Video Article

Generation of Microtumors Using 3D Human Biogel Culture System and Patient-derived Glioblastoma Cells for Kinomic Profiling and Drug Response Testing

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Abstract

The use of patient-derived xenografts for modeling cancers has provided important insight into cancer biology and drug responsiveness. However, they are time consuming, expensive, and labor intensive. To overcome these obstacles, many research groups have turned to spheroid cultures of cancer cells. While useful, tumor spheroids or aggregates do not replicate cell-matrix interactions as found *in vivo*. As such, three-dimensional (3D) culture approaches utilizing an extracellular matrix scaffold provide a more realistic model system for investigation. Starting from subcutaneous or intracranial xenografts, tumor tissue is dissociated into a single cell suspension akin to cancer stem cell neurospheres. These cells are then embedded into a human-derived extracellular matrix, 3D human biogel, to generate a large number of microtumors. Interestingly, microtumors can be cultured for about a month with high viability and can be used for drug response testing using standard cytotoxicity assays such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and live cell imaging using Calcein-AM. Moreover, they can be analyzed via immunohistochemistry or harvested for molecular profiling, such as array-based high-throughput kinomic profiling, which is detailed here as well. 3D microtumors, thus, represent a versatile high-throughput model system that can more closely replicate *in vivo* tumor biology than traditional approaches.

Video Link

The video component of this article can be found at https://www.jove.com/video/54026/

Introduction

The most common primary intracranial malignant brain tumors are grade III astrocytomas and grade IV *glioblastoma multiforme* (glioblastoma or GBM). These tumors offer poor prognoses with median one-year survival between 12 - 15 months with current therapies for GBM in the US[1-3]. Multimodality therapies include surgery, radiation, and chemotherapy including temozolomide (TMZ) and kinase-targeted agents. Kinase signaling is frequently dysregulated in GBM, including subsets of tumors with amplification or activating mutations in the Epidermal Growth Factor Receptor (EGFR), increases in Platelet Derived Growth Factor Receptor (PDGFR) signaling, increased Phosphatidyl-Inositol-3 Kinase (PI3K) and tumor supporting angiogenic signaling through Vascular Endothelial Growth Factor Receptor (VEGFR) as well as other kinase driven pathways[4-6]. Current *in vitro* and *in vivo* models frequently lose these representative alterations[7]. Additionally, genetic profiling has not offered the anticipated benefits that may reflect the fact that genetic and epigenetic changes do not always predict changes at the level of protein activity, where most kinase targeting agents act directly, and where therapies with other mechanisms of action may act indirectly.

The traditional immortalized cell line that can be passaged ad infinitum has long been the standard for drug testing due to their ease of maintenance and reproducibility. However, this model suffers from a high nutrient (and artificial) growth environment that selects for fast growing cells that differ greatly from the original tumor. As such, there has been considerable interest in developing more realistic model systems that reflect a more complex tumor biological system as is present in the patient. Tumor xenografts developed directly from a primary tumor grown in mice ("xenoline," patient-derived xenograft or PDX) provide a more reflective model system, particularly in the setting of cancer therapeutics, as they are felt to more reliably predict clinical success.[8] Despite the more reflective biology, these models are expensive and are difficult to establish and maintain. Moreover, they are not amenable to high-throughput studies. The need to better develop biologic models that more

accurately reflect molecular alterations in the primary tumors, and to profile and test these models using direct measures of kinase activity, not surrogate genetic markers, is clear.

It is well recognized that unlike two-dimensional (2D) monolayer cultures, 3D or multicellular assay models can provide more physiologically relevant endpoints[9-11]. Common 3D culture approaches involve matrix-coated microcarriers and cell spheroid formation. Tumor spheroids can be generated via cellular aggregation using spinner flask, pHEMA plate and hanging drop techniques. Limitations for these approaches include: inability for some cells to form stable spheroids, variability in growth and challenges with mixed cell types. Alternatively, many synthetic (hydrogel, polymer) and animal-derived Engelbreth-Holm-Swarm (EHS) matrix from mouse sarcomas, bovine collagen) matrices have been developed for 3D culture studies[12-14]. Mouse EHS matrix is extensively used but known to promote cell growth and differentiation *in vitro* and *in vivo*[15].

In order to replicate 3D tumor biology, a human biomatrix system was developed by Dr. Raj Singh *et al.*[16]. The natural, growth factor-free human biogel allows 3D culture scaffolds (beads, discs), which support long-term cultivation of multiple cell types. A series of 3D human biogel culture designs are established for studying tumor growth, adhesion, angiogenesis and invasion properties. Advantages and properties of human biogel as compared to common mouse EHS gels are summarized in **Table 1** and **Table 2.**

Source:	**Human Amnions** (Pooled tissue) Pathogen-free, IRB-exempt/approved
ECM nature:	**Non-denatured Biogel** (GLP-production)
Key Components:	Col-I (38%), Laminin (22%), Col-IV (20%), Col-III (7%), Entactin & HSPG (< 3%)
GF-free:	**Undetectable** EGF, FGF, TGF, VEGF, PDGF (Non-angiogenic, Non-toxic)

Table 1: Properties of Human Biogel as Compared to Common EHS Gels.

Human Biogel	EHS gels
Natural human matrix	Reconstituted mouse matrix
Controlled cell growth & differentiation	Can promote cell growth & differentiation
Physiologic gene expression	Variable gene expression
3D tissue-like culture model	Plate-based culture model

Table 2: Advantages of Human Biogel as Compared to Common EHS Gels.

Protocol

NOTE: All xenograft therapy evaluations were done using an orthotopic tumor model for glioblastoma on a protocol approved by the Institutional Animal Care and Use Committee.

1. Isolation of Patient-derived GBM Xenograft Cells

1. **Preparation of Reagents**
    1. Re-constitute collagenase-I in sterile water to a concentration of 5 mg/ml and sterile filter. Store in 1 ml aliquots at -20 °C (final concentration is 50 µg/ml in 100 ml enzyme solution).
    2. Dissolve 100 µg Epidermal Growth Factor (EGF) in 2 ml sterile Phosphate-Buffered Saline (PBS). Store in 100 µl (5 µg/100 µl) aliquots at -20 °C for a final concentration of 10 ng/ml.
    3. Dissolve 100 µg FGF-β in 2 ml sterile PBS. Store in 100 µl (5 µg/100 µl) aliquots at -20 °C for a final concentration of 10 ng/ml.
    4. Add the following to one 500 ml bottle of Neurobasal Media (NBM) to prepare complete NBM: 10 ml B-27 supplement without vitamin A, 5 ml N2 supplement, 100 µl EGF, 100 µl Fibroblast Growth Factor (FGF)-Basic, 5 ml amphotericin B, 0.5 ml gentamycin, 5 ml L-glutamine.
    5. Prepare fresh enzyme solution by combining: 98.5 ml PBS, 1 ml collagenase-1, 0.5 ml 10x trypsin/EDTA and sterilize by filtration through 0.22 µm pore filter.
    6. Prepare or obtain a small volume sterile Dulbecco's Modified Eagle Medium (DMEM)/F12 cell culture medium with 7% fetal bovine serum (FBS) and 1x PBS without calcium or magnesium.

2. **Tumor Disaggregation**
    NOTE: Athymic nu/nu mouse previously injected with tumor cells in the right flank and allowed to from a palpable tumor mass is necessary for this procedure. In the examples shown here, we used patient-derived xenograft GBM cells, JX10 and JX12[17].
    1. Sterilize work area by spraying 2% chlorhexidine and set up work area with necessary instruments/supplies including disposable chuck, forceps, scalpel, glass Petri dish, enzyme solution, PBS, and chlorhexidine (See **Figure 2A**).
    2. Euthanize mouse/mice harboring a flank tumor. Aim for a 20%/min displacement rate of CO_2 using 30% CO_2. After the mouse shows respiratory arrest, maintain CO_2 flow for about 1 min (typically 3 min total). Mice are removed from the chamber and cervical dislocation is performed as a secondary euthanasia confirmation. Spray the animal with 3% chlorhexidine to sanitize skin.
    3. While holding the mouse steady, make semi-circular skin incision ~ 1.5 cm from tumor mass (flank implanted tumor) using a sterile disposable scalpel with #11 blade beginning cranial to the tumor mass and incising toward the belly of the mouse and around to the caudal end.

4. Reflect skin over tumor mass using fingers with gentle traction on caudal end of the incision and then push on skin surface to elevate tumor to have best access without touching the tumor (See **Figure 2B**).
5. Use blunt dissection with forceps to gently free tumor mass from peritoneal wall and from skin (See **Figure 2C**).
6. Transfer tumor mass with forceps to sterile glass Petri dish (DO NOT use plastic dishes to prevent breaking during mincing) and properly discard carcass and place these instruments aside.
7. Use new set of sterile instruments (scalpel with #11 blade, semi-curved forceps) to debride tumor tissue of necrotic tissue and membranous host connective tissue covering tumor.
8. Place 15 ml enzyme solution in a sterile vented-trypsinizing flask with stir bar and begin stirring slowly in the hood.
9. In a sterile glass Petri dish, wash harvested tumors 3 - 5 times with sterile PBS to remove excess blood (may pour or use syringe to rinse them with the PBS). Gently tilt dish and pipet or aspirate the wash material. Mince finely using two #11 scalpel blades (See **Figure 2D**).
10. Add 14 ml of enzyme solution to minced tumor and pipet gently up and down 2 - 3 times. Transfer mixture to vented-trypsinizing flask and stir slowly for 20 min. Prepare 5 50 ml conical tubes by adding 1.5 ml FBS to each in order to neutralize the trypsin in step 1.2.11.
11. After 20 min, collect 14 ml of cell solution from trypsinizing flask and add to one tube with FBS. Add 14 ml fresh enzyme solution to flask and continue stirring.
12. Centrifuge collected cells at 150 x g for 8 min at room temperature and discard the supernatant. Add 45 ml of complete NBM to the pellet, mix gently, and repeat centrifugation to rinse out serum. Re-suspend pellet in 5ml complete NBM and hold on ice.
13. Repeat cell harvest steps for a total of 5 harvests, combining all harvested-cells in a single conical tube on ice.
14. Place a 40 µm cell strainer on the top of a 50 ml conical tube. Prep the cell strainer by passing 10 ml of DMEM/F12 + 7% FBS through and then washing with 10 ml PBS.
15. Slowly add cell suspension to strainer, allowing it to drip through (See **Figure 2M**). Gently lift the tab of the cell strainer in between each new addition to break any vacuum and allow the suspended cells to pass freely (See **Figure 2N**).
16. Centrifuge the filtered cell suspension as above. Re-suspend in 10 ml complete NBM and count to determine total number of viable cells using 0.04% trypan blue and a hemocytometer. Hold cells on ice until microtumor generation.

2. Alternate Tissue Disaggregation Protocol

1. Automated tissue dissociator and disaggregation system.
    1. Place the disaggregation tube into the cellular dissociator, select the program "Tumor _02_02" and press start and run for 32 sec. Transfer tube to rotator, place in 37 °C incubator for 40 min.
    2. Centrifuge suspended-cells at 150 x g for 8 min at room temperature and discard the supernatant. While centrifuging the cells, set up reagents for the processing of the cell suspension. Add 10 ml serum-free NBM and gently triturate suspension.
    3. After centrifugation, obtain a cell pellet containing ~ 10 - 30 x 10^6 viable cells (~ 0.3 ml). Re-suspended the pellet in 10 ml serum-free NBM (See **Figure 2O**). Determine the viable cells by exclusion assay (See Step 3.2.2).

3. Microtumor Generation

1. Preparation of Reagents
    1. Prepare complete NBM as in 1.1.4 and prepare neutralized High Density human biogel (HuBiogel) (HDHG at 3 mg/ml) per internal protocol. Similar biogel matrices can be used as well.

2. Microtumor Production and Culture
    1. Obtain freshly dissociated PDX cells as single cell suspension in complete NBM, on ice.
    2. Mix the cell suspension with an equal volume of trypan blue solution (0.4% in PBS) and analyze using hemacytometer to determine cell number and viability by trypan blue exclusion[18]. Remove volume necessary to generate 50,000 cells/microtumor where volume = (50,000 cells/tumor * # tumors)/(viable cell count / 1 ml) and place into a fresh conical tube.
    3. Concentrate cells by centrifugation at 150 x g, for 8 min, at room temperature. Discard the supernatant and resuspend the cell pellet with ice-cold HDHG solution in a final ratio of 1 part cells in FBS and 4 parts HDHG.
    4. Use an electronic multichannel pipette to dispense 10 µl per pin cell-HDHG mixture onto a 96-pin steel plate (with hydrophobic coating) to generate microtumors (2 mm beads each containing 50,000 cells).
    5. Place 3D tumor beads inside tissue culture incubator (37 °C, 5% CO_2, humidified) for 15 min to gelate the beads.
    6. After gelation, transfer the microtumors (10 µl) to a custom suspension culture chamber (50 ml) or large volume culture dish (10 cm) containing complete NBM using the electronic multi-channel pipette and custom pin-device.
    7. After 1 - 2 days in tissue culture incubator, transfer microtumors to 96-well culture plates containing 50 µl NBM/well using a wide-mouth dispensing pipette and perform various assay and analysis protocols.

3. Drug Treatment and Maintenance of microtumors
    1. Select final concentrations for drug testing.
    NOTE: If the drug has known efficacy in 2D culture, select 2x the IC_{50} as the middle dose concentration and select 3-fold serial dilutions above and below for a total of 5 dose levels. For example, if the IC_{50} is 9 µM for the drug in 2D culture, select 2 µM, 6 µM, 18 µM, 54 µM, and 162 µM as final concentration for drug testing.
    2. Prepare 2x drug dosing solution in complete NBM from dimethyl sulfoxide (DMSO) stock. Dilute dosing solution in 1% DMSO medium to prepare a 5 dose, 3-fold serial dilution.
    3. Add 50 µl of dosing solution to the microtumor well containing 50 µl in assay plates to achieve a final DMSO concentration of 0.5%. Repeat for each replicate (*e.g.,* 4) at each drug dose determined in 3.3.1.

4. Maintain cultures in 37 °C, 5% CO_2, humidified tissue culture incubator for 1 - 14 days. Feed cultures twice weekly by refreshing media and drug solution as above.

4. Morphological and Phenotypic Analysis of Microtumors

1. Determine cell morphology in microtumors using standard live-cell staining.
    1. Prepare 1 mM stock of Calcein-AM in DMSO. Aliquot and store at -20 °C.
    2. At desired culture intervals (1, 7, and 14 days), add Calcein-AM solution prepared in PBS (without Ca/Mg) to 96-well plate for 1 µM final concentration.
    3. Incubate 20 min in a 37 °C, 5% CO_2, humidified incubator, and image using a fluorescence microscope at 2X, 4X, and 10X (excitation: 450 - 490 nm band pass, emission: 515 nm long pass, dichroic 500 nm).

2. Microtumor Growth/Proliferation Assay
    1. Dissolve MTT powder in PBS (without Ca/Mg) to prepare 12 mM stock. Sterile filter, aliquot, and store at -20 °C.
    2. At desired culture intervals (1, 7, and 14 days), add 20 µl of MTT solution to 96-well plate per 100 µl culture volume. Incubate 2 hr in tissue culture incubator.
    3. Prepare fresh lysis solution of 10% SDS in 0.01 M HCl and add equal amount to culture volume to plates. Incubate sealed plates overnight in 37 °C incubator.
    4. Read absorbance at 570 nm using a multi-plate reader.

3. Microtumor Preparation for Kinomic Analysis
    1. Prepare lysis buffer by pre-chilling to 4 °C and then adding a 1:100 ratio each of 100x Protein Phosphatase Inhibitor (PPI) and 100x Protein Protease Inhibitor (PI). Mix well and keep on ice.
    2. Transfer 2 microtumors to each of three 1.5 ml microcentrifuge tube. Remove supernatant and add 40 µl of lysis buffer containing PPI and PI to each tube and lyse for 30 min at 4 °C.
    3. Pipette samples vigorously to break tumor beads. Centrifuge at 16,000 x g for 10 min at 4 °C and store samples at -80 °C until kinomic analysis.

5. Kinomic Profiling of Microtumors

1. **Protein Tyrosine Kinase (PTK) profiling**
    1. Thaw reagents (10x Protein Kinase (PK) buffer and 2% bovine serum albumin (BSA)) to 4 °C Load PK buffer (300 µl 10x PK buffer stock in 2.7 ml dH_2O) into syringe in position # 2 on the profiling platform.
    2. Open the kinase assay software program and load the PTK protocol file and press 'START', annotating samples within software program after scanning chips. Place chips onto profiling platform and load 25 µl of 2% Bovine Serum Albumin (BSA) per array and then press LOAD to begin the software-controlled protocol blocking step.
    3. Dilute 6 µM of 100 mM ATP stock with 54 µl dH_2O to make 10 mM adenosine triphosphate (ATP). When the software begins the 3rd, and final wash step (visible on screen) bring 15 µg of lysate brought up to 28 µl with dH_2O, mix and add to array.
    4. Prepare PTK master mix (MM) (for up to 12 samples). Add 32.6 µl dH_2O to reconstitute dithiothreitol (DTT) and add 6 µl DTT, and 60 µl 10x PK solution, to PTK-MM1 tube (already contains 60 µl 10x BSA).
    5. Wait until 'Load' prompt in the kinase assay software appears then add 126 µl of PTK-MM1, 60 µl PTK additive, and 60 µl 10 mM ATP to MM2 (contains previously aliquoted 4.5 µl PY20 FITC antibody) and mix. Add 16 µl of this PTK master mix to each sample lysate tube and pipette mix 5 times.
    6. Ensure cover glass is clean and then add 35 µl lysate/master mix per array, close carousel lid, and press LOAD button.

2. **Serine/Threonine Kinase (STK) Profiling:**
    1. Thaw reagents (10x PK buffer, 10x STK buffer, and 2% BSA) to 4 °C. Load PK buffer (300 µl in 2.7 ml dH_20) into syringe position # 1, 1:10 and STK buffer (300 µl in 2.7 ml dH_20) into syringe position # 2.
    2. Open the kinase assay computer software program and load the STK protocol file and press START, annotating samples within the program after scanning chips. Place chips onto profiling platform and load 25 µl of 2% BSA per array, and then press LOAD to begin the software-controlled protocol blocking step.
    3. Dilute 6 µM of 100 mM ATP with 54 µl dH_2O. During final (3rd) wash step mix 2 µg of lysate and bring up to 32.6 µl with dH_2O, mix and add to array.
    4. Make STK master mix: Add 70 µl 10x PK to STK-MM1 tube (contains 7 µl 100x BSA), and then add 42 µl dH_2O to STK-MM1 tube.
    5. Wait until 'Load' prompt in the kinase assay software then: Add 18 µl 10 mM ATP to STK-MM1, and add 9 µl MM1 to each sample lysate. Mix well.
    6. Ensure cover glass is clean and add 35 µl lysate/master mix per array, close carousel lid, and press LOAD.
    7. During Final (3rd) Wash Step add 1.05 µl STK-FITC secondary antibody to DMAB tube (contains 3.03 µl STK primary antibody mix), add 39.6 µl AB Buffer to DMAB, add 356.0 µl dH_2O to DMAB, add 30 µl to DMAB to each array, and press LOAD.

3. **Analysis of Kinomic Data** [19,20]
    1. Open analysis software and load the Image Analysis App. Select image folder containing barcoded and time-stamped whole array images for PTK or STK data, select article number matched array layout file (86312 for PTK and 87102 for STK), and load previously generated array annotation File.
    2. Verify correct gridding of all images, and run Exposure Time Scaling App. Statistically compare exposure/slope log2 transformed values, and validate with prewash kinetic curves[19-21].

3. Run upstream kinase prediction software to query altered kinomic profiles between conditions for upstream kinases[22,23].

Representative Results

We have shown that 3D biogel culture system supports long-term growth and function of multiple cell types. In this collaborative project, patient-derived GBM xenolines (PDX) are used for producing hundreds of microtumors. Dissociated cells (3 x 10^5) or neurospheres (40 - 50) were embedded in biogel beads (2 mm) and after quick gelation they are cultured in a NB-media filled custom bioreactor. Cellular viability (Calcein-AM), growth profile (MTT), and kinomic activity array-based analysis were determined. PDX microtumors maintained multicellular organization and high viability (> 80%) for > 3 weeks. We believe this *in vivo*-like biology is due to maintenance of 3D cell-matrix architecture (not possible with 2D or spheroid culture). It is also observed that 3D tumors are difficult to produce with fragile gelatinous protein scaffold, possibly due to lack of collagen I[14]. A working scheme for microtumor production using PDX cells and 3D biogel system is summarized in **Figure 1** and the dissociation process is depicted in **Figure 2**.

Through live cell imaging via Calcein-AM, we determined cell growth and viability as well as effects of the drugs on the GBM cells derived from the PDX tumor JX10, as indicated by a decrease in fluorescence, signaling cell death. Tumor growth was measured at day 0, 7 and 14 for microtumors displaying continuous growth as shown in **Figure 3A**. This microtumor, JX10, is known to be resistant to TMZ. When exposed to 1 - 10 µM TMZ, minimal growth suppression was noted (**Figure 3B**). However, testing PDX tumor JX12, which is known to be sensitive to TMZ, demonstrated sensitivity by MTT assay at 14 days that was confirmed by Calcein-AM imaging (**Figure 4**).

In order to explore potential mechanisms of drug resistance, we measured kinase signaling in the TMZ resistant microtumors (JX10). Kinomic profiles for 144 tyrosine and 144 serine/threonine phosphopeptide targets were captured for DMSO and 10 µM TMZ treated microtumors. Kinomic phosphorylation intensity for all peptide targets, per cycle, and per multiple exposure times was captured (Data not shown). A representative heatmap displaying peptides that had significantly altered intensities with 10 µM TMZ treatment ($p < 0.05$, unpaired students T-test) is displayed in **Figure 5A**. Kinomic profiles that were altered in TMZ treated JX10 microtumors were analyzed using the upstream kinase prediction software that identified SYK, LCK, and CTK kinases as increased in TMZ treated samples relative to DMSO (**Figure 5B**). Serine/threonine kinome upstream kinase analysis was also performed (**Figure 5C**).

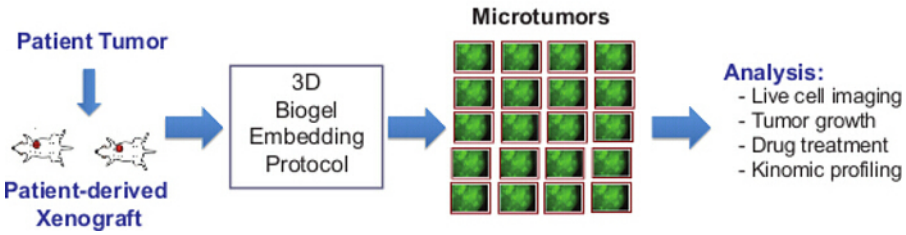

Figure 1: Working Scheme for Microtumor Production. GBM-PDX tumors are dissociated from the murine host, and single cells are embedded in biogel matrix to form microtumors. Analyses are then performed on the microtumors. Please click here to view a larger version of this figure.

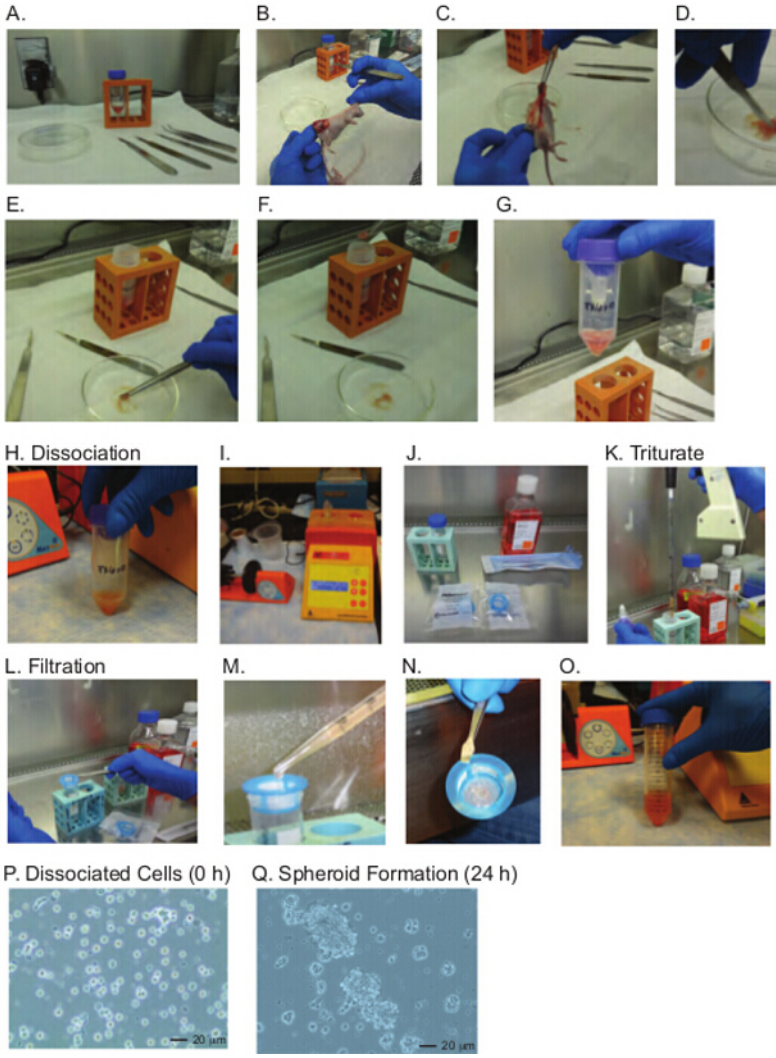

Figure 2: Dissociation of PDX Tumor Cells. Tumors are removed from host mice and minced prior to mixing with enzyme solution **(A-G)**. Dissociation is shown after 40 min **(H)**. Tumor cell dissociator is shown **(I)**. Trituration in media and filtration **(J-N)** is shown with an end result of 0.3 ml pellet **(O)** containing 29.5 x 10^6 viable cells **(P)** that form spheroids in NBM in 24 hr **(Q)**. Scale bar is 20 µm. Please click here to view a larger version of this figure.

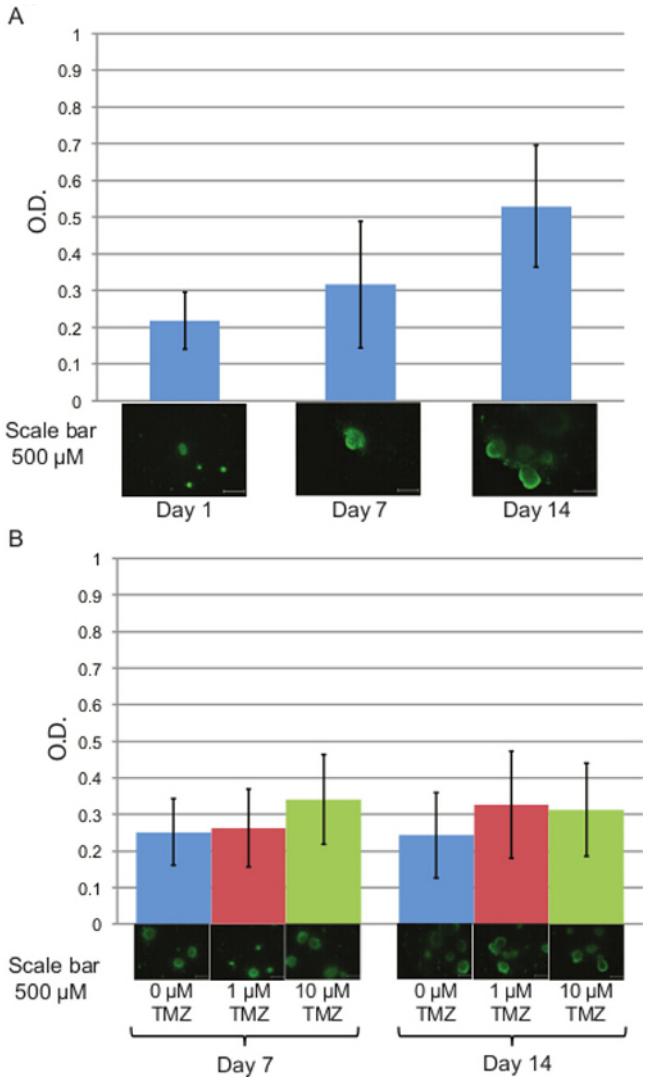

Figure 3: Growth of Microtumors. JX10 microtumor growth measured by optical density (O.D.) of MTT and representative images over 14 days, both basally **(A)**, and in response to 1-10 µM TMZ treatment **(B)**. Images are shown at 4X magnification (scale bar = 500 µm). Standard deviation indicated. Please click here to view a larger version of this figure.

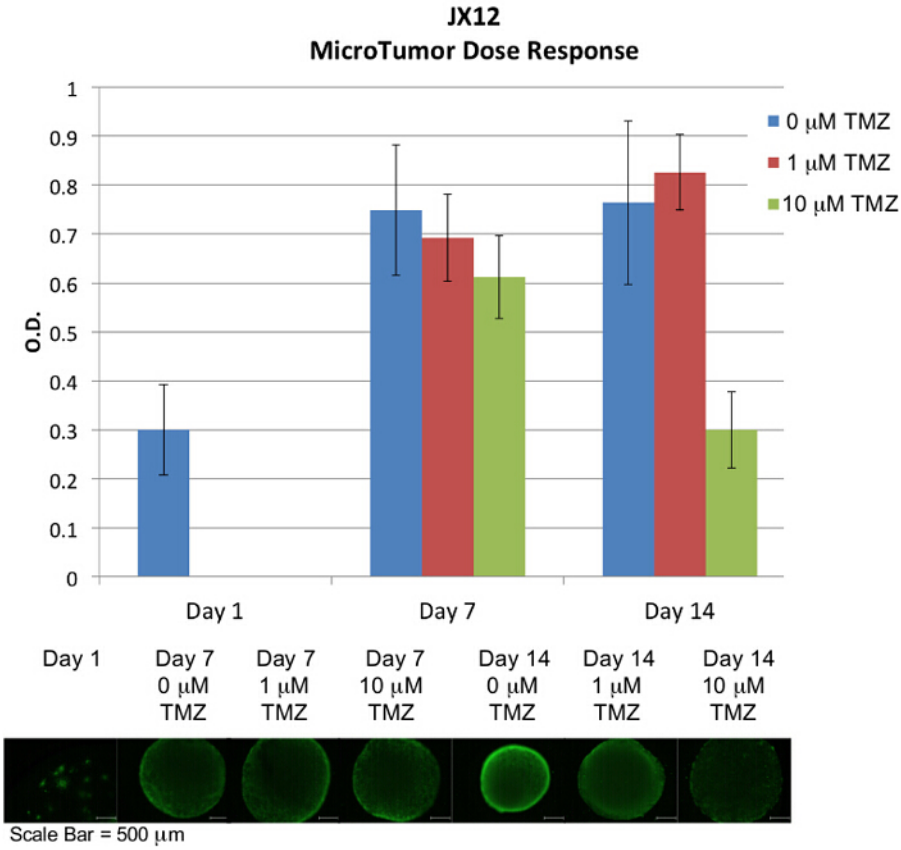

Figure 4: Growth of Microtumors. JX12 microtumor growth measured by O.D. of MTT and representative images over 14 days, both basally (Day 1), and in response to 0, 1, or 10 μM TMZ treatment (scale bar = 500 μm). Standard deviation indicated. Please click here to view a larger version of this figure.

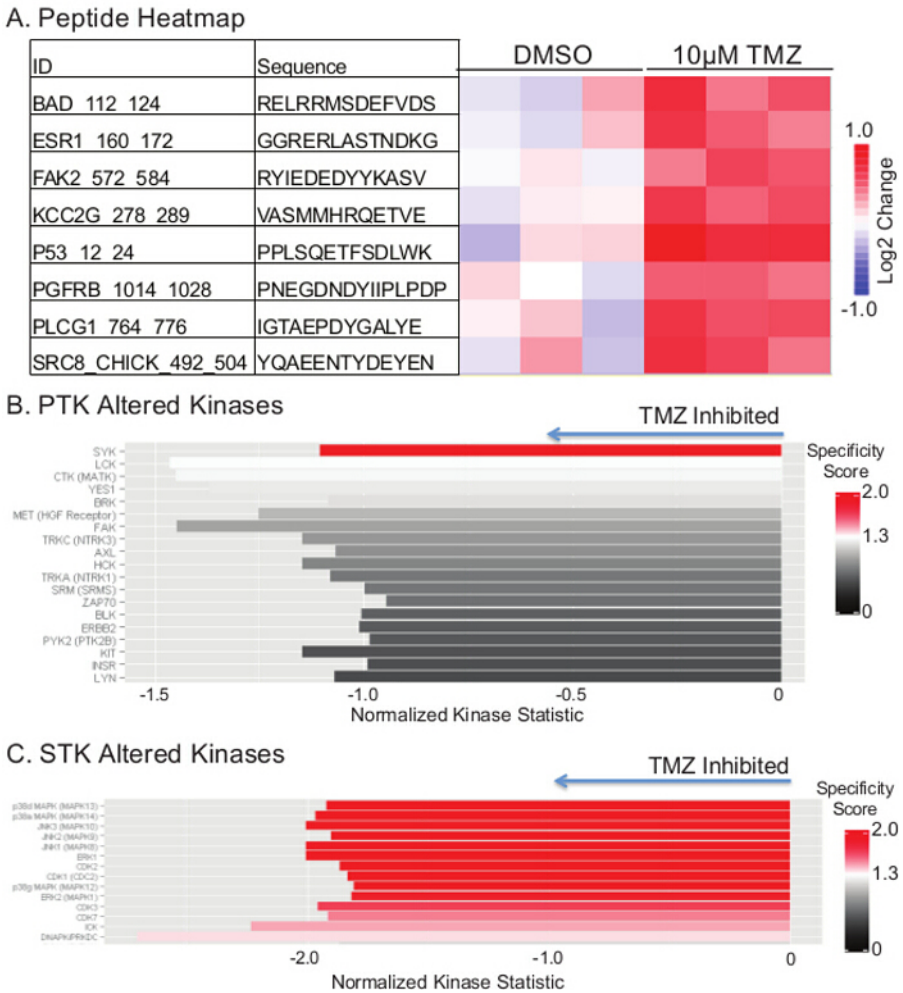

Figure 5: Kinomic Profiles of TMZ Treated Microtumors. Heatmap of exposure-integrated values (log2 transformed slopes (x 100) of 10, 20, 50, 100, and 200 ms median signal minus background exposure values) are displayed for significantly TMZ-altered altered peptides ($p < 0.05$). Red indicates increased, and blue indicates decreased relative to DMSO mean per peptide. TMZ altered profiles were compared using the upstream kinase prediction software and altered kinases. Normalized Kinase Statistic (NKS) score > 1.0 and specificity score > 1.3 (red) are considered highly altered. Please click here to view a larger version of this figure.

Discussion

Critical steps within the protocol predominantly relate to microtumor generation, as well as drug dosing and maintenance. Because the microtumor beads are fragile and easily torn, extreme care is needed in both developmental stages of an assay and maintenance. If an error occurs during either of these processes, experimental interpretation can be compromised, causing extension or unnecessary repetition of the experiments or even exclusion of data.

Modifications and troubleshooting, especially of the microtumor development process, included the design and production of a custom hydrophobic tool for the use of making the microtumor beads. This tool allows for faster and more accurate production of microtumors. Additionally, small modifications to the maintenance of the microtumors helped to speed up the process of changing medium and dosing the cells. Several of these modifications included, but were not limited to, using a multichannel electronic pipette to remove and replace medium from the 96-well plates and pre-mixing fresh drug dosing solutions and arranging the 2x solutions in a corresponding 2 ml 96-well plate.

Major optimizations for culture conditions in 3D modeling includes determining appropriate dosages for drug compounds, as there is a poor relation between 2D modeling and 3D modeling. Therefore, serial dilutions to establish a dose titration curve is often necessary for moving a 2D model system to the 3D microtumor model.

Potential issues to consider with microtumor generation from xenoline tumor cells harvested from a mouse include bacterial or fungal contamination, inconsistent availability of primary xenolines, as unique growth properties in mice can produce differences in harvest time and variance in cell yields from separate dissociations. With each cell line, the number of cells received, and similarly, how many microtumors can be produced will vary. As such, there is a need to prioritize which assays are required and which ones are "optional" should the microtumor yield be insufficient. Potential limitations with the kinomic profiling of microtumors include the difficulty in correcting for inert proteinaceous material loading, as samples are loaded in a manner to correct for BCA determined protein levels, that may not distinguish between biogel based proteins (ECM proteins) and those of the cellular kinase component that is intended to be measured. Measuring of gross microtumor mass via Calcein-

AM, or using a housekeeping protein as a correction factor may mitigate this, although issues with housekeeping protein levels in tumors being altered may confound this. For short time-course signaling experiments, assuming equally sized (kinase containing live cell number) and paired treated and untreated samples, this is less of an issue.

With this research, the goal is to provide a more cost-effective and disease-representative preclinical model for accurate drug therapy testing in comparison to orthotopic patient-derived xenografts, the current gold standard model for GBM testing. In recent years, several groups have attempted to model the *in vivo* GBM environment for drug testing purposes. Some groups have simply tried to grow GBM tumor cells in non-differentiating conditions to preserve GBM stem cells or at least brain tumor initiating cells (BTIC)[24,25]. These tumorsphere or neurosphere cultures can be used for drug testing and are likely superior to traditional models. However, since they still lack the tumor-stroma interplay that is preserved in our microtumor model, the tumorsphere approach is still limited. Other groups have attempted to apply engineering principles to improve GBM models[26-28]. These approaches have included flow chambers, variable ECM proteins and tumor cell/normal cell mixtures. While promising, almost all of these reports have used immortalized cells lines with the caveats mentioned previously. As such, we believe that the PDX-based microtumor model described here is more clinically relevant.

In the future, the microtumor model could be used as either a true tumor avatar or a 'proband' system. With the proband system, the PDX developed from a particular patient does not directly inform the clinician regarding that specific patient's therapy, as with the tumor avatar system. Instead, a patient's tumor is "matched" to a pre-existing, well-characterized (both molecularly and phenotypically) PDX[29]. Indeed, this model could serve as a 'go-to' avatar or reside in a proband library as a comparative profile. With traditional avatars, growing a patient's tumor cells in mice in parallel to the patient's treatment is not only time consuming, as it may take several months for the primary passage, but also costly, as testing several drug therapies in mice generate an overwhelming price tag. To combat this, the primary patient tumor can be implanted directly into the biogel matrix. With the microtumors, results could be generated quickly and at a lower cost.

As a proband model, the microtumor kinome data profiles and drug response could be added into a proband 'library,' along with profiles and data from previous 3D *in vitro* models, existing PDXs, animal studies, other patients, *etc.* In theory, the patient's tumor cells could then be 'omically' (genomically, kinomically, transcriptomically, *etc.*) matched to a similar existing microtumor profile to determine accurate treatment for the best possible outcome.

Summary: Here we identify that these microtumor models can be used to measure both phenotypic growth and can be queried at both the basal kinase activity level and in response to drug treatment that may be useful as a translational tool for further preclinical research. Developing accurate, and molecularly valid testable models for human tumors is imperative for effective drug development.

Disclosures

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