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Crossroad of ovarian cancer organoid culture: Single cell suspension and mechanically sheared fragment

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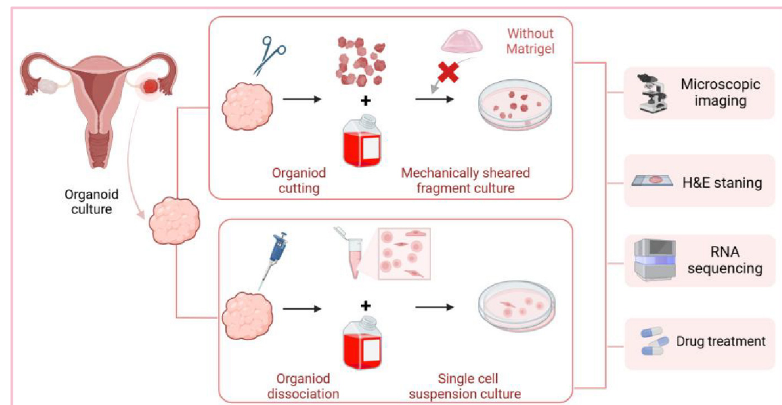
Graphical abstract



In Brief

This study explores the cultivation of uniformly-sized ovarian cancer organoids from single-cell suspensions, addressing challenges in high-throughput drug screening and providing insights into personalized treatment strategies against chemotherapy resistance.

Highlights

- Rapid and efficient generation of uniformly-sized organoids from single cell suspensions
- Consistent gene expression in two high-fidelity ovarian cancer organoid models
- Uniformly-sized ovarian cancer organoids show consistent, reliable drug responses for personalized chemotherapy resistance tests

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Crossroad of ovarian cancer organoid culture: Single cell suspension and mechanically sheared fragment

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ABSTRACT

Ovarian cancer, a common gynecologic tumor, is associated with a high mortality, due to challenges in early detection within the reproductive system. According to our previous research, cultivating patient-specific organoids from mechanically sheared tissues can be utilized for drug response evaluation but has limitations for high-throughput screening efficiency due to their inconsistent size. In this research, we focused on organoids developed from single-cell suspensions to address the critical requirement for uniformity in organoid size. By the day 3 of culture, single-cell suspensions rapidly and spontaneously aggregated into spherical structures with a more consistent size. Notably, the organoids of sample OVA-37 were ten times larger after 8 days of culture. Transcriptomic analysis was used to compare the two organoid culture techniques, demonstrating that the variations between different organoid culture methods were minimal, with higher variability observed among patients. Gene set enrichment analysis (GSEA) revealed only minor discrepancies in specific pathways, such as TGF-β and tight junctions. Furthermore, treatment with carboplatin in a 96-well plate setup resulted in reproducible drug responses, as evidenced by coefficients of variation lower than 40%. This finding suggests that single-cell suspension-cultured organoids can be employed for reproducible high-throughput drug screening. This approach holds potential for personalized drug screening in ovarian cancer and may contribute to the development of novel therapeutic strategies.

KEYWORDS

ovarian cancer, organoid, drug sensitive, personalized medicine

Introduction

Ovarian carcinoma represents a significant fraction of female reproductive system malignancies, posing a global health hazard. Its early detection remains elusive, chiefly due to the indistinct nature of early symptoms, thus complicating the diagnostic process [1]. The disease exhibits a heterogeneity marked by varied histological subtypes and a wide range of molecular expression patterns, which contributes to disparate drug responses and the emergence of drug resistance [2]. The intricate genetic mutation landscape in ovarian cancer presents formidable challenges to efficacious treatments [3]. Personalized treatment strategies, pivotal for enhancing patient outcomes, rely on advanced laboratory techniques that accurately predict individual drug responses [4]. Progress in organoid technology, particularly the development of self-organizing, three-dimensional tissue cultures that emulate the complexity and functionality of *in vivo* tissues, has become indispensable in drug response evaluation. Exploiting patient-derived tumor organoids for identifying potent chemotherapy agents enables personalized medicine to devise treatment regimens congruent with unique genetic and physiological profiles of individuals, potentially augmenting therapeutic efficacy and diminishing adverse effects [5]. Our research, focusing on ovarian cancer within our organoid-based drug screening model, addresses the imperative for more nuanced, precise, and effective treatment methodologies. We have pioneered novel techniques in establishing ovarian cancer organoids, thereby enhancing the relevance of organoid models and facilitating the establishment of high-throughput screening platforms. This approach heralds a promising avenue for personalized oncology treatment.

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The generation of patient-specific organoids from tissue fragments is a crucial advancement in personalized healthcare, primarily due to its efficacy in evaluating drug responses [6]. However, this technique faces challenges related to the inconsistent sizes of organoids, which impairs their applicability in high-throughput drug screening [7,8]. Variations in organoid size can lead to inconsistent drug penetration and response, affecting the robustness of results [9]. Phenotypic evaluation of models is complicated by intra- and inter-patient differences in organoid size, cellular heterogeneity, and dynamic responses [10]. Our previous methods, utilizing tissue fragments to cultivate organoids, encountered limitations due to these size inconsistencies, complicating the observation of clear drug responses [6]. The importance of uniform organoid size is underscored, as it ensures consistent drug exposure and more clinically relevant results [9,11,12].

In our research, we have pioneered a novel approach for the cultivation of uniform-sized organoids from single-cell suspensions, a technique particularly advantageous for high-throughput drug screening in ovarian cancer models. This process involves the generation of single-cell suspensions from digested cultured organoids, which subsequently self-organize into organoids of uniform size. We conducted meticulous assessments of their growth and histological attributes, confirming the retention of tissue characteristics similar to those derived from tissue fragment cultures. Notably, these organoids displayed significant responsiveness to drug treatments, as evidenced by comprehensive cell viability assays. Furthermore, when embedded into a matrix gel, these organoids maintained sustained growth and preserved their distinctive features, highlighting the adaptability of our cultivation method, especially for matrix gel-requiring experiments. This approach significantly enhances reproducibility and robustness in chemotherapy drug screening, while also showing immense potential in revolutionizing personalized drug screening for ovarian cancer. It opens a promising path for developing tailored therapeutic strategies, specifically tailored to the nuances of ovarian cancer treatment in the realm of oncological research.

Methods and materials

Tissue collection

The ovarian cancer tissues were obtained after patients' post-surgery. None of the patients underwent chemical therapy prior to surgery. Samples from four patients were utilized for the study. Subsequent to surgery, the ovarian cancer tissues were promptly transported under cryogenic conditions to LiSheng Biotech Co., Ltd., and stored at 4 °C until processing, typically within 3 days.

Mechanically sheared fragment culture

The cancer tissue samples were initially washed in washing solution (LSNO00100201; Shanghai LiSheng Biotech, China) to remove any adherent blood or mucus, with this process repeated three times. Subsequently, the samples were cut into fragments of no more than 3 mm using ophthalmic surgical scissors and cultured in ovarian cancer culture medium (LSTO001004; Shanghai LiSheng Biotech, China) in a 10 cm culture dish. The culture medium was replaced every five days, with half of the medium aspirated and an equivalent volume of fresh culture medium added and mixed by pipetting. For passaging, the organoids and adherent cells were collected using cell scrapers, the supernatant was removed by centrifugation, and the collected organoids were fragmented with scissors to a size of no more than 3 mm. They were then cultured in fresh culture medium and mixed by pipetting. Irregular edges became rounded were saw as a criterion for successful organoid culture. The growth and morphological changes of the organoids were regularly observed using an inverted microscope (DMi1, Leica, USA), and the organoid size and area were measured using Image J software.

Single-cell suspension culture

After successfully culturing organoids from tissue fragments, we aimed to expand the application field of our culture method for high-throughput drug screening by digesting the organoids into single cell suspensions. Then the organoids obtained from fragment organoid culture were collected using cell scrapers and centrifuged to obtain all organoids. The collected organoids were then incubated with Organoid Dissociation Reagent I (LSNO00100501; Shanghai LiSheng Biotech, China) at 37 °C for 15 min. The digested liquid was aspirated repeatedly through 1 mL pipette tips. Digestion was terminated if smooth aspiration was achieved; otherwise, digestion was continued for an additional 10 minutes if there was a noticeable blockage during aspiration. The digested liquid was passed through a cell strainer (352340, Corning, USA) and collected. Any undigested organoids on the cell strainer underwent a second round of digestion for 10 minutes. Subsequently, the liquid from the second digestion was passed through a cell strainer again and centrifuged together with the suspension collected from the first round at 600 g for 10 min. The supernatant was removed, and 2 mL of ovarian cancer culture medium was added and mixed. The mixture was then centrifuged at 600 g for 10 min to completely remove residual digestion solution. Next, 2 mL of ovarian cancer culture medium was added and mixed to prepare the single-cell suspension. 1 ml of the suspension was used for culturing single-cell organoid spheres in a 6-well Clear TC-treated Multiple Well Plates (3516, Corning Incorporated, USA). The remaining 1 mL was added to 3 mL of ovarian cancer culture medium, mixed, and evenly distributed into 96-well Clear Round Bottom Ultra-Low Attachment Microplate (7007; Corning Incorporated, USA) for single cell automatically assembly observation experiments. The growth and morphological changes of the organoids in 96-well microplate were regularly observed through a stereo microscope (YZ39; ShanghaiYuehe, China).

Embedding organoids in matrigel

The Matrigel Matrix (354263, Corning, China) was placed on ice and liquefied by incubating at 4 °C for 1 h. The organoid suspension was mixed with the liquefied Matrigel at a 3:2 ratio. The mixture was placed in a 10 cm culture dish and allowed to solidify in a 37 °C incubator, then supplemented with culture medium for continued cultivation. The growth and morphological changes of the organoids were regularly observed using an inverted microscope (DMi1, Leica, USA).

Hematoxylin and eosin (H&E) staining

Organoids were fixed in 4% formaldehyde (BL539A, Biosharp, China) for 24 h and embedding with Tissue-Tek O.C.T. compound (4583, Sakura Finetek, USA). 10 μm thick sections were then obtained using a cryostat microtome (CM 1950, Leica,

USA) and stained with hematoxylin solution (BL700A, Biosharp, China) for 5 min. Excess stain was removed by washing with distilled water, and the sections were differentiated in a H&E differentiate solution (G1862, Solarbio, China) for 10 s, followed by a 2-min rinse in running tap water. Eosin staining solution (BL700B, Biosharp, China) was then added for react 30 s. Finally, the sections were mounted with neutral balsam (G8593, Solarbio, China) and subjected to image acquisition through upright microscope (YI21, ShanghaiYuehe, China).

RNA sequencing

Organoid samples were collected and immediately submerged in TRIzol Reagent (15596018, ThermoFisher, USA) for RNA stabilization. The samples were then stored at – 80 °C until further processing. The samples were then sent to Honsun, a sequencing company, in dry ice for RNA extraction and sequencing. Honsun performed the RNA extraction and subsequent RNA sequencing using their established protocols and equipment. The raw sequencing data obtained from Honsun was further analyzed. RNA expression levels were quantified using fragments per kilobase transcript mapped reads per million (FPKM). The data was processed using R and Morpheus online software to perform differential gene expression analysis and other relevant analyses.

Drug treatment

The organoids cultured in a 96-well plate were used to drug treatment. Organoids were treated with different concentrations (0, 5, 25, and 50 μmol/L) of paclitaxel injection (H20067345; Taxus Pharma, China) and carboplatin injection (HJ20171063, Corden Pharma Latin a S.P.A., Italy), with each treatment group having 4 replicates to reduce random error. On Day 8, additional drug was added to maintain continuous treatment. Regular monitoring was conducted to record organoid growth and morphological changes. CellTiter-Glo®3D Cell Viability Assay (G9683, Promega, USA), as per the manufacturer's instructions, was used to assess cell viability to evaluate the efficacy of drug treatment.

Statistical analysis

The data were collected from three or more replicates, and data are presented as mean ± standard deviation from independent experiments. Statistical analysis was performed using GraphPad 8.

Results

Organoids derived from mechanically sheared tissue fragments and single-cell suspensions exhibit comparable characteristics

In our preceding study, we established that organoids derived from small tissue fragments closely resemble native tissues.[6] However, the significant size variability of these organoids, often exceeding 3mm in diameter, presented challenges for high-throughput drug screening in standard 96 or 384 well plates. To overcome this, we refined our culture technique to produce organoids suitable for high-throughput applications (Fig. 1a). This refinement involved enzymatically digesting organoids from tissue fragments to create single-cell suspensions for subsequent organoid cultures. Regular monitoring showed consistent organoid growth, and their structural integrity was verified via H&E staining. Remarkably, we observed that within 8 days, single cells in the culture medium spontaneously formed compact organoids (Figs. 1b and 1c), indicating the success of our modified method in maintaining key organoid characteristics suitable for high-throughput analysis. Over time, we observed increased adhesion among organoids from sample OG-OVA-37 through our microscopic observations of brightfield images. H&E staining corroborated that both the original and modified methods yield organoids with comparable histological structures (Fig. 1d)

The RNA-seq sequencing results, analyzed through principal component analysis (PCA) and correlation studies, distinctly highlight that inter-patient sample variability supersedes the differences attributable to the organoid culture methods (Figs. 2a and 2b). Particularly, the OG-OVA-39 and OG-OVA-40 samples displayed notable similarities. Our in-depth heatmap analysis of gene expression profiles across both single-cell suspension and mechanically sheared fragment methods revealed remarkable transcriptional congruence, particularly in genes with high and low expression levels (Fig. 2c). This striking similarity underscores the effectiveness of both methodologies in preserving the heterogeneity inherent in ovarian cancer tissue, a critical aspect for realistic disease modeling. Differential gene expression analysis further supported this, as no significant variations were observed in key pathways between the two methods. This is particularly evident in cell cycle pathways, pivotal in the tumorigenesis and progression of ovarian cancer. The lack of significant fold change differences (Fig. 2d) between the two methods not only affirms their individual efficacy but also suggests that organoids derived from single-cell suspensions are equally capable of simulating the authentic pathological state of ovarian cancer, akin to the previously validated minced tissue method. Our further gene set enrichment analysis (GSEA) transcriptome pathway analysis suggested enhanced TGF-β signaling and tight junction pathways in organoids derived from minced tissue compared to single-cell suspensions. This can be attributed to the preserved tissue architecture and cellular dynamics in minced tissue-derived organoids, which likely support more robust cell-cell and cell-matrix interactions, essential for pathways such as TGF-β and tight junctions. TGF-β signaling, vital for cell differentiation, proliferation, and apoptosis, is influenced by the structural integrity of the microenvironment [13,14]. Similarly, tight junction pathways, crucial for maintaining cellular polarity and barrier functions, depend on the physical organization of cells [15]. Conversely, organoids from single-cell suspensions, lacking this initial complex structure, rely on the self-organizing capacity of individual cells, potentially leading to less pronounced TGF-β and tight junction signaling. Thus, the stronger signals in minced tissue-derived organoids underscore the significant influence of tissue architecture and cellular interactions on these pathways, highlighting the importance of the organoid derivation method in accurately modeling disease pathology and cellular behavior (Fig. 2e).

Optimizing uniform organoid formation from single-cell suspensions for high-throughput screening

In high-throughput drug screening, the use of 96- or 384-well plates is crucial for testing multiple drugs across varied concentrations. Assessing whether single-cell suspensions could form organoids within 96-well plates was essential for integrating this method into high-throughput screening. Upon seeding single-

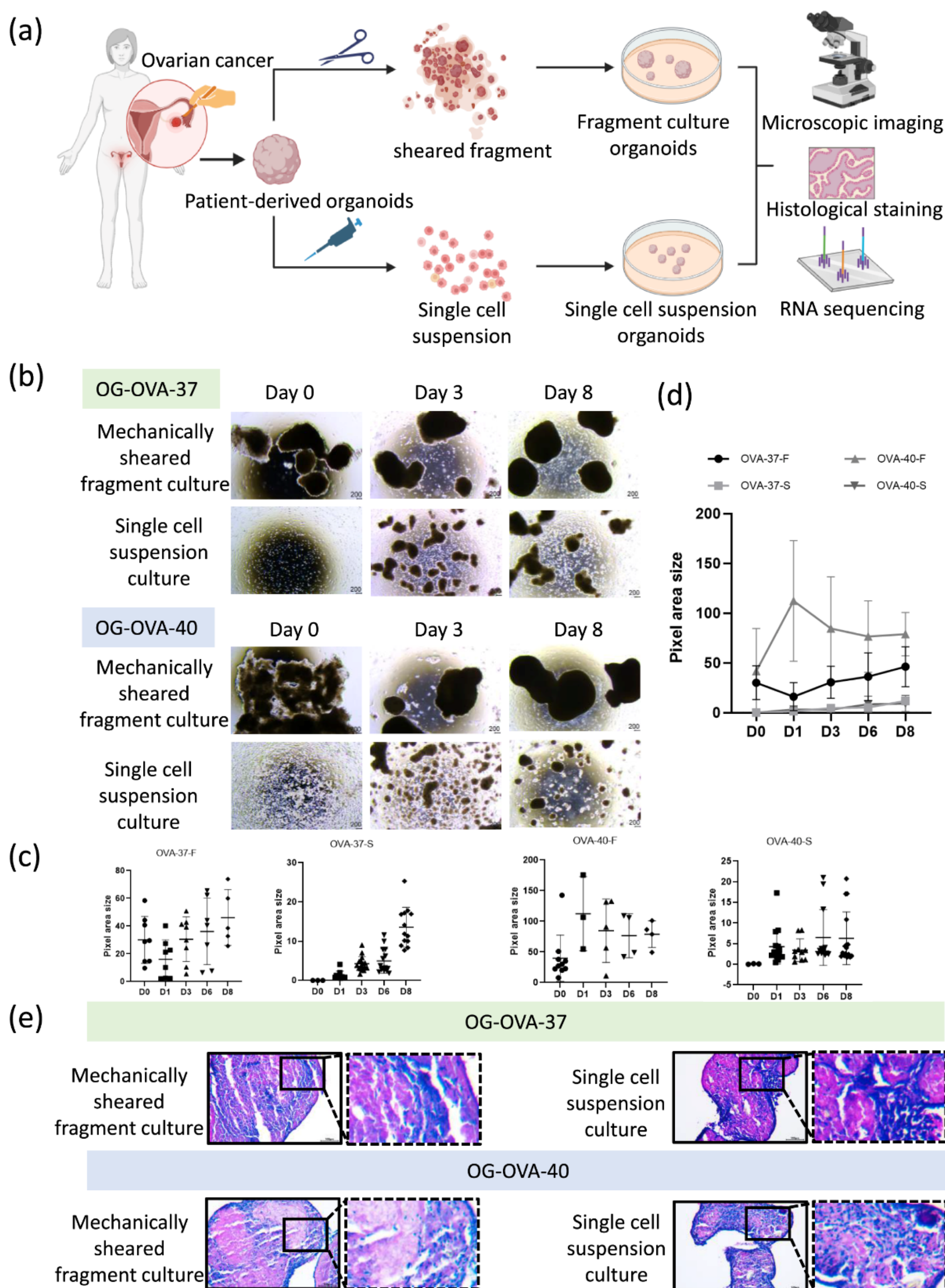

Figure 1. Comparative analysis of ovarian cancer organoid cultivation methods. (a) Diagram illustrating the methodology for generating organoids from single-cell suspensions versus mechanically sheared fragments, highlighting procedural differences. (b) Brightfield microscopy visualization of ovarian cancer organoids 8 days post-culture, with scale bars for size reference. (c) Quantitative results of organoid size evolution over time, utilizing brightfield imaging to document growth dynamics across culture methods. The *y*-axis represents the pixel area size calculated from ImageJ, while the *x*-axis indicates the number of days in culture. The F denotes the fragment culture method, while S represents the single cell suspension culture method. (d) Quantitative comparison of organoid size evolution over time, utilizing brightfield imaging to document growth dynamics across culture methods. (e) Histological comparison via H&E staining of organoids derived from each method, demonstrating morphological consistency with scale bars provided.

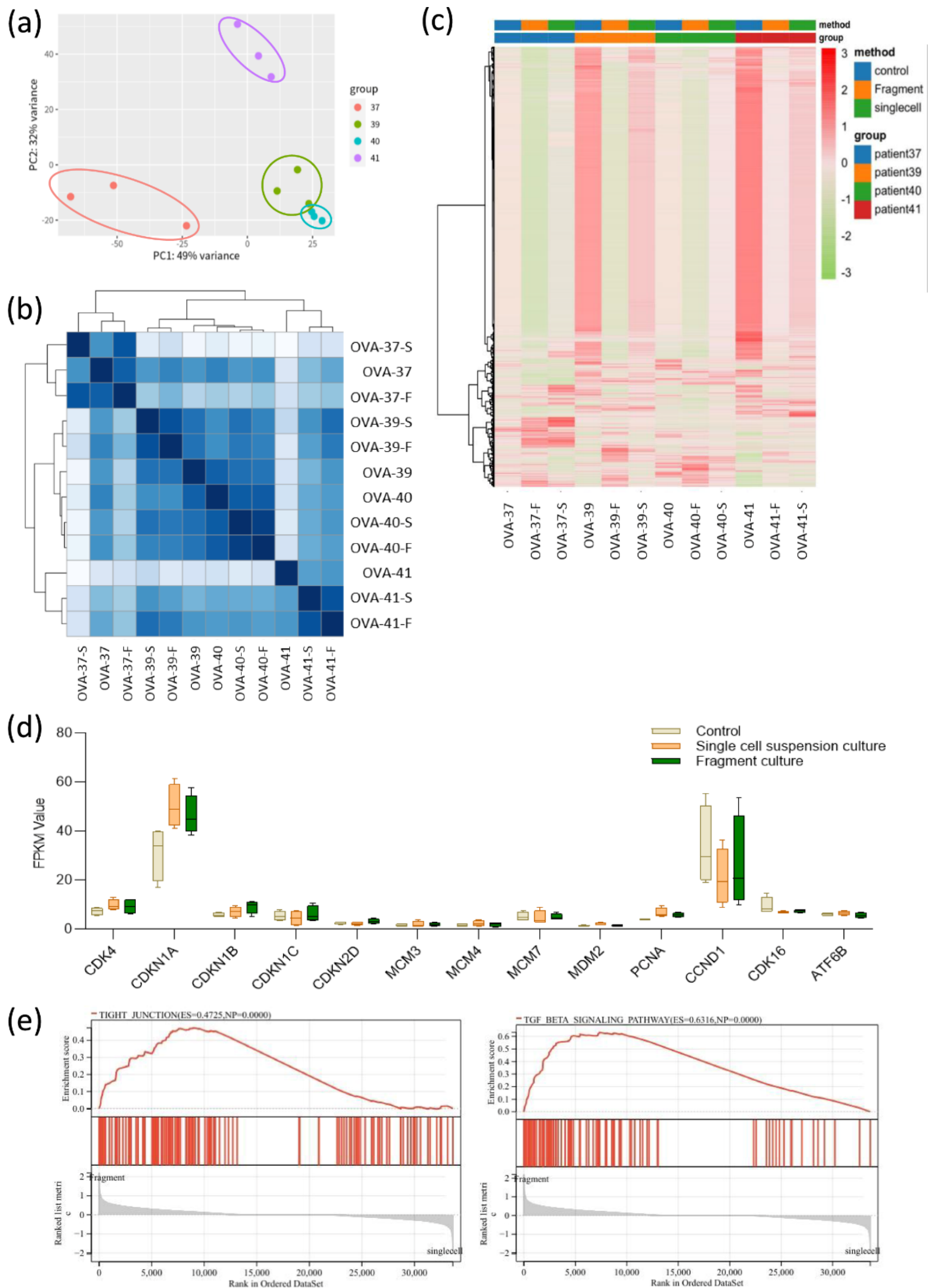

Figure 2. Transcriptomic profiling of ovarian cancer organoids cultured by different methods. (a) PCA showcasing the distinct genomic landscapes of organoids derived from single-cell suspensions and mechanically sheared fragments, with clustering by patient sample origin. (b) Sample correlation matrix highlighting the relationship between organoid culture methods, where intensity of color correlates with the degree of transcriptomic similarity. (c) Heatmap displaying differential gene expression patterns across samples, with normalization of raw counts to TPM and subsequent Z-score transformation for direct comparison. (d) Expression analysis of pivotal genes within cell cycle pathways, comparing their relative expression levels across the two organoid culture techniques. (e) GSEA revealing pathway variances between organoids derived via single-cell suspension and mechanical shearing, underscoring methodological impact on cellular function.

cell suspensions, derived from digested organoids, into 96-well plates (Fig. 3a), our daily monitoring identified that cells settled at the well bottoms and spontaneously formed spherical structures with defined boundaries (Figs. 3d and 3e). Comparatively, mechanical shearing of OVA-41 organoids into fragments followed by seeding in 96-well plates resulted in uneven organoid formation and size distribution. In stark contrast, single-cell suspension-derived organoids demonstrated superior uniformity in size, although initial seeding density influenced some variation (Fig. 3b). Quantitative analysis of organoid sizes further validated that single-cell derived organoids maintained a more consistent size profile than those from pre-formed fragments (Fig. 3c).

Evaluating carboplatin efficacy on ovarian cancer organoids within a high-throughput screening framework

Investigating carboplatin efficacy on ovarian cancer organoids, derived from single-cell suspensions, we conducted a meticulous evaluation within a 96-well plate framework. Organoids were exposed to carboplatin concentrations reflective of clinical application. Due to the CellTiter-Glo® 3D Cell Viability Assay's reliance on consistent input amounts across different wells for reliable results, the size variability of the minced tissue culture method in input material precludes its reliable use for drug testing. Over 14 days, organoid morphology was closely monitored, revealing no significant alterations within the initial six-day period. Subsequently, a discernible onset of peripheral disintegration was observed, intensifying by day 14, particularly at 50 μmol/L carboplatin concentration (Fig. 4b). This structural degradation contrasted markedly with the untreated controls. Intriguingly, a concentration-dependent biphasic response was noted. At 5μM, an unexpected augmentation in metabolic activity suggested a stimulatory carboplatin effect, which subsided with increasing concentrations, yielding to a predominant cytotoxic impact (Figs. 4c and 4d). Crucially, the reproducibility of these findings was affirmed across organoids derived from individual patients. Consistent drug-response patterns were observed in quadruplicate wells at each concentration, with coefficient of variation (CV) values aligning with the standards for high-throughput screening (Fig. 4c). This consistency demonstrates the robustness of these patient-specific organoids as a viable platform for high-throughput pharmacological screening.

Synergizing single-cell suspension cultivation with Matrigel embedding for enhanced organoid growth and analysis

Next, we explored the growth dynamics of organoids derived from single-cell suspensions in matrix gels, particularly focusing on Matrigel, a composite of extracellular matrix components like laminin and collagen IV, enriched with growth factors. Traditional organoid cultivation often employs Matrigel embedding of stem cells, which, while facilitating detailed longitudinal observations, might limit cellular heterogeneity compared to self-assembling organoids from single-cell suspensions. To optimize this process, we developed a novel hybrid technique. Initially, organoids are cultivated from single-cell suspensions to promote diverse cellular representation. Subsequently, these organoids are embedded in Matrigel, enabling their sustained growth and detailed monitoring (Fig. 5a). This approach has the potential to synergize the cellular diversity of self-assembly with the observational advantages of Matrigel embedding.

To assess the influence of Matrigel on organoid morphology, we performed histological analyses on organoids post-15 days of Matrigel embedding compared to those maintained in suspension (Figs. 5b and 5c). Our results indicated no significant morphological differences between the two conditions, suggesting that Matrigel embedding does not compromise the intrinsic structural integrity of the organoids.

Discussion

In this study, we have not only optimized single-cell derived organoid technology for high-throughput screening applications but also bridged this approach with conventional matrix gel-based organoid culture methods. This integration facilitates the amalgamation of various technical advantages, offering an optimized strategy for personalized therapeutic screening in ovarian cancer.

Our findings underscore the importance of adapting 96-well and 384-well plate formats for drug testing, given their suitability for high-throughput applications. The ability of single-cell suspensions to form uniformly sized organoids in these plates and their responsiveness to drug treatment is a critical advancement in organoid-based drug screening methodologies. Moreover, the uniform size and consistent growth patterns of these organoids address previous limitations in organoid cultivation, enhancing the reliability of drug response assessments.

While our RNA-seq analysis indicates that mechanically fragmented tissue-derived organoids exhibit higher TGF-β pathway expression, suggesting a closer mimicry of the original tissue's microenvironment, this does not diminish the value of single-cell suspension-derived organoids. The latter's utility in representing a physiologically relevant model for drug discovery, especially in the realm of immunotherapy, is noteworthy. Further investigations are needed to explore the potential benefits of mechanically fragmented organoids in immune-related drug screening.

Overall, this research paves the way for personalized medicine in ovarian cancer treatment, potentially improving patient outcomes. By offering a physiologically relevant platform for drug discovery and development, this approach could lead to the identification of novel therapeutic targets, customization of therapy, and reduction of adverse effects, thereby enhancing treatment efficacy for ovarian cancer and other diseases.

Research ethics and patient consent

This study adhered to the ethical standards set forth by the Obstetrics and Gynecology Hospital of Fudan University. Informed consent was obtained from all patients prior to collecting their samples, and they were made aware of the intention to use their samples for future research. Participants were enrolled in the study only after providing their informed consent, thereby ensuring voluntary involvement. The research was granted ethical approval under the number kyy2023-06.

Availability of data and material

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

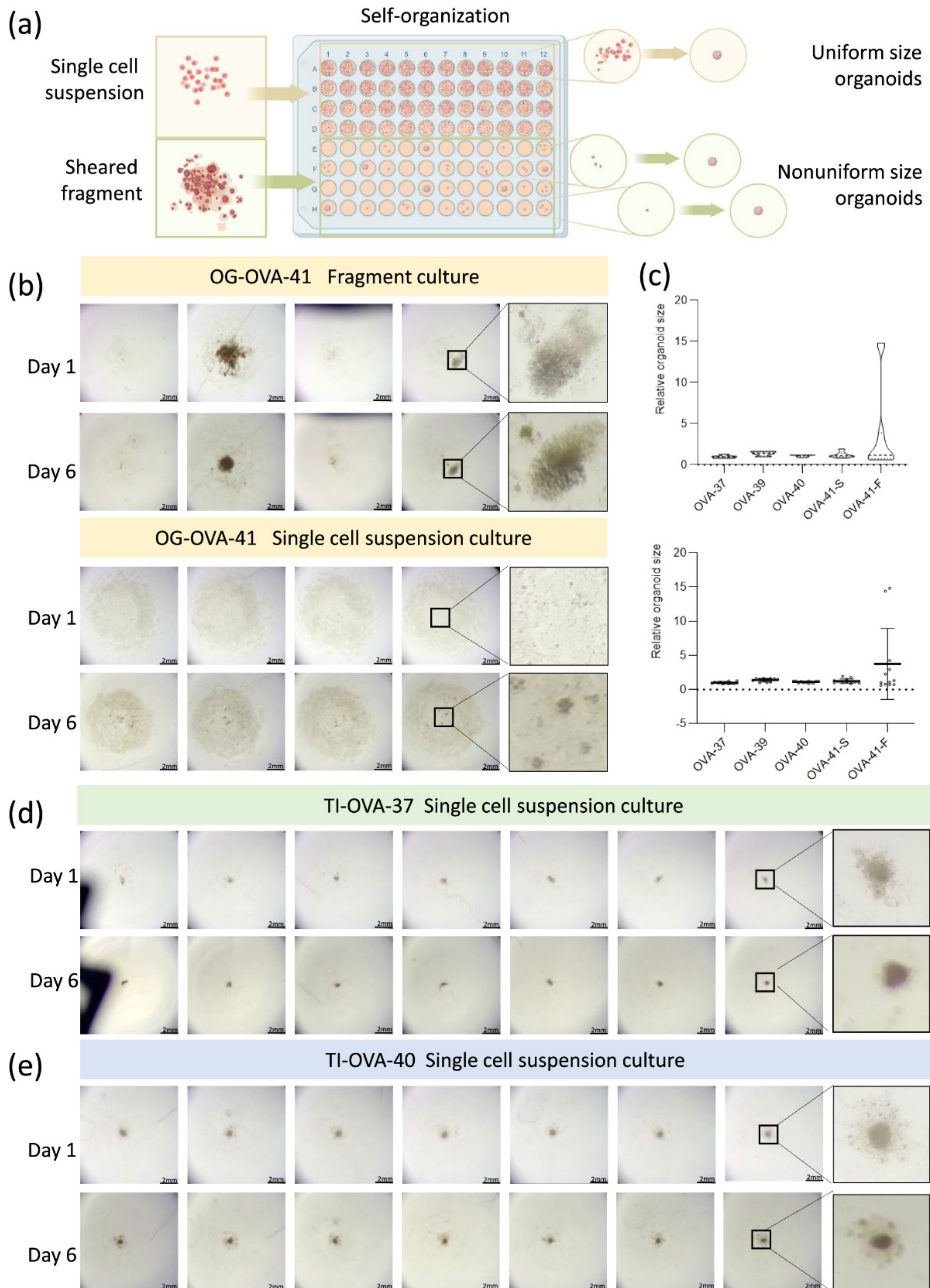

Figure 3. Establishment and growth dynamics of ovarian cancer organoids in 96-well plates. (a) Detailed schematic showcasing the formation process of organoids from single-cell suspensions within the microenvironments of 96-well plates, emphasizing the self-aggregation mechanism. (b) High-resolution brightfield microscopy images displaying the comparative growth patterns of ovarian cancer organoids, cultured from single-cell suspensions and tissue fragmentation methods, at day 6, with the OG-OVA-41 sample serving as a case study. (c) Quantitative image analysis to evaluate the size uniformity of organoids developed from single-cell suspensions, underscoring the methodological advantages in promoting consistent organoid formation.

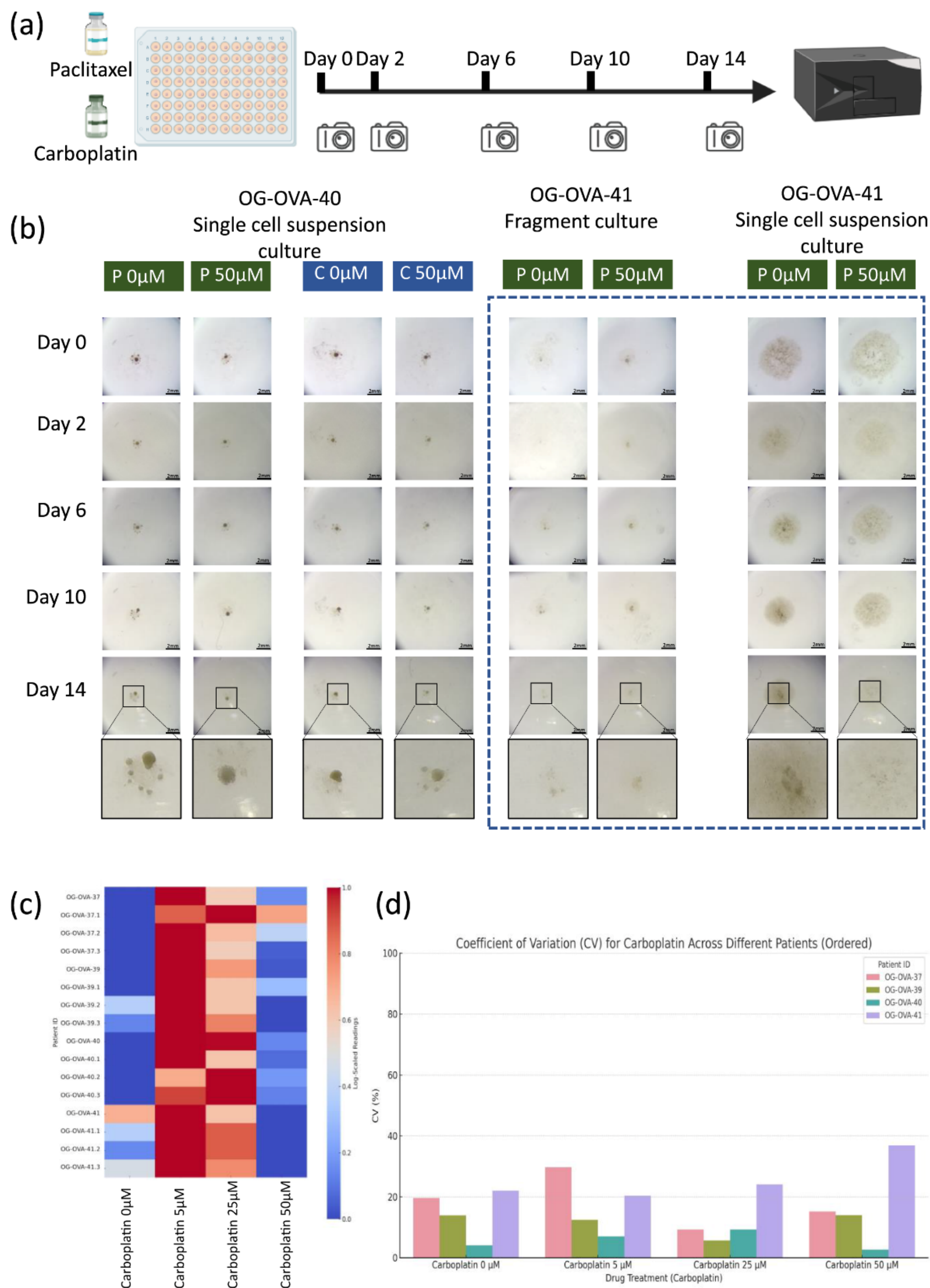

Figure 4. Organoid responses to chemotherapeutic treatment. (a) Detailed schematic outlining the protocol for administering drug treatment to organoids, highlighting key observation intervals for assessing cellular health and viability. (b) Comparative brightfield microscopy visuals of ovarian cancer organoids post-chemotherapy, emphasizing the differential impact of treatment on organoids cultured via single-cell suspensions and those from tissue fragments. (c) A heatmap illustrating the quantitative cell viability, as indicated by log-transformed luminescence intensities, across varying concentrations of carboplatin, with data from quadruplicate samples underlining the consistency of response. (d) Analysis of variability in carboplatin response, presented as CV, across organoids derived from different patient samples, demonstrating the assay's reliability for drug sensitivity testing.

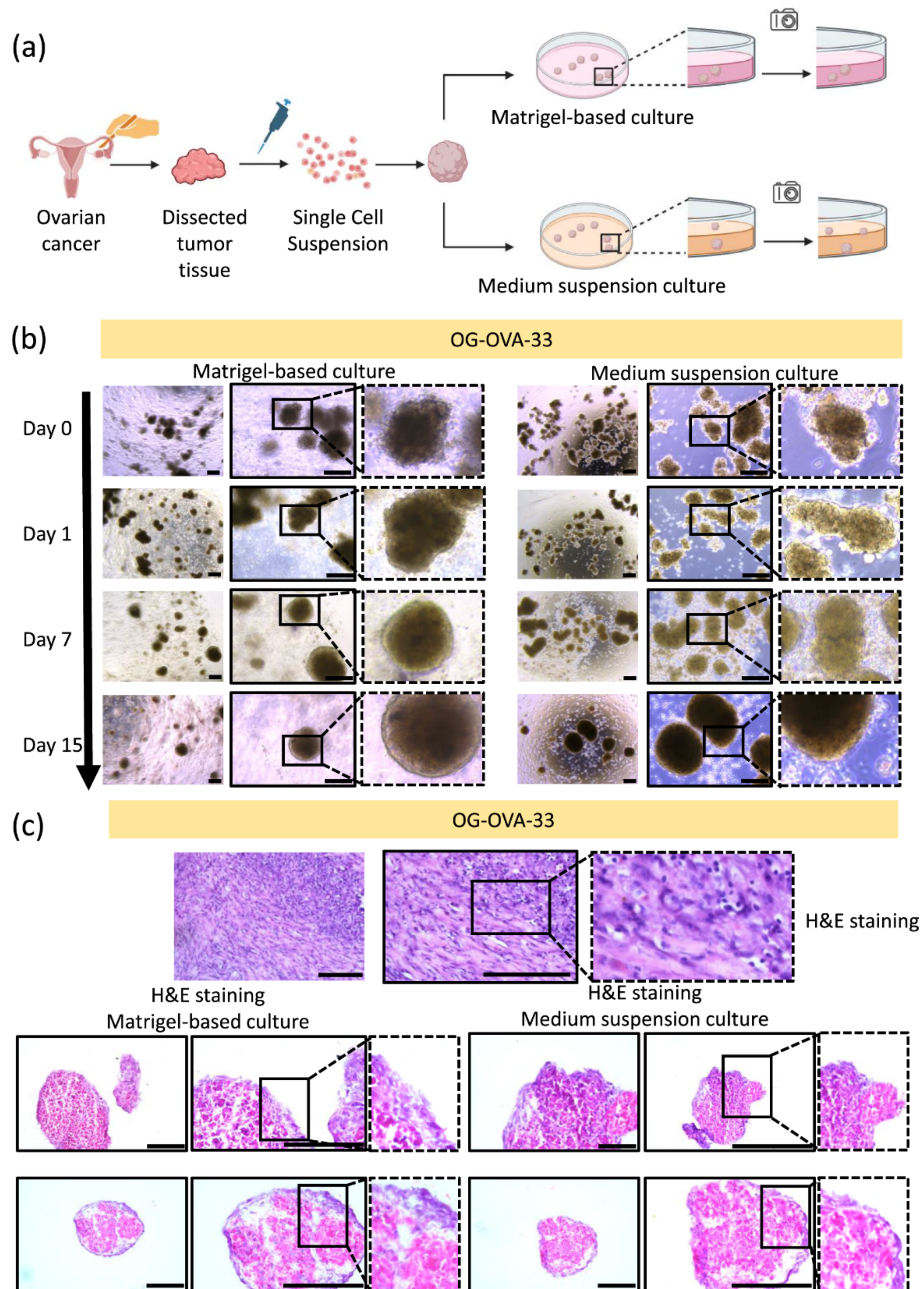

Figure 5. Cultivation dynamics of single-cell suspension-derived organoids in varied environments. (a) Detailed schematic showcasing the methodology for continued cultivation of single-cell derived organoids, comparing suspension cultures against matrix gel environments. (b) Brightfield microscopy provides a visual comparison of organoid growth and morphology in suspension versus matrix gel conditions, with precise scale measurement for accurate size assessment. (c) Comparative histological analysis using H&E staining highlights the structural integrity and cellular detail of organoids cultivated under both conditions, with scale bars ensuring consistent magnification across samples.

Declaration of conflicting interests

This work was sponsored by Shanghai Lisheng Biotech Ltd (Lisheng). The manuscript was written in a responsible and ethical manner. X.X.H. is a shareholder of Lisheng, as a founder. Y.H.S., L.Y.L., C.W., J.Z., M.J.R., J.P.L and C.H.C. are senior scientists of Lisheng. All authors declare no competing financial interests.

X.X.H. and C.H.C., both members of the Editorial Board for Cell Organoid, have abstained from any roles in the journal's evaluation, review, or decision-making processes regarding this manuscript to ensure impartiality.

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Author contributions

C.H.C. and X.X.H. conceived and designed the study. L.Y.L. Q.C., Y.Q.Z., X.T., C.W., J.Z., Y.H.S., and M.J.R. were responsible for sample collection and experimental procedures. J.P.L. conducted the analysis of the RNA sequencing data. L.Y.L., Q.C., D.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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