specific to the particular cell line (as described in the section ''Cell lines'') was supplemented with 20% methylcellulose stock solution. For preparation of methylcellulose stock solution 6 g of autoclaved methylcellulose powder (M0512; Sigma-Aldrich) was dissolved in preheated 250 mL basal medium (60°C) for 20 min. Thereafter, 250 mL of medium (room temperature) containing double the amount of FBS for the particular cell line was added to a final volume of 500 mL and the whole solution was mixed overnight at 4°C. The final stock solution was aliquoted and cleared by centrifugation (5000 rpm for 2 h at room temperature). Only the clear, highly viscous supernatant was used for the spheroid formation, which was approximately 90–95% of the stock solution.

Cell lines

Five human pancreatic cancer cell lines, Panc-1, AsPc-1, BxPC-3, Capan-1, and MIA PaCa-2 cells were obtained from American Type Culture Collection (ATCC) (Table 1).

Table 1. Donor Patient Information and Cell Line Characteristics

Cell line	*Source*	*Derivation*	*Metastasis*	*Proliferation*	*Differentiation*	*Ref.*
Panc-1	56 y.o, female	Primary tumor	Yes	52 h	Poor	38
AsPc-1	62 y.o, female	Ascites	Yes	38–40 h	Poor	39
MIA PaCa-2	65 y.o, female	Primary tumor	Not described	40 h	Poor	40
Capan-1	40 y.o, male	Liver metastasis	Yes	Not described	Well	41
BxPc-3	61 y.o, male	Primary tumor	No	48–60 h	Moderate to poor	42

Panc-1 and AsPc-1 cells were maintained in DMEM with 10% FBS. BxPC-3 was maintained in RPMI-1640 medium with 10% FBS. Capan-1 was maintained in IMDM medium with L-4 mM glutamine and 20% serum. MIA PaCa-2 was maintained in DMEM medium with 10% FBS and 2.5% horse serum. About 2% penicillin-streptomycin solution was added to the media of all cell lines. All cells were cultured in 5% CO_2 at 37°C.

A modified and improved hanging drop method for 3D pancreatic cancer cell spheroids formation

Spheroids were created using a novel approach that combined two traditionally used techniques: the hanging drop technique and the use of methylcellulose in the medium. For spheroid generation we used 20% of the stock solution and 80% culture medium corresponding to final 0.24% methylcellulose. Twenty microliters drops of the 0.24% methylcellulose-culture medium solution containing 20,000 cells were pipetted onto the lid of 100 mm dishes and were inverted over dishes containing 10 mL phosphate buffer solution (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tec). Cells were counted using the Countess Automated Cell Counter according to the manufacturer's recommendations (Invitrogen). Hanging drop cultures were incubated under standard culture conditions (5% CO_2, at 37°C) for 7 days, which allowed adequate sedimentation time. The resultant cell aggregates were harvested by pipetting 10 mL of DMEM plus 10% FBS gently onto the lid where the hanging drop spheroids lay, which caused them to become suspended in the media. Each spheroid was gently caught by a sterile spatula and transferred to a well in a 12-well plate for treatment or imaging. To evaluate the effect of agitation, spheroids grown in the presence of methylcellulose where transferred to the corresponding media without methylcellulose. The spheroids were left to equilibrate for 2 h and the plates were gently manually agitated for 10 s (five agitations) with an amplitude of 3–5 cm.

The viscosity of each cell medium with and without methylcellulose was evaluated using Vibro Viscometer SV-10 (A&D Company LTd.).

Histological and immunohistochemical evaluation

Tumor spheroids were fixed in formalin, embedded in histogel™ (Thermo Scientific Richard-Allan Scientific), processed in paraffin blocks, and sectioned using standard techniques. Tumor spheroid slides were stained with hematoxylin and eosin (H&E), rabbit monoclonal antibody Ki67 (RM-9106-R7; Thermo Fisher Scientific) that stains positive for proliferating cells, rabbit polyclonal antibody hypoxia-inducible factor 1-alpha (HIF-1α, IW-PA1041; IHCWorld) for hypoxic regions, and rabbit polyclonal antibody cleaved poly-ADP ribose polymerase (PARP, PA5-16452; Thermo Fisher Scientific), which stains positively for apoptotic cells. Positive control staining regimens for Ki67 and PARP were performed on human tonsil tissue and HIF-α positive control was performed on breast cancer tissue and are included in the supplementary section.

Scanning electron microscopy

Spheroids were fixed by washing thrice with 0.1 M sodium cacodylate buffer (CDB) followed by incubation in 2.5% glutaraldehyde for 25 min at room temperature. Spheroids were washed thrice again in 0.1 M CDB and subjected to an ethanol series for dehydration. The spheroids were then incubated in 1:1 t-butanol:ethanol mixture for 5 min and mounted on carbon tape upon an SEM stub. Immediately before imaging the samples were sputter coated with 50% platinum 50% palladium at a thickness of 5 ± 0.2 nm to ensure good electrical conductivity.

Light microscopy

Brightfield imaging for analysis of size distribution and shape of spheroids was captured at the desired time points using an Nikon Eclipse TE2000-U microscope fitted with a Nikon digital sight DS-Fi1 video camera.

Generation of orthotopic PDAC tumors

Animal studies were performed in accordance with the guidelines of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals based on approved protocols by Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC). Orthotopic pancreatic tumors were grown in 6–7 week old athymic nude (FOXn1 nu) female mice (Harlan Sprague Dawley). Under sterile conditions an incision of ~ 2 cm was made in the left flank to expose the pancreas. Panc-1 or Capan-1 cells (1×10^6 in 40 μL of PBS) were injected into the pancreas. Abdominal wound was closed in two layers. Tumors were allowed to grow for 3 weeks before tumors were excised. The tumor weight was measured before fixing in 4% formaldehyde for histology preparation.

Results and Discussion

We first evaluated the formation of the spheroids using the proposed technique. The viscosity of the medium increased four to five-fold for all cell-culture media tested when methylcellulose was added (Table 2).

The 3D tumor spheroids were characterized via standard light microscopy, and electron microscopy. Figure 2A and B compare the difference in robustness to gentle agitation between spheroids produced using the method proposed and spheroids that have been created in the hanging drop method without the methylcellulose constituent added. As can be seen from the figure, in the absence of methylcellulose the spheroids are easily dismounted following a mild agitation. The variability in the optical density of the spheroids and their shape is high and the spheroids are generally significantly less dense and more irregular in the absence of methylcellulose.

We have tested commonly used pancreatic cancer cells and proved the technique proposed is capable of growing uniform and reproducible spheroids across these cell lines. Without methylcellulose, we failed to create unifrom

Table 2. Viscosity of Various Media With and Without Methylcellulose

Media	*Viscosity without MC (MPa (±0.1)*	*Viscosity with MC (MPa) (±0.1)*
RPMI	1.03	4.23
IMDM	1.03	5.30
DMEM	1.10	5.25

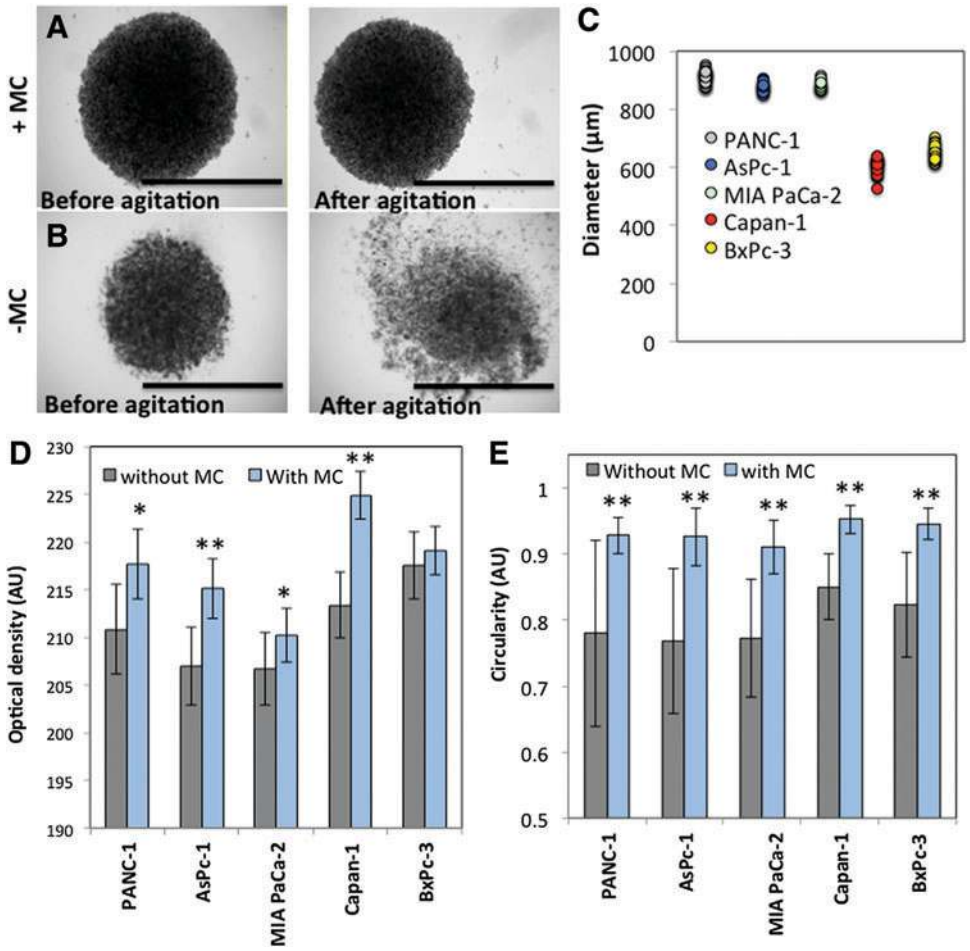

FIG. 2. Characterization of PDAC spheroids. Spheroids grown in hanging drop with 0.24% methylcellulose DMEM media are easily handled and highly reproducible. Effect of agitation on Panc-1 spheroids grown in hanging drop for 7 days produced with **(A)** 0.24% methylcellulose DMEM and **(B)** DMEM (Bar 1000 μm). **(C)** Diameter of spheroids grown in media + MC, **(D)** optical density and **(E)** circularity of spheroids grown with and without 0.24% MC hanging drop for 7 days ($n = 40$ per group, $*p < 0.01$ $**p < 0.05$). MC, methylcellulose; PDAC, pancreatic adenocarcinoma cell. Color images available online at www.liebertpub.com/tec

spheroids in all tested PDAC cell lines. It was previously reported that PDAC cells are difficult to cohere into spheroids capable of being used for high-throughput drug assays.[45] Data in Figure 2C and D show that the spheroids formed using the proposed method are highly reproducible. Light microscopy shows that these spheroids are uniform in shape, have smooth/defined boundaries, and are dense enough to be used for various assays (Fig. 2B).There was a difference in the characteristics of the spheroids between different cell lines, as can be expected from the different characteristics of cells. (Fig. 2C, D). Interestingly, BxPC-3 and Capan-1 showed similar morphology and growth pattern; both cell lines formed extremely dense spheroids that were approximately one-third to a half smaller in size than AsPc-1, Panc-1, and MIA PaCa-2 spheroids. After gentle agitation, these spheroids also avoided dissembly, whereas the spheroids without methylcellulsoe were prone to disaggregation. This means the proposed method yields spheroids that are more robust and can be easily handled during multistep processing that is commonly required for various imaging techniques and hisology analysis. Besides PDAC cell lines, the practicability of the techniques described herein have also been validated on the human hepatocellular cancer cell lines Hep G2, Hep 3B, and SNU 449 (Supplementary Fig. S2).

As can be seen from Figure 3, spheroids displayed a formation pattern that consisted of three phases: cell sedimentation, a size-dependent growth phase, and a plateau of size and density, which is consistent with literature regarding spheroids formed via other methods.[12,46] The observation of a plateau in growth after 5 days indicates that spheroid development is hampered by decay in media conditions, such as spontaneous degradation of glutamine, photo-degradation of some vitamins and amino acids, and increased osmolarity due to evaporation. Another possibility is a formation of hypoxic zones, which limit growth inside the dense spheroids as observed *in vivo*. It is possible to replenish the hanging drop with 10 μL of fresh methylcellulose-media solution between days 7 and 10 without losing the droplets on inversion if more mature spheroid cultures are desired. Alternatively, homogeneously sized spheroids can be harvested during the exponential phase before onset of hypoxia and necrosis, and then used in secondary culture. Both approaches lead to extended growth with no loss in homogeneity.

Brightfield light microscopy (Fig. 4A–E) was used to assess the morphology of all PDAC spheroids. Panc-1, AsPc-1, and MIA PaCa-2 spheroids displayed a similar phenotype and BxPc-3 and Capan-1 spheroids displayed a similar phenotype.

Spheroid robustness meant that they were able to survive numerous steps involved with SEM microscopy preparation. SEM micrographs were able to characterize, at a high resolution, the overall shape of spheroids from several PDAC cell lines and also highlight individual cell interactions on and near the surface of the tumor (Fig. 5). Panc-1 (Fig. 5A),

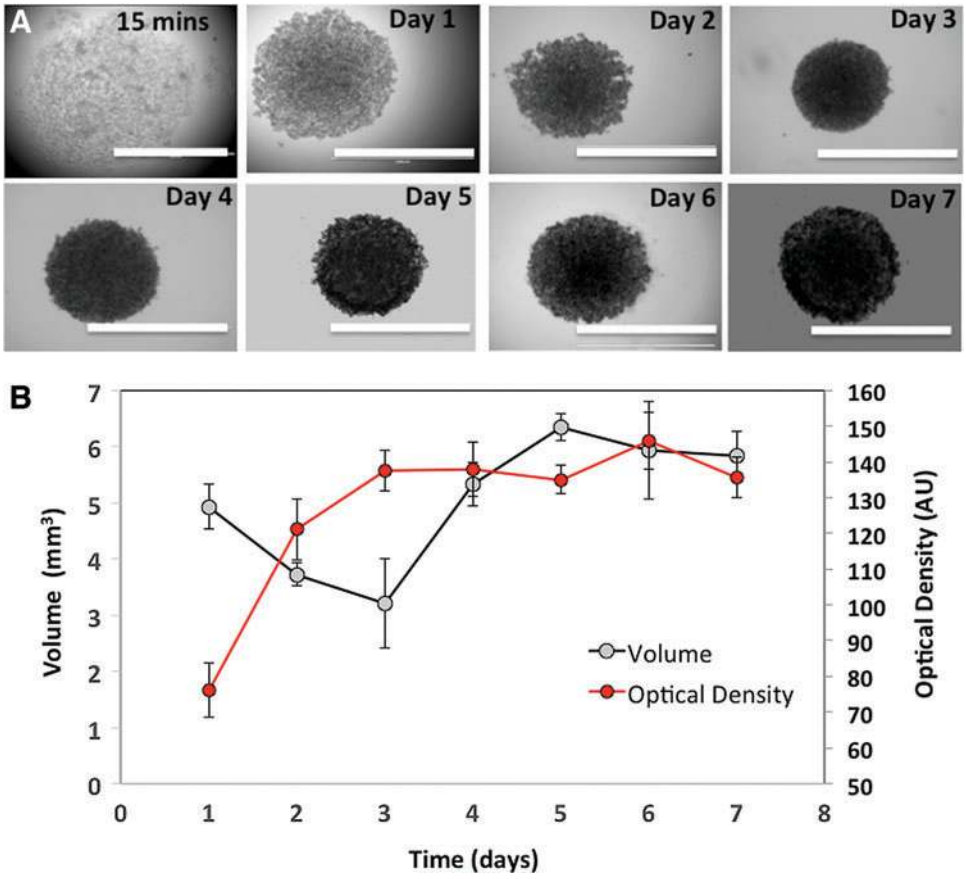

FIG. 3. Formation of Panc-1 spheroids in hanging drops seeded with 20,000 cells per 20 μL drop and cultivated in DMEM +10% FBS and 0.24% methylcellulose at 37°C. **(A)** Light micrographs showing the growth of Panc-1 spheroids. Scale bar 1000 μm **(B)** Phases of 3D tumor spheroid formation. Average spheroid volume and optical density 0–7 days after seeding in 0.24% methylcellulose hanging drop. Color images available online at www.liebertpub.com/tec

AsPc-1 (not shown), and MIA PaCa-2 (Fig. 5B) spheroids displayed a similar phenotype with a ruffled surface and lightly packed cells with deep pore-like structures. Cells on the surface of these spheroids were mostly spherical, except the cells expressing tunneling nanotubes (TNTs). TNTs are a mode of intercellular communication commonly seen in many cell lines, including PDAC cells *in vitro*, but have not yet been described in PDAC in a 3D setting. Capan-1 and BxPc-3 represented another phenotype (Fig. 5C, D, respectively). These spheroids contained two distinct layers of cells; the inner layers of cells were densely packed whereas the outer layers of cells were much more loosely interacting. Interestingly, Capan-1 and BxPc-3 spheroids expressed a large number of TNTs on and near their surface and other vessel-like structures. TNTs were not yet documented in PDAC in a 3D setting. Thus, the proposed technique can shed more light into cell–cell interactions and communication in a more biologically relevant 3D setting.

Next, we wanted to compare the structure of the produced spheroids to that of tumors grown *in vivo*. Immunohistochemistry confirmed that the PDAC spheroids grown via our improved hanging drop method possess features and micro-regions relevant to tumors relevant to human-derived, orthotopically implanted pancreatic tumors grown in mice models. For example, their densely packed nature and relatively large size meant that hypoxic (HIF-1α) and hence apoptotic (Cleaved PARP) micro-regions had started to form. The physiological state of spheroids depends on the spheroid size, the individual and cell type-specific behavior of pancreatic cancer cells, the cell packing density, and also the culture time. Small spheroids with a size of up to 200 μm are often used for drug testing and while may be sufficient to reflect 3D cell–cell and cell–matrix interaction,[47,48] do not exhibit clear pathophysiological gradients and hypoxia that more accurately represent the *in vivo* tumor microenvironment. Hypoxia is not only a well-established radiation- and drug-resistance factor, but also leads to numerous effects in tumor cells by modulating expression patterns.[6,49] Larger spheroids (diameter >400 μm) or spheroids that are tightly packed do develop hypoxic region, and hence these spheroids more accurately model the pathophysiology found in the PDAC tumor microenvironment. This is particularly pertinent as PDAC has high levels of radiation- and drug resistance.[50–52] Furthermore, spheroids displayed heterogeneous proliferating regions (Fig. 6 and Supplementary Fig. S3). A tumor model such as PDAC tumor spheroids is not a population of exponentially growing cells that cycle from G1 to S, G2 and mitosis without any constraint and therefore models tumor cell proliferation more accurately than 2D culture. In spheroids, regionalization and heterogeneity of the cell cycle parameters and distribution within the layers constituting the different proliferating zones are recapitulated.

Immunohistochemistry showed that our spheroid model possess many hallmarks commonly found in PDAC and suggests our spheroids represent a useful tool to investigate how micro-regions found in solid tumors change over multiple time points when dosed with chemo or radiation therapy. Such research may play a vital role in the optimization of therapy to this disease. We have found that the expression of such hallmarks of cancer as Ki-67 and HIF-1alpha is similar between

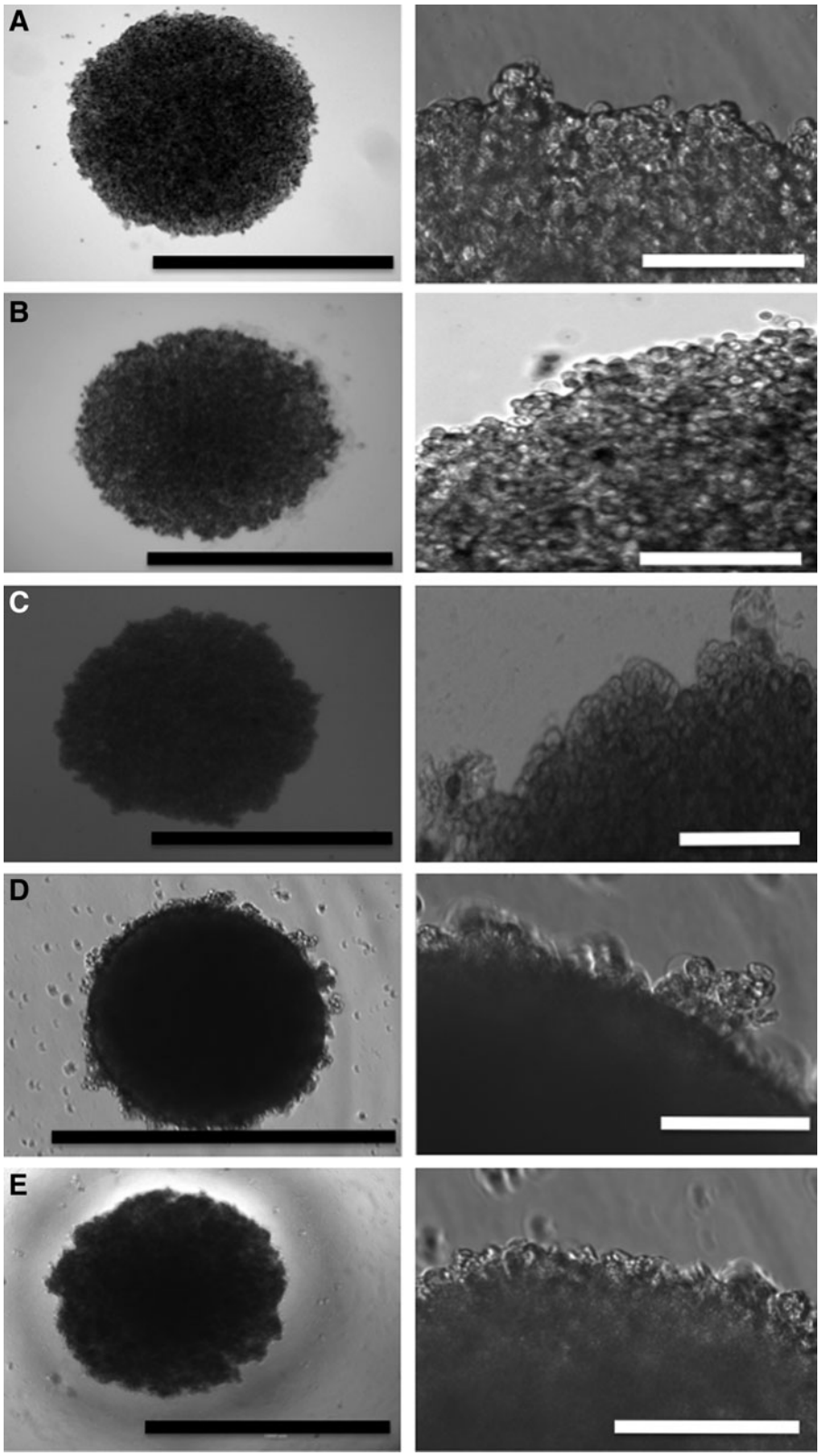

FIG. 4. Imaging spheroids of various pancreatic cancer cell lines grown in hanging drop with 0.24% Methylcellulose. Brightfield images of **(A)** Panc-1, **(B)** AsPc-1, **(C)** MIA PaCa-2, **(D)** Capan-1, **(E)** BxPc-3 spheroids (*Black* scale bars 1000 μm, *white* scale bars 100 μm).

the 3D spheroids and the tumors grown orthotopically in the pancreas of mice.

The expression of the human monoclonal Ki-67 antibody is strictly associated with cell proliferation. Ki-67 protein can be exclusively detected in the nucleus during interphase, whereas in mitosis most of the protein relocates to the chromosomes' surface. Ki-67 protein is present during all active phases of the cell (G1, S, G2, and mitotic phases) but is absent from resting cells (G0 phase), which makes it a reliable marker for determining the growth fraction of cells

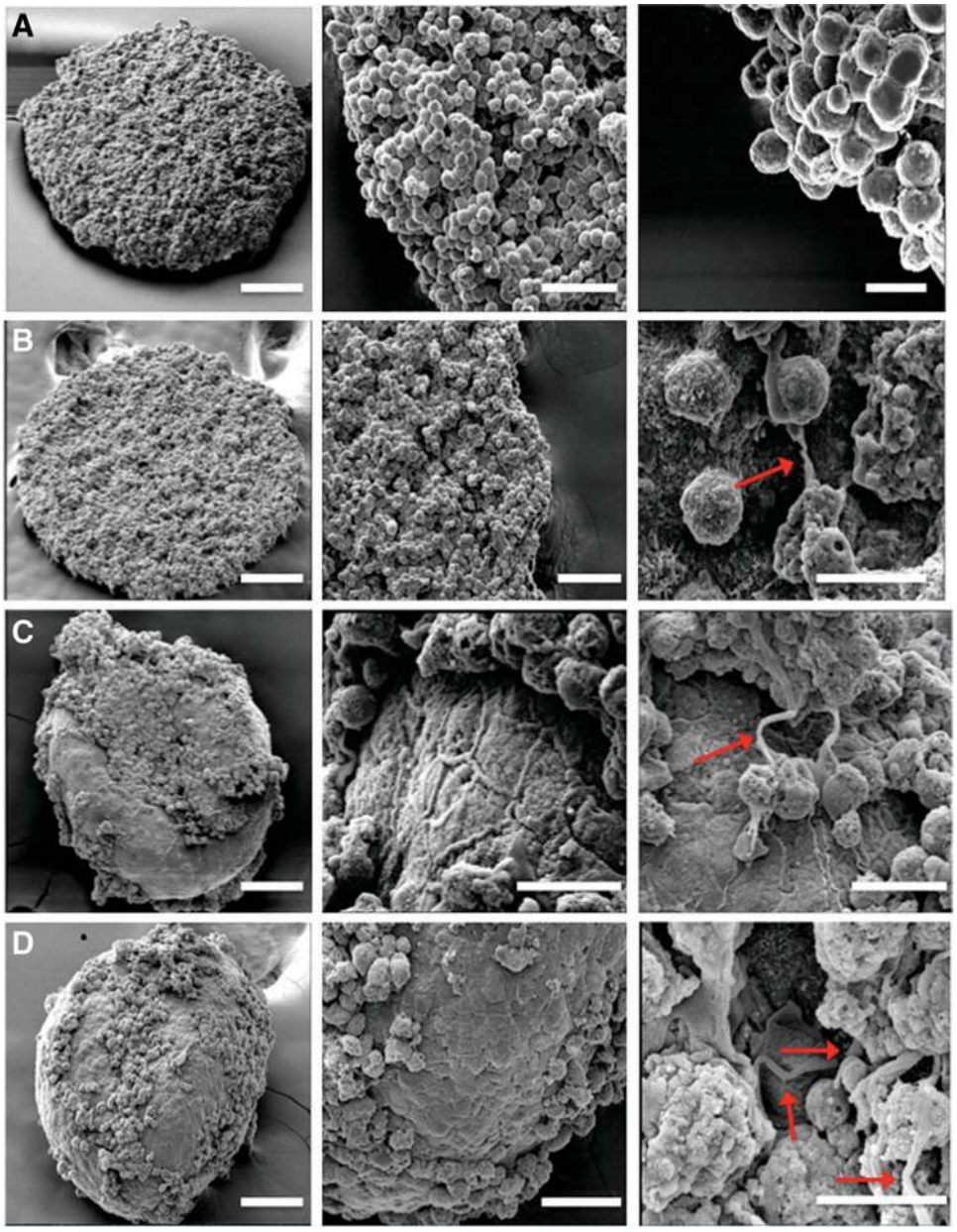

FIG. 5. SEM characterization of spheroids. **(A)** Panc-1 spheroid with increasing magnification, (scale bars *left* to *right*, 100 μm, 40 μm, and 10 μm). **(B)** MIA PaCa-2 spheroid (scale bars *left* to *right*, 100 μm, 40 μm, and 10 μm); **(C)** Capan-1 spheroid (scale bars *left* to *right*, 100 μm, 40 μm, and 10 μm); **(D)** BxPC-3 spheroid (scale bars *left* to *right*, 100 μm, 40 μm and 10 μm) *Red arrows* indicate TnT-like stuctures. Color images available online at www.liebertpub.com/tec

in a given cell population.[53] The fraction of Ki-67-positive tumor cells, also known as the Ki-67 labeling index, is often correlated with the clinical course of the disease.[53] The best-studied examples in this context are carcinomas of the prostate and the breast. However, Ki67 has also been used in combination with other biomarkers and clinicopathological predictors to predict patient survival post surgery in pancreatic cancer.[54]

HIF-1alpha (Hypoxia-inducible factor 1a, HIF1A) is a transcription factor that mediates cellular and systemic homeostatic responses to reduced O2 availability in mammals, including angiogenesis, erythropoiesis, and glycolysis. HIF-1a transactivate genes are required for energy metabolism and tissue perfusion and is necessary for embryonic development and tumor explant growth. HIF-1alpha is overexpressed during carcinogenesis, myocardial infarction, and wound healing. It is crucial for the cellular response to hypoxia and is frequently overexpressed in human cancers, resulting in the activation of genes essential for cell survival. HIF-1a regulates the survival and function in the inflammatory microenvironment directly. It is a transcription factor that plays a pivotal role in cellular adaptation to changes in oxygen availability.

HIF-1 controls oxygen delivery (via angiogenesis) and metabolic adaptation to hypoxia (via glycolysis).[55] HIF-1 consists of a constitutively expressed HIF-1β subunit and an oxygen- and growth factor-regulated HIF-1α subunit. In xenografts, tumor growth and angiogenesis are correlated with HIF-1 expression. In human cancers, HIF-1α is overexpressed as a result of intratumoral hypoxia and genetic alterations affecting key oncogenes and tumor suppressor genes. HIF-1α overexpression in biopsies of brain, breast, cervical, esophageal, oropharyngeal, and ovarian cancers is correlated with treatment failure and mortality.

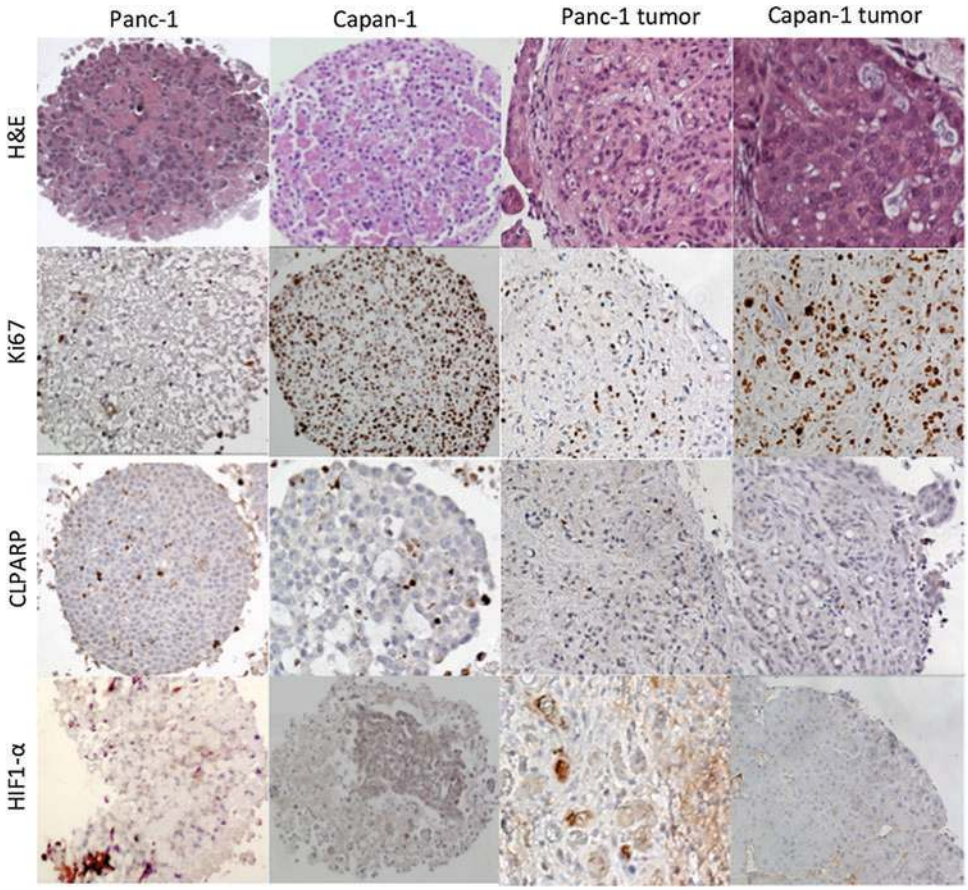

FIG. 6. Histology of Panc-1, Capan-1 spheroids, and Panc-1 and Capan-1 orthotopic tumors. The center section of the spheroids and tumors were stained with H&E, Ki67, CLPARP, and HIF-1α for a comparison of hallmarks of solid tumors existing in the spheroid culture. Color images available online at www.liebertpub.com/tec

Increased HIF-1 activity promotes tumor progression, and inhibition of HIF-1 could represent a novel approach to cancer therapy[55]

Cells traditionally grown as monolayers for investigating the mechanisms and treatment of cancer fail to reproduce phenomena observed *in vivo.*[16] Three-dimensional culture models are believed to reflect more accurately many aspects of the *in vivo* tumor microenvironment and to represent valuable *in vitro* models for the preclinical evaluation of anticancer drug or radiation treatment.[2,5,56] Therefore, spheroids are considered a superior model compared to the traditional monolayer culture for *in vitro* research.[4,8,12,57–61] A high-throughput screening that involves the use of 3D cultures is limited due to the difficulty in obtaining homogenous spheroid cultures.[62] The susceptibility of these cultures to disaggregation during treatment poses another constraint for using spheroids in high-throughput screening. Additionally, some traditional methods of spheroid formation include biologically active components that can affect the assay results. For instance, Matrigel is a commonly used ECM substitute, but it has many different constituents with relatively unknown effects on dose–response systems.

Conclusions

Herein, we have introduced a modified hanging drop method by adding methylcellulose to the hanging drop, which yielded a straight forward production of spheroids in PDAC cells. Crucial for effective experiments designed to test drug therapy in growth assays is a homogeneous size distribution, reproducibility, and a short formation time for the spheroids. PDAC cells formed well-rounded spheroids after 5 days in hanging drops. Moreover, homogenous distribution means that the spheroids can be used directly, whereas other techniques call for either handpicking of equally sized and shaped spheroids under the microscope or sieving through nylon meshes.[24,59] Appealing to the need for preventing disaggregation during physical handling of specimens, the proposed technique yield spheroids that are compact, rigid, and have high tolerance to mechanical force, thus enabling standard manipulations, such as medium substitution, transfer for imaging, and so on. without affecting the physical characteristics of the cultures. Additionally, the spheroids display some hallmarks of solid tumors, such as hypoxia, proliferating cells, and apoptotic regions (Fig. 6 and S3). It is noteworthy that many cell lines we used were unable to form homogenous and robust tumor spheroids using the traditional hanging drop method.

Furthermore, the proposed modified method does not rely on the use of alginate coats to increase mobility or polyelectrolytes to enhance affinity, and thus possible material artifacts[36] can be avoided. The yielded uniform and highly reproducible spheroid geometry lends itself to more accurate modeling of growth,[63,64] oxygen, nutrient, and metabolite transport,[60,65] movement of cells within the spheroid,[66] and the effect of anti-cancer treatments.[4,67]

Although the histological analysis of the spheroids is terminal, these spheroids are easily accessible to microscopic

methods, such as confocal or multiphoton microcopy, and can provide a platform to study the diffusion of probes, fluorescent drugs, and nanomaterials through 3D tumor tissue architecture in the real time setting and the changes in the tumor morphology/architecture due to the introduction of various agents.

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Disclosure Statement

No competing financial interests exist.

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