Generation of matched patient-derived xenograft in vitro-in vivo models using 3D macroporous hydrogels for the study of liver cancer

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ABSTRACT
Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide, often manifesting at the advanced stage when cure is no longer possible. The discrepancy between preclinical findings and clinical outcome in HCC is well-recognized. So far, sorafenib is the only targeted therapy approved as first-line therapy for patients with advanced HCC. There is an urgent need for improved preclinical models for the development of HCC-targeted therapies. Patient-derived xenograft (PDX) tumor models have been shown to closely recapitulate human tumor biology and predict patient drug response. However, the use of PDX models is currently limited by high costs and low throughput. In this study, we engineered in vitro conditions conducive for the culture of HCC-PDX organoids derived from a panel of 14 different HCC-PDX lines through the use of a three-dimensional macroporous cellulosic sponge system. To validate the in vitro HCC-PDX models, both in vivo and in vitro HCC-PDX models were subjected to whole exome sequencing and RNA-sequencing. Correlative studies indicate strong concordance in genomic and transcriptomic profiles as well as intra-tumoral heterogeneity between each matched in vitro-in vivo HCC-PDX pairs. Furthermore, we demonstrate the feasibility of using these in vitro HCC-PDX models for drug testing, paving the way for more efficient preclinical studies in HCC drug development.

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1. Introduction
Liver cancer is one of the leading causes of cancer death worldwide, with hepatocellular carcinoma (HCC) accounting for up to 90% of the disease [1]. While a third of HCC patients can benefit from resection, transplantation or local ablation, the remaining majority are diagnosed at advanced disease stages and are ineligible for such potentially curative treatments [2]. Given that advanced HCC is refractory to conventional chemotherapies, molecularly targeted therapies have been the mainstay treatment approach. However, at present, there are only two approved targeted therapies – multi-kinase inhibitors, sorafenib and regorafenib. Since the approval of sorafenib in 2007, several phase III trials conducted in the past decade investigating first-line and second-line agents have all failed to demonstrate superiority or
cells in vitro for preclinical studies. One possible solution is to grow PDX-derived time-consuming and costly, making them less than effective and thought to hold the potential to revolutionize anti-in vitro culture in animals. However, primary HCC cells are notoriously challenging to culturing HCC-PDX cells involving growing the cells as adherent monolayers on patient-derived xenograft (PDX) models. A recent paradigm shift away from the use of cancer cell lines to tumors. [14]. Since then, we established 85 different HCC-PDX models of which a select group of 14 lines was used in this study. Importantly, all established models can be cryopreserved for subsequent re-engraftment and propagation, constituting a living HCC biobank that can be readily employed for drug screening. Using a bioengineering approach, we established robust in vitro conditions for the culture of cells derived from these HCC-PDX models as tumor organoids (henceforth referred to as HCC-3DPDX) using a 3D macroporous sponge fabricated from hydroxypropylcellulose (HPC). The 3D sponge system developed for this study is a modification of a previously published system designed for the culture of normal hepatocytes [15,16]; this is a cellulose-based hydrogel with interconnected macropores fabricated by leveraging the ability of HPC derivatives to undergo thermal-induced phase separation (TIPS) and photo-crosslinking. We previously demonstrated that the sponge macropores enable spheroid size control through physical constraint while conjugated galactose ligands and in vivo-like mechanical stiffness provide cues to normal hepatocytes to form spheroids with preserved hepatic morphology and functions [15]. However, fabrication of this sponge system is challenging as it involves the use of moisture-sensitive reagents (anhydrous chloroform) necessary for grafting allyl groups onto HPC to render the polymer photo-crosslinkable, generating batch-to-batch variations. In this study, we addressed this problem by replacing allyl as the photo-crosslinkable group with methacrylate (MA), as well as replacing the use of chloroform with dichloromethane as the solvent for the reaction to synthesize MA-HPC. Hypothesizing that this modified sponge system would be able to support the culture of cancerous hepatocytes, we report that majority of the HCC-PDX cells cultured within this bioengineered sponge remain viable, proliferative, and preserve the genomic and transcriptomic profiles as well as intra-tumoral heterogeneity of their in vivo counterparts.

2. Materials and methods

2.1. Synthesis of MA-HPC

In this study, instead of using allyl groups as the crosslinker for sponge fabrication [15,16], we used MA groups. HPC (M₉₈ = 10,000 g/mol) and methacrylic anhydride (99%) were purchased from Sigma-Aldrich (Singapore), triethylamine (99%) was purchased from Merck (Singapore), dichloromethane (99.6% AR grade) and anhydrous diethyl ether (99%) were purchased from Fisher (Singapore). HPC (4 g, 11.75 mmol repeating units) was dissolved in dichloromethane (100 mL) and stirred rigorously. Following dissolution, methacrylic anhydride (3.48 mL, 2 equiv.) was added dropwise into the reaction mixture, followed by triethylamine (1.14 mL, 1 equiv.). The reaction mixture was stirred for 24 h, then quenched with 400 mL anhydrous diethyl ether. After washing twice with anhydrous diethyl ether, resulting fibers were filtered, collected and dried under rotovap for 30 min. The dried fibers were then re-dissolved in 200 mL deionized water and poured into dialysis tubes (MWCO = 14 kDa) for dialysis against running water for 48 h before lyophilization for 1 day. NMR characterization of MA-HPC is shown in Fig. 1A in Ref. [17].

2.2. Fabrication of sponge

MA-HPC was minced into small pieces and dissolved with water (10% w/v). The MA-HPC solution was then transferred into glass test tubes (6 mm diameter) and placed in a 1 L beaker filled with warm water (T = 42 °C). After 5 min, the beaker was placed in a rotating gamma radiator for 2.5 h (total dose = 5 kGy; Gammacell 220, MDS Nordion, Canada). Following irradiation, cross-linked MA-HPC hydrogel cylinders were obtained by breaking the test tubes. The hydrogel cylinders were sliced into thin pieces (1 mm) then lyophilized for 1 day. The MA-HPC sponge was then rinsed in dry acetone for 1 day, placed in vials filled with deionized water and lyophilized at -80 °C. The MA-HPC sponge slices were then washed in dry acetone (20 min each run, 3 times). Acetone in the vials was then removed and replaced with D-(t)-galactosamine HCl solution (0.08 g in 40 mL sodium bicarbonate buffer, pH = 10), and placed overnight at a shaker at 4 °C. The MA-HPC sponge slices were then washed in DPBS buffer (20 min each run, 3 times), followed by deionized water (20 min each run, 3 times) and then lyophilized.
2.3. Physicochemical characterization

2.3.1. Morphology

Top and cross section views of the sponge surface morphology were captured using SEM (JEOL JSM-5600, Japan) at 5 kV. Prior to imaging, the dried sponge was sputter-coated with platinum for 60 s.

2.3.2. UV transmission

The temperature-mediated phase behavior of MA-HPC in deionized water (10% w/v) was investigated using a UV/VIS/NIR spectrophotometer (Jasco, V-570, Japan) by measuring the optical densities at 480 nm as a function of temperature. The temperatures were controlled (heating rate of 0.1 K/min) using a Jasco PSC-498 temperature controller.

2.3.3. Porosity

Porosity of MA-HPC sponge was measured using Micromeritics AutoPore IV 9500 mercury intrusion porosimeter; value obtained was 94.8%. The porous morphology of hydrated sponge was visualized using propidium iodide staining. Sponge was incubated in 100 mg/mL propidium iodide in phosphate-buffered saline (PBS) overnight. Stained sponge was washed with PBS for 5 times before imaging was performed using the Olympus FluoView FV1000 confocal microscope.

2.3.4. Young's modulus

The mechanical properties of hydrated sponge were evaluated by compression tests using an Instron Micro-Tester 5848 (Instron Co., U.S.A.), at a speed of 0.5 mm/min and 298 K. The Young's modulus was calculated from the slopes of the initial linear portion of stress-strain curves.

2.4. Generation and maintenance of HCC-PDX models

This study received ethics board approval at the National Cancer Center Singapore and SingHealth. All mice were maintained according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA. We previously reported the capability to reliably generate primary HCC-PDX models using SCID mice for drug testing applications in HCC [14]. Since then, these HCC-PDX models have grown in numbers to comprise a living PDX biobank at the National Cancer Center Singapore, representative of HCC patients in the region and have been employed to evaluate the efficacy of various chemotherapeutic and targeted therapies against HCC [18–21]. Primary HCCs have previously been used to create PDX lines [14], of which 14 lines [25-0705A, 01-0207, 26-0808B, 25-0914, 26-0808A and 09-0913] were used to establish tumors in male SCID mice (In Vivo, Singapore) aged 9–10 weeks. For ease of reference, these 14 lines will be referred to as HCC 1-14.

2.5. Isolation and culture of HCC-PDX cells

HCC-PDX cells were harvested from the animals as previously published [20]. Tumors were finely minced and washed three times with modified Eagle medium (MEM). The minced tissue was incubated with MEM medium containing 5% fetal bovine serum (FBS) and 5 mg/mL collagenase (Roche Diagnostics Corporations, Indianapolis, IN, USA) at 37°C for 12 h. Cells were harvested by centrifugation at 800 g for 10 min and then passed through a 70 μm strainer. Cells were centrifuged again for 3 min and then counted. Each 3D sponge placed in a 24-well plate was seeded with 3 x 10^5 cells in 20 μL of medium, comprising DMEM supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. 45 min after seeding, 500 μL of medium was added to submerge the samples. Medium was replaced the next day with 1 mL of medium and every 2–3 days after.

2.6. Cell viability and growth assessment of HCC-3DPDX cultures

HCC-3DPDX samples were assessed for viability using calcein-AM (2 μM) and propidium iodide (25 μg/mL). Following a 30 min incubation with calcein-AM and propidium iodide, samples were immediately imaged using an Olympus Fluoview FV1000 or Zeiss LSM 710 confocal microscope. Samples were assessed for growth using CellTiter-Glo® (Promega) as described by the manufacturer.

2.7. Immunofluorescence staining of HCC-3DPDX

HCC-3DPDX samples were fixed with 4% paraformaldehyde for 30 min and stored in PBS at 4°C. Samples were permeabilized with 0.3% Triton X-100 for 30 min, and blocked with 2% bovine serum albumin (BSA) in 0.1% Triton X-100 for 30 min. Following which, samples were incubated with primary anti-Ki-67 antibody (1:200, Merck) in 2% BSA overnight and subsequently washed with 0.1% Triton X-100 thrice with 15 min for each wash. Samples were then incubated with goat anti-rabbit secondary antibody (1:200, Abcam), phalloidin and DAPI in 2% BSA for 2 h and subsequently washed again thrice with 15 min for each wash. Samples were then mechanically torn to release the tumor organoids for imaging. All imaging was performed using the Olympus Fluoview FV1000 or Zeiss LSM 710 confocal microscope.

2.8. Whole exome sequencing and RNA-sequencing data analysis

HCC-PDX and HCC-3DPDX samples were both sequenced by Auragen Pte Ltd, Singapore. For whole exome sequencing (WES) (using HiSeq 4000-150PE), short reads were aligned to hg19 with BWA [22] and duplicated reads were removed with Picard (Broad Institute). Improvement of alignments and genetic variants calling were completed using Genome Analysis Toolkit (GATK) [23]. Overlaps of SNP and INDEL between HCC-PDX and HCC-3DPDX were analyzed using VCFtools [24]. To investigate whether HCC-3DPDX retains key mutations in HCC, we referred to 32 known HCC driver genes [10] for genetic variant annotation achieved by ANNOVAR [25]; mutation types are divided in two groups, synonymous mutation and nonsynonymous/indel mutation, latter of which includes frameshift deletion, frameshift insertion, nonframeshift deletion, nonframeshift insertion, nonsynonymous SNV, stopgain and stoploss. To investigate whether intra-heterogeneity is retained in HCC-3DPDX, we used variant allele frequency as a surrogate measure. For each genetic variant locus, variant allele frequency is the ratio of altered allele number to detected total number.

For RNA-sequencing (RNA-seq) data (using HiSeq 2500-100PE), raw reads were aligned to the human reference genome using STAR [26]. Fragment per kilobase of transcript per million mapped reads (FPKM), a gene expression measure, was calculated for each gene by HOMER [27]. To investigate whether HCC-PDX and HCC-3DPDX share similar gene expression profiles, we focused on 219 up-regulated and 514 down-regulated genes known to be dysregulated in HCC [28]. Comparative analysis was performed using Pearson correlation (gene expression measure FPKM underwent asinh transformation). To verify this, using the same strategy, we also compared the similarity in gene expression of known HCC dysregulated pathways between HCC-PDX and HCC-3DPDX. Based on KEGG [29], nine cancer related pathways, namely AKT, Hedgehog, Hippo, Notch, p53, Ras, TGF-beta, VEGF and Wnt pathways, as...
well as the overall pathways in cancer, were analyzed.

2.9. Drug treatment

HCC-3DPDX samples were cultured for 5 days before treatment with Sorafenib (Nexavar) or BGJ-398 for 48 h. Following which, samples were collected for CellTiter-Glo® assay. To determine changes in indicated proteins, HCC-3DPDX samples were collected following 48 h of drug treatment. Lysates of 4–5 samples from each group were pooled. Each lane represents one protein pool (30 mg of proteins). Antibodies used for immunoblot analyses included for p-Akt (Ser 473) (1:1000, Cell Signaling), total Akt (1:1000, Cell Signaling), p-Erk1/2 (1:1000, Cell Signaling), total Erk1/2 (1:1000, Cell Signaling) and β-actin (1:5000, Sigma). The blots were then visualized with a chemiluminescent detection system as described by the manufacturer.

3. Results

3.1. Synthesis and characterization of cellulosic sponge

The ability of HPC to undergo TIPS from an isotropic aqueous to meta-stable bi-phasic state enables the unique fabrication of 3D macroporous sponge-like hydrogels by crosslinking its derivatives during phase separation [16]. To render HPC photo-crosslinkable, we reacted the polymer with MA and verified the grafting of MA groups (degree of substitution, 5.8%) using NMR (see Fig. 1A in Ref. [17]). The resulting MA-HPC derivative retains the ability to undergo TIPS without any noticeable sedimentation (Fig. 1A). The colloidal structure that forms during TIPS is then fixed through the use of gamma-irradiation, giving rise to a macroporous sponge (Fig. 1B).

As shown in the scanning electron micrograph in Fig. 1C, the sponge has a highly macroporous structure (pore size: 80-180 μm, see Fig. 1B in Ref. [17] for pore size distribution; porosity 94.8%) which is consistent throughout the sponge cross-section, enabling the formation of cellular organoids within diffusion limits [30]. This macroporosity is maintained whether the sponge is dry or hydrated (Figs. 1C and D). Besides having mechanical properties (modulus of 9.7 kPa) similar to in vivo liver [31], the sponge is also conjugated with galactose moieties (see Fig. 1C in Ref. [17]) to promote organoid formation and tethering to the sponge surface. Receptor-mediated interaction between asialoglycoprotein receptor on hepatocytes and galactose moieties is known to guide hepatocyte adhesion [32]. To test whether the sponge supports the organoid formation of HCC-PDX cells, we harvested HCC-PDX tumors grown in the animal, dissociated the tumor, and seeded single HCC-PDX cells onto the sponge. As hypothesized, these cells aggregated to form organoids within the physical constraints of the sponge macropores, suggesting that the sponge may be conducive for the culture of HCC-PDX cells in vitro (Fig. 1E).

3.2. HCC-3DPDX cells remain viable and proliferative in vitro

Given that the 3D sponge supports the formation of HCC-PDX organoids, we next asked if this finding is universal to most HCC-
PDX lines and whether cells remain viable and proliferative in culture, the latter being critical for assessing drug candidates that target proliferating cancer cells. To this end, we assessed the viability of 16 different HCC-PDX lines using a viability stain comprising calcein-AM and propidium iodide, which stains viable cells green and dead cells red, respectively. Fig. 2A shows the viability of six representative HCC-PDX lines; cells rapidly formed organoids by Day 2 (see Fig. 2A in Ref. [17]) and remained largely viable through one week in culture; the same was found for approximately 75% of the HCC-PDX lines (see Fig. 3A in Ref. [17], data not shown for 2 of the 16 lines which did not survive) evaluated. For a few lines, viability could be maintained for almost three weeks in culture (see Fig. 2B in Ref. [17]). We verified this quantitatively with CellTiter-Glo® (Fig. 2C and Fig. 4 in Ref. [17]) by using ATP content as a surrogate measure of cell number and found that cell numbers were largely maintained for the HCC-PDX lines that appeared viable by staining. Moreover, by probing for Ki-67 expression, we found that even though there was a lack of significant measurable growth during two weeks of culture, all HCC-PDX lines formed organoids with varying proportions of Ki-67⁺ cells, indicating that the in vitro conditions maintain the proliferative potential of the cultured HCC-PDX cells (Fig. 2B and Fig. 3B in Ref. [17]).

In summary, the inability to support all HCC-PDX lines in culture reflects the expected inter-tumoral diversity that exists among HCC patients and the immense difficulty of developing conditions that are conducive for the culture of heterogeneous primary HCC cells, as is well-recognized in the field. In subsequent studies, we only characterized 14 matched in vitro-in vivo HCC-PDX lines that remained viable in our culture system. Matched pairs were comprehensively characterized using WES and RNA-seq to determine the degree of genomic and transcriptomic correlation, as well as retention of intra-tumoral heterogeneity.

3.3. HCC-3DPDX retain global genomic profile and mutational signature of in vivo counterpart

The extent to which these matched in vitro-in vivo HCC-PDX...
lines is useful for the study of HCC is dependent on the fidelity of the HCC-3DPDX models in recapitulating the molecular phenotype (key genomic aberrations and signaling pathways) of their in vivo counterparts. We first determined the degree of genomic concordance by subjecting matched in vitro and in vivo models to WES, followed by SNP and INDEL calling. SNPs are genetic variations that occur naturally in the human genome. An insertion/deletion polymorphism, or INDEL, is a type of genetic variation in which a specific nucleotide sequence is present or absent. The extent of SNP and INDEL overlap was used to determine the degree of genomic concordance between matched pairs. As shown in Fig. 3A and B (also see Table 1 in Ref. [17]), high SNP and INDEL concordance was observed between matched in vitro-in vivo pairs for all HCC-PDX lines, except one line (HCC4-PDX). Furthermore, mutational signatures [33] were also conserved (see Fig. 6 in Ref. [17]). This indicates that culture of HCC-PDX cells within the sponge enables the retention of global genomic features of their in vivo counterpart.

Several groups have probed into the genomic landscape of HCC to identify genes that are recurrently altered in HCC and play critical role in pathways involved in hepatocarcinogenesis, including tumor suppressor genes TP53, RB1 and AXIN1 that are inactivated, the Wnt pathway oncogene CTNNB1 and chromatin remodeling genes ARID1A and ARID2, amongst several others [10]. Focusing specifically on these key HCC mutations, we demonstrated that HCC-3DPDX cells largely recapitulated these genomic alterations found in their corresponding in vivo counterpart (Fig. 3C). In summary,
our findings robustly demonstrate preservation of genomic profile when HCC-PDX cells are cultured in our 3D sponge.

3.4. HCC-3DPDX retain gene expression profile of their in vivo counterpart

Given the strong genomic concordance between HCC-PDX cells grown in our sponge system and in vivo, we next asked how in vitro culture affects gene expression, considering that several tumor microenvironmental cues, including vasculature and other stromal components, are absent in culture. Subjecting both sponge-cultured HCC-PDX cells and matched in vivo tissue to RNA-seq, we determined the degree of correlation between the matched pairs by focusing on a set of known HCC dysregulated genes. Ho and colleagues previously evaluated The Cancer Genome Atlas (TCGA) whole transcriptome sequencing data of HCC by comparing the global gene expression profiles of tumor and corresponding normal liver tissue; gene set enrichment analysis (GSEA) indicated that various genes associated with cell cycle processes were frequently up-regulated, while genes associated with metabolic processes such as metabolism of retinol, amino acids, and carbohydrates were preferentially down-regulated in HCC [28]. Focusing on these reported up- and down-regulated genes, we performed Pearson correlation analysis on matched in vitro-in vivo HCC-PDX pairs and found that the correlation coefficient was on the average 0.8 (Fig. 4A, and Figs. 8A and B in Ref. [17]), suggesting strong correlation in gene expression between the two models despite the lack of supporting stromal components.

Variations in the degree of in vitro-in vivo gene expression correlation (Fig. 4A) amongst the different HCC-PDX lines indicate that the in vitro conditions established in this study support the preservation of in vivo gene expression to different extents (PDX line-dependent). This suggests that different HCC-PDX lines may have unique requirements for growth, as would be expected given that
each of these PDX lines were derived from individual patients (an indication of inter-tumoral heterogeneity). Noting significant fold changes in gene expression comparing the two groups in certain genes (represented by red and blue lines in the heatmaps, Fig. 4A), we asked if these differences in gene expression between matched HCC-PDX in vitro and in vivo models might potentially influence important cancer-associated pathways. Pathway analysis was performed using hypergeometric test with adjusted p-value (Benjamini) threshold set as 0.01, to associate the differentially expressed genes reported by Ho et al. [28] with signaling pathways (Fig. 4B). We found that in the set of up-regulated genes, the p53 signaling pathway was significantly enriched with 7 genes involved (adjusted p-value < .01, Fig. 4Bi), but 6 out of these 7 genes preserved their expression patterns when HCC-PDX cells were grown in our sponge system (Fig. 4A and Bi). However, we also found that the majority of the enriched pathways in the set of down-regulated genes were metabolic processes-related, and most of the genes in these pathways were different in expression levels between matched HCC-PDX in vivo and in vitro models (Fig. 4A and Bii). This suggests that metabolic adaptation of the HCC-PDX cells might have occurred in our sponge system. Comparing the expression of 395 genes in cancer-associated pathways in vitro and in vivo, Pearson correlation coefficient was high at approximately 0.9 (Fig. 4C). Furthermore, analysis of genes in specific HCC-associated pathways such as tumor protein p53 (TP53), Wnt, and Ras amongst others indicate a tight range in the degree of correlation (all greater than 0.8) between HCC-PDX grown in vitro and in vivo (Fig. 4C), confirming that HCC-PDX grown in vitro closely match the gene expression profile of the corresponding in vivo HCC-PDX. In summary, characterization of genomic aberrations and gene expression of the various HCC-PDX lines grown in vitro confirms these models have a striking molecular resemblance to their corresponding matched in vivo counterpart.

3.5. Intra-tumoral heterogeneity of HCC-PDX cells is retained in culture

HCC is a disease with high intra-tumoral heterogeneity in which several signaling cascades are altered [9]. We used WES data to determine whether HCC-PDX cells grown in our sponge system retain the intra-tumoral heterogeneity inherent in their matched in vivo counterpart. Quantification of variant allele frequency as a surrogate measure of intra-tumoral heterogeneity revealed that the in vivo HCC-PDX models had a range of scores, as expected given their diversity (indicated by the genomic and transcriptomic analyses above). A score of 100% indicates that there is no heterogeneity present while a score of less than 100% indicates the presence of sub-clonal populations. As shown in Fig. 5, the variant allele frequency scores in matched in vitro and in vivo models were similar for 9 out of 11 HCC-PDX lines, highlighting that in vitro HCC-PDX models largely capture the intra-tumoral heterogeneity of their in vivo counterparts. Notably, the mismatch in variant allele frequency for HCC4-PDX corroborates with the poor SNP and INDEL overlap (Fig. 3) as well as gene expression correlation (Fig. 4A) observed, suggesting that for this particular line, the sponge system is not able to maintain the clonal dynamics of the in vivo tumor. Nevertheless, contrary to what is expected of in vitro culture (that there would be clonal selection resulting from adaptation to in vitro culture conditions), overall, our data suggests that the sponge system well-preserves the intra-tumoral heterogeneity of the corresponding in vivo counterpart for the majority of HCC-PDX lines.

3.6. HCC-PDX cultures are amenable for drug testing in vitro

To evaluate the feasibility of using these HCC-PDX cells grown in our 3D macroporous sponge for drug assay development, we
employed the use of Sorafenib (Nexavar) and BGJ-398. Sorafenib is an FDA-approved multi-kinase inhibitor for advanced HCC patients [34] whereas BGJ-398 is a pan-fibroblast growth factor receptor (FGFR) inhibitor which has been explored for use in certain sub-classes of HCC [35]. In vitro drug treatment on HCC-3DPDX organoids showed dose-dependent decrease in cell viability, with cell kill of 87% and 37% at the highest concentration for Sorafenib and BGJ-398, respectively (Fig. 6A). In addition, an immunoblot analysis on these treated cells showed dose-dependent decrease in the levels of phosphorylated-Erk and a significant decrease in phosphorylated-Akt levels in response to Sorafenib and BGJ-398 respectively (Fig. 6B) as previously reported [20,36]. Our data suggests that HCC3-3DPDX is sensitive to both Sorafenib and BGJ-398, and that HCC-PDX cells grown in our sponge system can potentially be used for HCC drug discovery and development.

4. Discussion

The recent paradigm shift away from the use of cancer cell lines has catapulted PDX models as the gold standard for use in pre-clinical studies and drug development [4,37]. The use of PDX models is particularly important for cancers such as HCC, which has extensive heterogeneity of which individual cancer cell lines are largely unable to recapitulate. While PDX models are highly relevant, the use of these models for high throughput drug studies is significantly limited by the demanding nature of the model, requiring high costs and lengthy experimental durations. To overcome this problem, efforts have been made to develop in vitro counterparts to PDX models, which not only increase the throughput of experiments, but potentially also reduce costs and alleviate animal welfare concerns [38,39]. However, the same has yet been achieved for HCC as primary HCC cells are notoriously challenging to culture in vitro; there are only very few reports in the literature describing in vitro culture conditions for HCC cells derived either directly from patients [9,40], or from HCC-PDX models [12,13]. Specifically, Cheung et al. [12] and Xin et al. [13] reported the establishment of cell lines from HCC-PDX models. However, phenotypic characterization and correlation with the corresponding in vivo PDX models were limited in these studies, making it challenging to assess the fidelity of these 2D HCC-PDX models in recapitulating the HCC-PDX in vivo phenotype. Furthermore, these methods generally involve the use of adherent monolayer culture, which has been linked to clonal selection, acquisition of genetic alterations and changes in gene expression [5,6,40]. In the past few decades, numerous studies have shown that 2D cultures are not representative of the in vivo situation [41–43]. Particularly for HCC, studies have shown that drug response between 2D and 3D cell cultures are different, where cells in 3D cultures were found to be more resistant than those in 2D cultures [44]. Additionally, HCC cells grown in 3D were also found to be more tumorigenic than those grown in 2D [45]. Hence, in this study, we chose to focus only on developing 3D conditions amenable for the culture of HCC-PDX organoids. To our knowledge, we are one of the first to establish robust 3D in vitro culture conditions that supports the culture of HCC-PDX lines, with retained viability, proliferative capacity, genomic profile, gene expression and intra-tumoral heterogeneity.

In seeking to develop in vitro conditions suitable for the culture of HCC-PDX organoids, we asked whether existing 3D platforms that have been shown to be conducive for the culture of normal hepatocytes would similarly support the culture of cancerous HCC-PDX cells. Various methods are available to culture hepatocytes in 3D [46]. However, these approaches generally do not provide appropriate biochemical and mechanical cues necessary to maintain hepatocyte function. We previously reported the development of a macroporous cellulose sponge system specifically engineered for the culture of normal hepatocytes as constrained spheroids with preserved hepatic morphology and functions based on in vivo-like mechanical and biochemical properties [15]. However, fabrication of this sponge system is challenging due to the use of moisture-sensitive reagents (anhydrous chloroform) which may generate batch-to-batch variations. Accordingly, in this study, we replaced allyl as the photo-crosslinkable group to MA, as well as replaced the use of chloroform with dichloromethane as the solvent for the reaction (Fig. 1B) to synthesize MA-HPC as the HPC derivative that undergoes TIPS and crosslinking. As shown in Fig. 1A, MA-HPC retains the ability to undergo TIPS, which we ‘fixed’ using photocrosslinking via gamma irradiation. The resulting sponge system remains highly macroporous, with porosity, mechanical and biochemical properties similar to our previously developed sponge system [15]. Notably, individual sponge pieces are fabricated thin to minimize drug adsorption and to support high throughput applications, similar to conventional multi-well plates. Importantly, we found that this sponge system was able to support the rapid

Fig. 6. Evaluation of standard and experimental drug response on HCC-3DPDX Response of HCC3-PDX to sorafenib and BGJ-398 as assessed by (A) CellTiter-Glo® and (B) western blot.
organoid formation of HCC-PDX cells and maintain the viability and proliferative capacity of the majority of HCC-PDX lines we tested (Fig. 2). Furthermore, each HCC-PDX in vivo model can give rise to tens to hundreds of HCC-3DPDX samples, greatly expanding the throughput of the PDX model. Extrapolation of in vitro conditions established for normal cells to cancer cells is an approach that has been explored for tumor engineering [41,47] and our data strongly supports this, suggesting that other cancers may also benefit from such an approach.

While we observed the presence of proliferative (Ki-67⁺) cells, the lack of measurable cell growth during culture was perplexing. We suspect that there is a sub-population of cells that are dying as well during culture. Hence, there is cell turnover which maintains cell number over time. A similar observation was previously reported with prostate cancer PDX cells in 3D culture [39]. Additionally, the physical constraint exerted by the macropores of the sponge may have limited the proliferation of the HCC-PDX organoids. Incorporating degradability into the sponge system, such as through the use of matrix metalloproteinase-sensitive moieties, may potentially resolve this issue. Beyond being able to maintain the culture of HCC-PDX organoids, our data clearly demonstrates the strong concordance in genomic and gene expression profiles between matched in vitro-in vivo HCC-PDX pairs (Figs. 3 and 4).

Using SNP and INDEL calling and mutational signatures, we found that 10 out of 11 of the in vitro-in vivo HCC-PDX pairs shared similar genomic profiles, except for HCC4-PDX. Not unexpected, HCC4-3DPDX also poorly recapitulated known HCC mutations such as NFI, CUL2 and ACVR2A. This was confirmed by subsequent analysis indicating that HCC4-3DPDX poorly correlated with its in vivo counterpart in terms of expression levels of known dysregulated HCC genes (Fig. 4), as well as its inability to maintain in vivo levels of intra-heterogeneity (Fig. 5). Comparing gene expression profiles, that metabolism-related genes surfaced as significantly different between the in vivo and in vitro models (Fig. 4B) suggests that our existing 3D sponge system is yet able to support in vivo-like metabolic activities. Schutte and colleagues reported that patient-derived colorectal cancer organoids grown in culture exhibited differential expression of genes involved in carbohydrate, steroid, retinoid and fatty acid metabolism as compared to the PDX in vivo model and original patient tumor [48], implying that metabolic adaption to cell culture conditions may be an intrinsic consequence of in vitro culture.

It is possible that clonal selection might have occurred for HCC4-PDX when grown in our sponge system. Indeed, Qi and colleagues showed that early-passage patient-derived HCC cells when grown in culture gain approximately half of total new mutations in vitro, attributing this to clonal selection of HCC cells during culture [40]. Notably, cancer cell adaptation resulting in changes in clonal architecture also occurs for organoid culture [49], and even PDX in vivo models [11], highlighting the challenge of developing in vitro conditions suitable for maintaining primary cancer cells. In contrast to conventional spheroid-generating methods such as the hanging drop technique or ultra-low attachment surfaces, our 3D bioengineered sponge enables control over biochemical and mechanical cues needed to recapitulate the tumor microenvironment.

Ongoing investigations are being undertaken in our laboratory to modify the biochemical and mechanical properties of our sponge system to accommodate the culture of HCC-PDX lines such as HCC4-PDX with unique growth requirements. For example, the mechanical properties of the sponge may be modulated by changing the degree of MA grafting onto the HPC polymer backbone, or changing the duration of gamma irradiation or concentration of the MA-HPC solution used for sponge fabrication. Nevertheless, despite the complexity of the HCC microenvironment—an ecosystem comprising various players (stromal cells and the dynamic extracellular matrix) that shape the cancer cell phenotype spatiotemporally [50]—that the majority of the HCC-PDX lines evaluated in this study conserved key molecular features including intra-tumoral heterogeneity uniquely positions our sponge system as a novel platform for primary HCC culture with high translational value for the study of HCC and drug development.

In summary, we demonstrated the feasibility of using a 3D macroporous sponge system to maintain HCC-PDX organoids in vitro, which increases the cost-effectiveness and throughput of current HCC-PDX in vivo models for preclinical drug development in HCC.

Potential conflict of interest

Pishon Biomedical Co. Ltd and Invitrogen Ltd have licensed technology relevant to the class of cellulose sponge materials from ETPL, A*STAR and H.Y. has equity in these companies. There is no other conflict of interest otherwise.

Author contributions

E.L.S.F. conceived the experiments and wrote the manuscript. E.L.S.F., T.T.B and T.H.H. carried out the experiments. Z.L. designed and fabricated the sponge. All bioinformatics analyses in this study were performed by T.B. and performed by X.L. L.H. and M.R. provided technical support. E.L.S.F., T.T.B, E.K-H.C, T.H.H. and H.Y. contributed to the interpretation of the results and provided critical feedback and helped shape the research, analysis and manuscript.

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References


