Cell Organoid

Article

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An advanced culture methodology suitable for the self-assemble and tissue-fragment derived intrahepatic cholangiocarcinoma organoids

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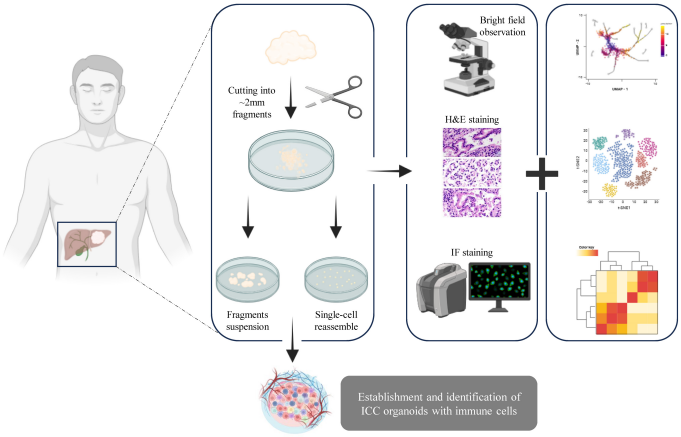
Graphical abstract



In Brief

We have established an efficient and rapid ICC organoid model that recapitulates and maintains the disease characteristics of ICC *in vitro*. Bulk RNA-seq revealed the presence of viable immune cells in this organoid model, which was not observed in previous established ICC organoid models. Our work provides a novel model for studying immunotherapeutic approaches in ICC.

Highlights

- An advanced and user-friendly organoid culture methodology
- Consistent genome stability and gene expression profiles throughout the culture process
- Potential for high-throughput drug screening without reliance on matrigel

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An advanced culture methodology suitable for the self-assemble and tissue-fragment derived intrahepatic cholangiocarcinoma organoids

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ABSTRACT

Intrahepatic cholangiocarcinoma (ICC) is a highly lethal malignancy associated with significant morbidity, necessitating the urgent development of an effective chemotherapeutic assay for ICC patients. In this study, we have successfully established an advanced culture method for ICC organoids that can be utilized with both single-cell assembly and tissue fragmentation initiation techniques. These ICC organoids maintain the morphological characteristics, including mutation profiles and frequency (46.9% in organoid and 48.5% in tumor tissue) of *IDH1* genes, and 1733 high-frequent overlapped mutated genes (94.2%). Additionally, ICC biomarkers such as CK7 and CK19 also maintain a similar pattern compared with the original tissue. Furthermore, RNA-seq analysis reveals upregulation of immune-related genes in single-cell assembly organoids. The significantly changed genes including *IL9R* (4.4-fold), *IL2RB* (3.2-fold), *CCR4* (3.5-fold), *TESPA1* (4.4-fold), *ZAP70* (4.3-fold) and *CD6* (4.3-fold) in log scale. These evidence both indicating the presence of viable and active immune cells. Overall, our findings present an advanced and user-friendly culture approach for generating ICC organoids adaptable to diverse experimental objectives.

KEYWORDS

intrahepatic cholangiocarcinoma, organoid, culture methodology

Introduction

ICC (intrahepatic cholangiocarcinoma) is one of the most lethal liver cancers, and the overall 5-year survival rate for patients after surgery is only around 9%[1]. Moreover, the incidence and mortality of ICC are increasing rapidly, endangering the lives and health of patients[2]. The most efficient therapy for ICC patients is curative-intent surgical resection; however, the 5-year overall survival rate after surgery is only 20%–35%[3]. Because of this, chemotherapy plays a critical role in ICC treatment, but the efficacy of drugs such as gemcitabine and oxaliplatin varies among different patients[4–8]. Therefore, it is important to establish a fast and precise drug testing method for clinical treatment.

Unfortunately, analysis of drug response features and biomarker investigation for precision therapy in patients with ICC is still lacking. Patient-derived organoids (PDOs) have been established and utilized in research related to clinical cancer treatment in recent studies, including liver, colon, and pancreas cancers[9–11]. However, these matrigel-based organoid culture methods are relatively difficult to handle for beginners, limiting their application in clinical therapy. While matrigel-based organoids can reflect molecular features of origin tissues, the small volume of single-cell derived organoids makes it difficult to reflect millimeter-scale morphological features accurately. Therefore, a more advanced culture method is needed for clinical treatment and scientific research purposes.

Here, we present an advanced robust ICC organoid culture method that is compatible with both single-cell derived and tissue-fragment derived organoids. Tissue-fragment derived organoids maintain morphology features due to their relatively large volume (millimeter scale). Both types of organoids maintain gene

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expression profiles compared with public ICC data, suggesting their high-throughput drug screening potential.

Methods and materials

Tissue collection

The ICC tumor tissues were obtained from patients undergoing clinical surgery. Samples from four patients were utilized for this study. If necessary, samples were temporarily stored at 4 °C until processed.

Microscopy observing

Daily observations were performed using an inverted microscope (DMi1, Leica, USA), and immunofluorescence was observed with a fluorescence microscope (K5, Leica, USA).

Culture of tissue-fragment derived organoids

ICC tumor tissues were washed at least 3 times with a washing solution (LSNO00100201, Shanghai LiSheng Biotech, China), then fragmented into diameters of 0.5–2 mm using scissors. Tissue-fragment derived organoids were collected and resuspended in medium (LSTO01000403, Shanghai LiSheng Biotech, China), then transferred into 10 cm dishes. The medium was semi-replaced every week. The information of washing solution and medium can be found on the website: http://www.sh-lise.com

Culture of single-cell derived organoids

Tissue-fragment derived ICC organoids were collected after 7–14 days and then digested into single cells using Organoid Dissociation Reagent I (LSNO00100501, Shanghai LiSheng Biotech, China) at 37 °C for 15–45 min. When the volume of organoids decreased by half, the digestion liquid was filtered with a 40 μm cell strainer (352340, Corning, USA) and centrifuged at 600× *g* for 10 min. The supernatant was removed and 2 mL of medium was added to resuspend the cells. The cells were centrifuged again at 600× *g* for 10 min and then resuspended in 1 mL of medium. Finally, the cells were cultured in Clear TC-treated Multiple Well Plates (3516, Corning Incorporated, USA).

Hematoxylin and Eosin (H&E) staining

Organoids were fixed with 4% formaldehyde (BL539A, Biosharp, China) for at least 24 h and embedded in Tissue-Tek O.C.T. compound (4583, Sakura Finetek, USA). Samples were sectioned into 10 μm slices using a cryostat microtome (CM 1950, Leica, USA). The sections were stained with hematoxylin solution (BL700A, Biosharp, China) for 5 min and eosin staining solution (BL700B, Biosharp, China) for 30 s. Finally, the sections were mounted with neutral balsam (G8593, Solarbio, China) and observed under a microscope (YI21, Shanghai Yuehe, China).

Immunofluorescence

Sections were permeabilized with PBS+0.25% TritonX-100 buffer for 20 min at room temperature, followed by blocking with a blocking buffer (E674004, BBI Life Science, China). Rabbit anti-CK7 (ab68459, Abcam, USA) and CK19 (ab76539, Abcam, USA) primary antibodies were diluted at a ratio of 1:200 and incubated with the sections overnight at 4 °C. The sections were then washed five times using 0.125% phosphate buffered saline with Tween 20 (PBST) and incubated with a secondary Cy3 antibody (711-165-152, Jackson ImmunoResearch Laboratories Inc., USA) diluted at a ratio of 1:500. Nuclei were stained with DAPI (diluted at a ratio of 1:1000). Fluorescence images were acquired using a Leica K5 microscope.

Whole exome sequencing

For each sample, 200 ng of genomic DNA was shared with 150–200 bp fragments to construct libraries. The whole exome was captured using AIExome® Human Exome Panel V3 with TargetSeq One® Hyb & Wash Kit v2.0 (iGeneTech Co., Ltd, Beijing, China) and sequenced on DNBSEQ-T7 with 150-bp reads.

RNA sequencing

RNA Isolation Kit (DP451, TIANGEN, China) was used to isolate cellular mRNA. The RNA concentration was measured using a Qubit4 fluorometer. Reverse transcription was performed using an RT Kit (KR118, TIANGEN, China) and libraries were generated using an RNA Library Prep Kit (E7530L, NEB, USA). Both libraries were sequenced on DNBSEQ-T7 with PE-150 bp reads for subsequent analysis.

Statistical analysis

Angle degree and size of organoids were measured with ImageJ and data analysis was performed using Prism software. All data are expressed as mean ± standard deviation. Expression level of immune genes was measured with TPM value, and visualized with Omicshare website tool. Mutations from WES were annotated with ANNOVAR. TPM value was generated with Salmon, and clustered with factoextra package, and DEGs were found with DESeq2. GO analysis were performed with ClusterProfiler website tool.

Results

Establishment and identification of tissue-fragment derived ICC organoids

We obtained primary tumor tissue from patients with intrahepatic cholangiocarcinoma (ICC) and generated ICC organoids using two distinct methodologies: single-cell isolation and tissue fragmentation (Fig. 1a). For the generation of tissue-fragment derived organoids, patient tissues were mechanically dissected into fragments measuring approximately 0.5–2 mm in diameter using surgical scissors, followed by suspension culture in a specialized medium. In contrast, for the production of single-cell derived organoids, patient tissues were enzymatically digested to obtain individual cells which were subsequently cultured under suspension conditions.

The mechanical fragmentation method used for organoid generation results in tissue-fragment derived ICC organoids exhibiting jagged edges at the beginning of the culture period. However, over time, these organoids acquire a smoother edge, indicating their growth and self-repair capabilities (Fig. 1b). We took the minimum angle degree to measure the growth of organoids. During the culture, the minimum angle degree of organoids increase to 180° (spherical), indicate that ICC organoids could repair and regenerate.

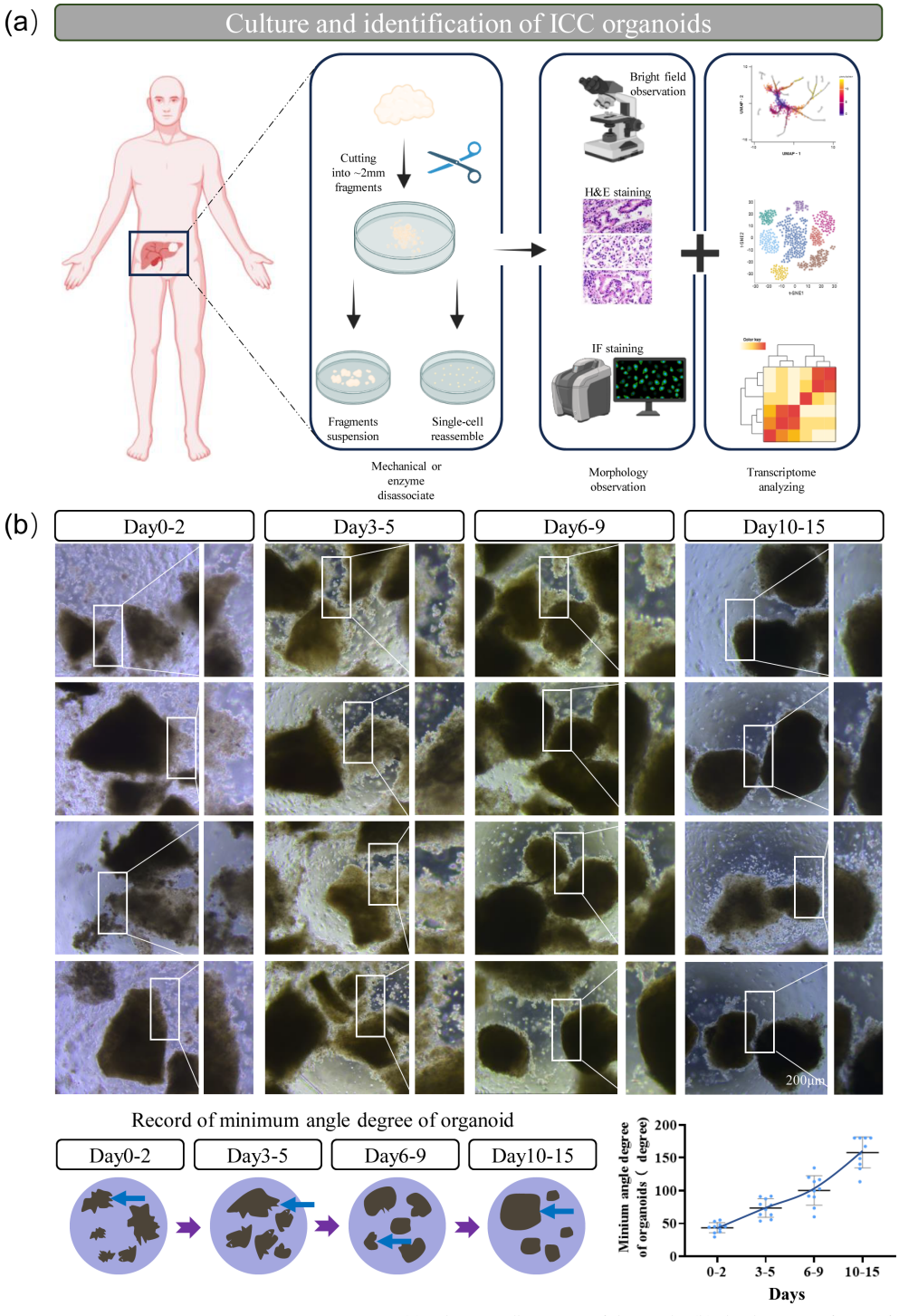

Figure 1. Refined establishment protocol for the culture of organoids: (a) Schematic illustration of this study; (b) development of tissue-fragment derived ICC organoids. During the culture period, the organoid edges exhibited a smooth appearance, indicating growth and self-repair. Top: microscopy observation of ICC organoids. Bottom: the degree of minimum angle of organoids (left), measurements of the growth of organoids (right). The scale bar represents 200 μm.

Morphology and molecular features of tissue-fragment derived ICC organoids

One of the key features of organoids is their ability to recapitulate characteristics of their tissue of origin. Therefore, we conducted immunocytochemistry experiments on ICC organoids derived from tissue fragments and compared them with the corresponding tissue source. CK7 and CK19 are biomarkers for biliary epithelial cells, which were found to be significantly upregulated in ICC tumor tissues[12]. The expression patterns of CK7, CK19, and CD45 in organoids and tissue were found to be similar, indicating that organoids maintain the pathological features of their origin tissue at a millimeter scale, and maintain immune microenvironment (Fig. 2a). Additionally, H&E staining of ICC organoids demonstrated the resemblance between ICC organoids and tissue (Fig. 2b).

To further validate the stability of organoids, whole exome sequencing (WES) was conducted to investigate the mutation

profile. The analysis revealed that the number and type of mutations in organoids remained consistent (1733 mutations affecting 94.2% genes, as shown in Fig. 2c), indicating the robustness of our culture method and medium. Additionally, we identified a mutation in *IDH1* (exon6:c.G532A:p.V178I), a key gene involved in intrahepatic cholangiocarcinoma (ICC) development, which was also maintained in the organoids (Fig. 2c). Therefore, both morphological characteristics and

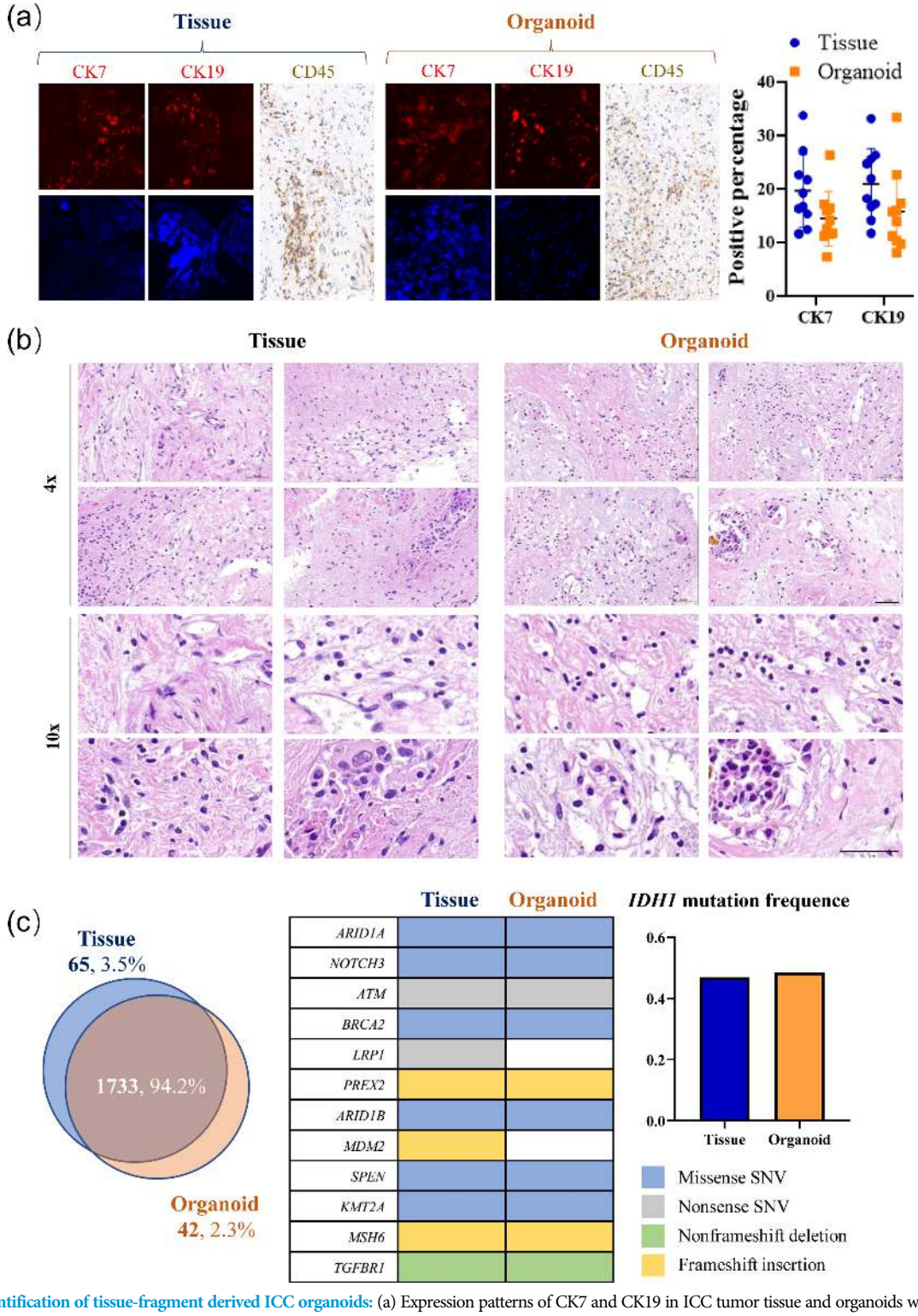

Figure 2. Identification of tissue-fragment derived ICC organoids: (a) Expression patterns of CK7 and CK19 in ICC tumor tissue and organoids were found to be similar, with a consistent pattern observed in both tissue samples and organoids. The scale bar represents 200 μm. (b) H&E staining revealed that the cancer region was preserved in the organoids compared to its original tissue. The scale bar represents 200 μm. (c) Comparison of gene mutation numbers between tumor tissue and organoids showed that mutations with an allele frequency > 0.25 were selected for analysis. Both tissue samples and organoids exhibited IDH1 exon6:c.G532A:p.V178I mutation, which remained stable even after 14 days of culture.

genomic features were preserved in tissue-fragment derived ICC organoids, demonstrating the feasibility of our advanced culture approach.

Establishment of single-cell derived ICC organoids

Although tissue-fragment derived ICC organoids can capture the diverse characteristics of ICC tumor tissue, their application potential in the industry field, such as high-throughput drug screening, is limited due to their relatively large volume and low throughput. In the matrigel-based organoid culture method, matrigel serves as a scaffold for single cells, facilitating the generation of self-proliferating organoids. However, handling matrigel can be challenging for beginners and both time and financial resources required are substantial. Therefore, we investigated the potential of ICC cells to form organoids in suspension culture without the use of matrigel. Intriguingly, after only 20 h of suspension culture in medium, the cells self-organized into organoids (Fig. 3a). These organoids exhibited growth and proliferation throughout the culture period, indicating their suitability for high-throughput screening applications (Fig. 3b). However, due to their small size and suspended state, obtaining sections of these compact organoids was challenging.

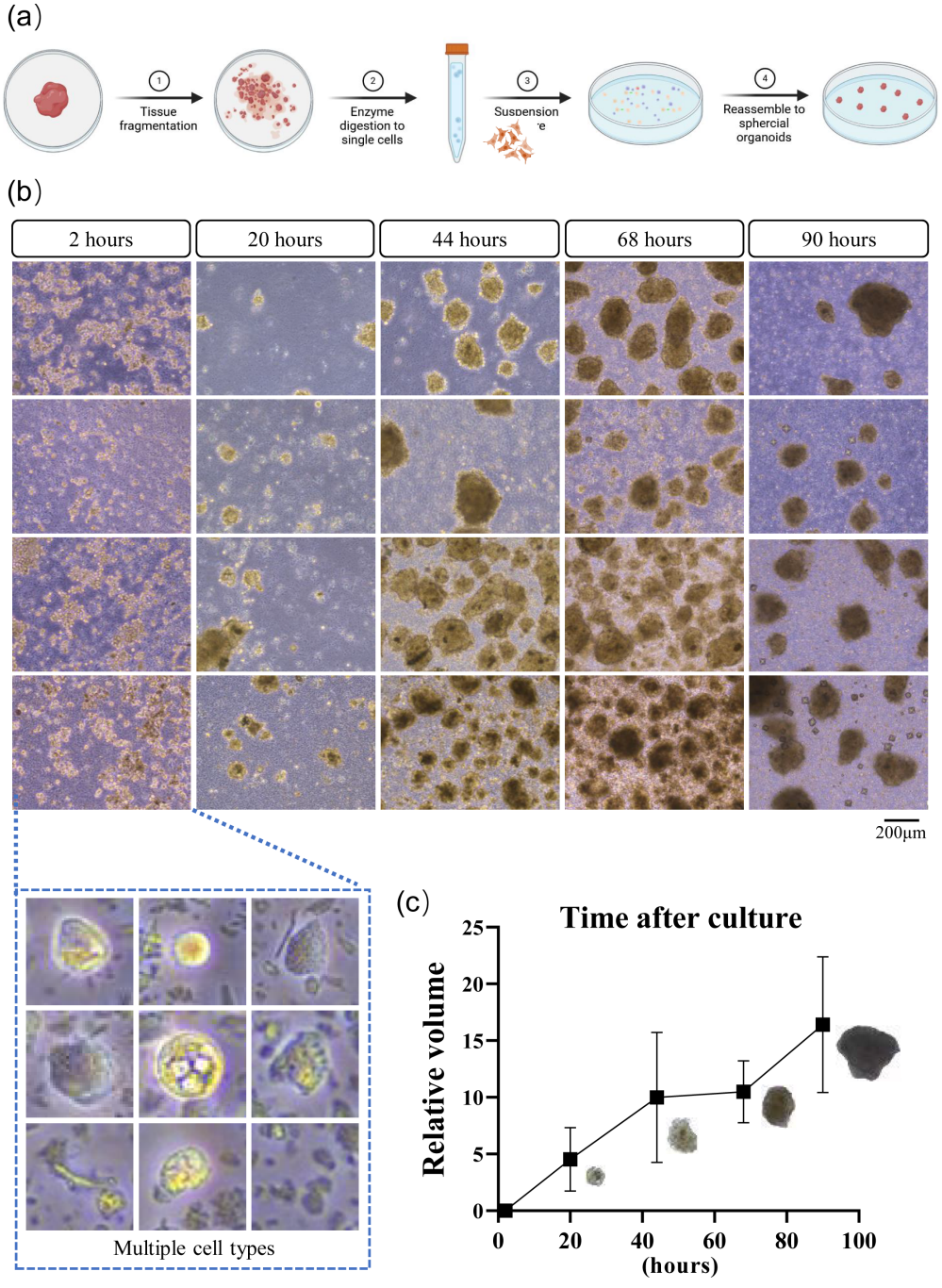

Figure 3. Observation of single-cell derived ICC organoids: (a) Schematic diagram of generation of single-cell derived ICC organoids. (b) Development and growth of single-cell-derived ICC organoids. The organoids exhibiting increased volume during culture after 20 h. Scale bar represents 200 μm. (c) The change of relative volume of organoids during culture.

Gene expression profile of ICC organoids and clinic tissues

To gain a comprehensive understanding of ICC organoids using our advanced methodology, we conducted RNA-seq analysis at day 3, day 6, and day 9. Furthermore, we also examined publicly available ICC tissue and organoid datasets, GSE107943[13,14] and GSE215997[15]. The cluster dendrogram reveals that large duct type ICC organoids (SRR21949015-21949019) are grouped together in a distinct branch. Interestingly, our organoids exhibit proximity to ICC tumor tissues (SRR6373759-6373787), as supported by the correlation heatmap analysis (Figs. 4a and 4b). This suggests that

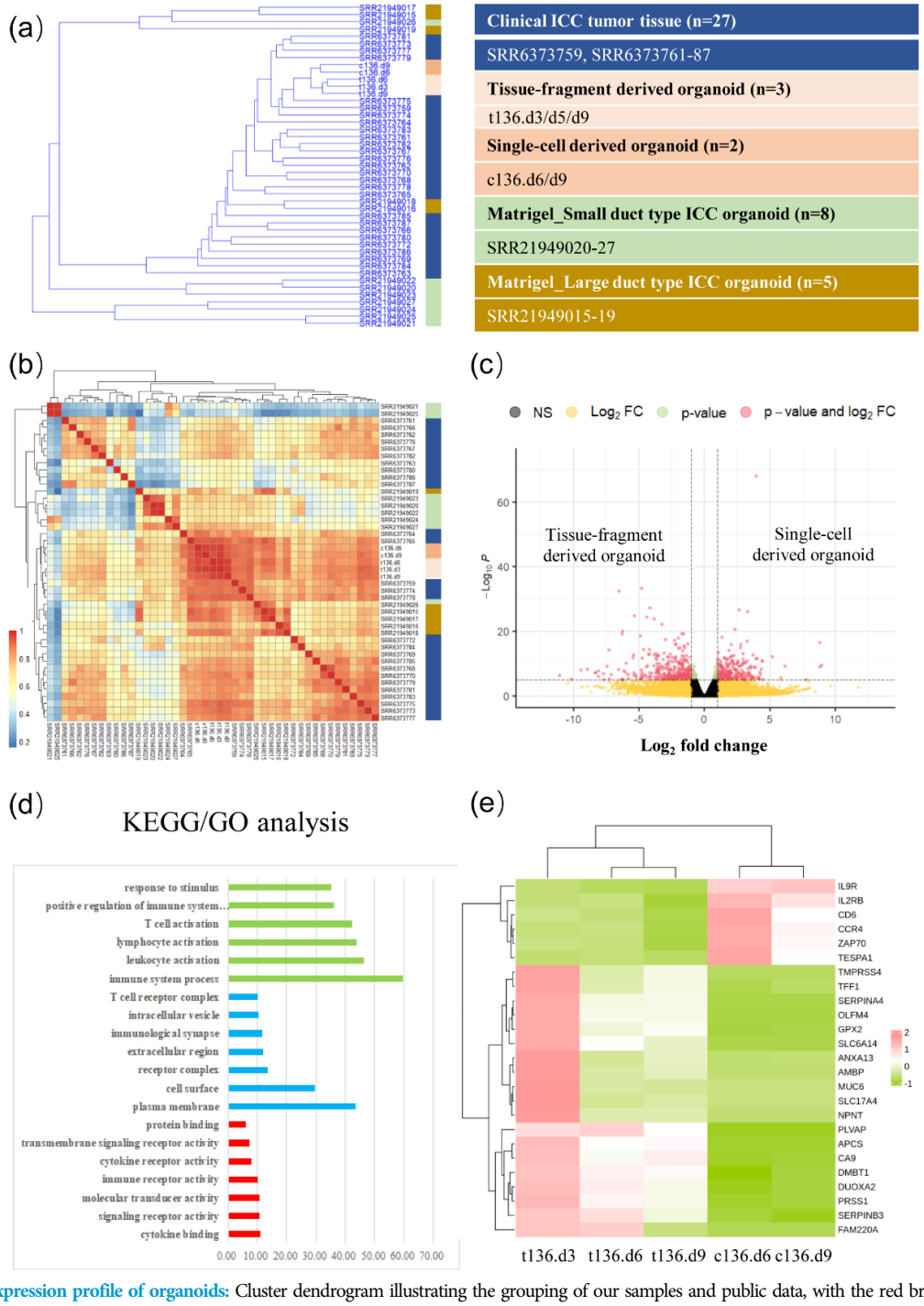

Figure 4. Gene expression profile of organoids: Cluster dendrogram illustrating the grouping of our samples and public data, with the red branch indicating the presence of large duct type ICC organoids. Our organoids clustered closely with clinical ICC tissues. (b) Correlation heatmap depicting the strong correlation between ICC tissues and both single-cell and tissue-fragment derived organoids, highlighting the robustness of our culture method. (c) Volcano map displaying differentially expressed genes (DEGs) between single-cell and tissue-fragment derived organoids. While most genes maintained stable expression levels, DEGs were still observed due to variations in culture protocols. (d) Gene Ontology (GO) analysis revealed up-regulated immune pathways in single-cell derived organoids. (e) Heatmap of DEGs express in tissue and single-cell-derived organoids, suggesting that different pathways were activate, and immune cells persisted within the organoid environment despite being under abnormal activation states.

our organoids share similarities with clinical ICC tumor tissues. However, it is worth noting that single-cell and tissue-fragment organoids are clustered into separate branches, indicating inherent differences between these two types. To quantify the impact of these dissimilarities, we examined differentially expressed genes (DEGs) between the two types of organoids. A total of 1638 genes (5.9%) were found to be up-regulated, while 2132 genes (7.7%) were down-regulated in single-cell derived organoids (Fig. 4c). Gene Ontology analysis revealed distinct alterations in pathways between the two types of organoids. Notably, immune-related pathways exhibited upregulation in single-cell derived organoids (Fig. 4d). Further investigation into these pathways identified several immune-related genes that were upregulated, including *IL9R, CCR4* and *IL2RB* etc. (Fig. 4e). This observation may be attributed to the stimulation caused by enzyme digestion; however, it was also confirmed that the presence of immune cells within the culture environment is crucial for immune drug testing.

Discussion

Although the rapid development of the organoid field has provided researchers with a novel model, certain limitations still persist. The widely-used matrigel-based organoid culture method poses challenges for beginners and is time-consuming, rendering it unsuitable for clinical practitioners. In this study, we present an advanced ICC organoid culture method that eliminates the need for matrigel, making it accessible to both single-cell and tissue-fragment derived ICC organoids. Our developed method ensures the preservation of molecular and morphological features such as CK7 and CK19 expression in these organoids, while exhibiting a gene expression profile similar to that of public ICC tumor tissue data. Additionally, the data from whole-exome sequencing indicate that organoids exhibit similarity in mutation sites and the number of genes compared to the tissue of origin. This suggests that organoids maintain genomic stability at the chromosomal level. These compelling findings validate the usability and robustness of our advanced ICC culture method.

Our future research will focus on investigating the microenvironment of organoids in greater detail. The fragmentation culture method we have employed has shown promise in preserving the tissue microenvironment and organization pattern, albeit temporarily (Fig. 5). However, there is still much to learn about how this preservation can be optimized and extended. One significant advantage of our tissue-fragment derived ICC organoids is their ability to retain the local tumor immune microenvironment that is lacking in matrigel-based organoid culture methods. This opens up new possibilities for studying the interactions between cancer cells and immune cells within a more physiologically relevant context. In particular, we are interested in determining whether this preserved microenvironment can maintain its biological function over time. Understanding how these organoids respond to various stimuli and treatments will provide valuable insights into the mechanisms underlying tumor-immune cell interactions. It is worth noting that immune drugs such as pembrolizumab have already demonstrated success in clinical therapy for ICC cancer patients. By utilizing our advanced organoid culture method, we hope to contribute significantly to drug screening efforts aimed at identifying novel immunotherapies or optimizing existing ones for a larger patient population. Furthermore, by studying the microenvironment of these organoids, we may uncover potential biomarkers or therapeutic targets specific to ICC cancers. This knowledge could lead to personalized treatment strategies tailored specifically for individual patients based on their unique tumor characteristics.

Research ethics and patient consent

The study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University (B2018-018(2)). Patient gave informed consent to this study.

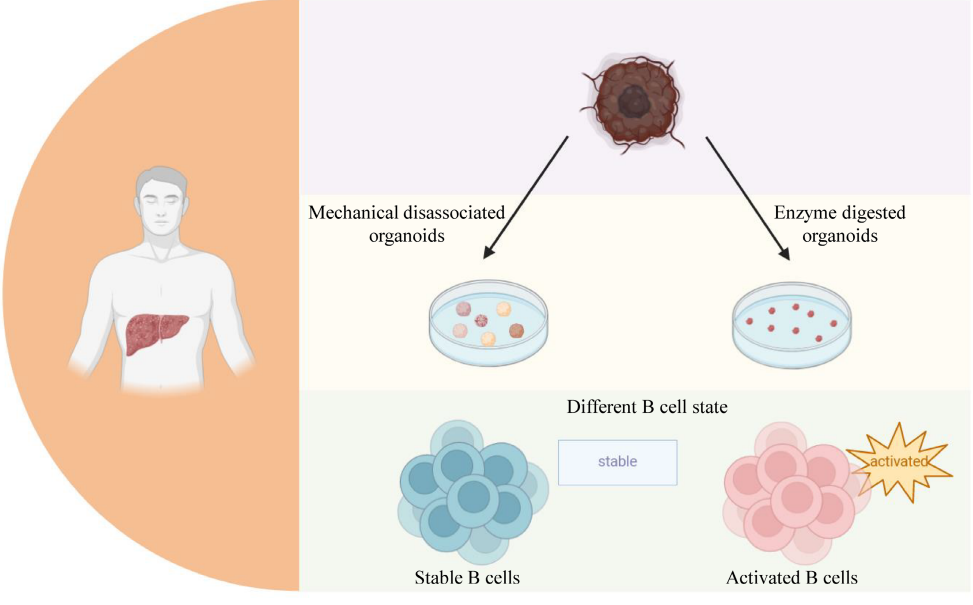

Figure 5. Different B cell state in two different protocols: Different organoid construction methods can lead to varying states of B cells. In organoids derived from single cells, B cells are activated, possibly due to the exposure of new antigenic epitopes during the enzymatic digestion process, which stimulates the immune cells. In contrast, in organoids from tissue fragments, B cells remain in a stable state.

Declaration of conflicting interests

This work was sponsored by Shanghai Lisheng Biotech Ltd (Lisheng). X.X.H. is a shareholder of Lisheng, as a founder. C.W., Y.H.S., J.P.L., L.Y.L., J.Z., M.J.R., and C.H.C. are senior scientists of Lisheng. X.X.H. and C.H.C. are members of the Editorial Board for *Cell Organoid*. They were not involved in the journal's review of, or decisions related to, this manuscript. All authors declare no competing financial interests.

Availability of data and material

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

X.Z, C.H.C. and X.X.H conceived and designed the study. C.W, Y.H.S, L.Y.L, J.Z, M.J.R and A.H were responsible for sample collection and experimental procedures. J.P.L conducted the analysis of the RNA sequencing data. C.W, C.H.C and X.X.H. wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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