

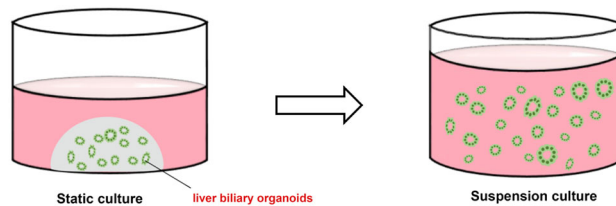


Facile suspension culture protocol of the liver biliary organoids

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Graphic abstract



Introduction

Why do we focus on human liver biliary organoids?

The three-dimensional (3D) organoid approach is emerging as a powerful tool for basic study and biomedical applications due to its excellent recapitulation of the structure and functions of the primary tissues [1–3]. Liver biliary organoids are derived from the biliary tree, which includes the intrahepatic bile duct (IHBD) and extrahepatic bile duct (EHBD). Disorders of the biliary tree system can cause inflammation, fibrosis, bile duct damage, and finally liver failure, which carries a considerable incidence. Liver transplantation is the last choice for the treatment of end-stage liver failure. However, the gap between donor and clinical requirements makes it impossible to meet the needs of patients for liver transplantation. Cell and tissue engineering has long held promise as an alternative to liver transplantation [4]. The implementation of cell therapy is hampered by the limited expansion of hepatocytes and their tendency to lose function *in vitro* [5, 6]. Liver biliary organoids have become a potential alternative for liver

transplantation. First, owing to the high proliferation capacity and the potential to differentiate into hepatocytes [7–11], liver biliary organoids are reliable candidates for hepatocyte sources. Moreover, several studies have shed light on the regenerative capacity of liver biliary organoids, which can rescue the bile duct structure in mice and humans, respectively [12–14]. In summary, liver biliary organoids have been playing an increasingly important role in the basic study and regeneration of the liver, which is why we focused on them.

What is the current culture method for liver biliary organoids?

The current routine culture method for liver biliary organoids is the static culture. The static culture method was as follows: First, thawed Matrigel was used to suspend the digested liver biliary organoid pellets. Then, the mixed drops were seeded on the bottom surface of the petri dish before Matrigel solidification. Later, the petri dish was transferred to a 37 °C incubator for 15–20 min until Matrigel solidification. Finally, the medium was added to the petri dish for culture. In this traditional culture method, liver biliary organoids are confined to a certain space in the petri dish by Matrigel; therefore, their interaction with the medium is relatively static. Matrigel is the basement membrane protein extract derived from Engelbreth-Holm-Swarm (EHS) tumors in mice, which mainly simulates the role of the matrix in regulating cell fate and tissue homeostasis [15]. In static culture, there is something to be concerned about. First, due to the limited

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space at the bottom of the petri dish, the number of organoids seeded on it is restricted, which makes it difficult to realize the scalable culture. Second, given the physical properties of Matrigel, which is liquid at low temperatures and becomes a 3D gel as the temperature increases to a certain degree, we need to pay attention to the speed of seeding and spend extra time on the solidification of Matrigel.

Why is it necessary to develop the suspension culture method for liver biliary organoids?

Liver biliary organoids are in great demand because of their wide range of applications, from basic studies to tissue engineering connected with animal models, as well as liver regeneration. For instance, it is estimated that 10% of liver mass, which can be helpful for curing liver failure, has approximately billions of cells [16]. Billions of cells require a great deal of time and labor to maintain in static culture, and frequent passage increases the risk of genetic instability in cells of liver biliary organoids. Moreover, the static culture process is tedious to some extent; that is, its steps can only be performed separately rather than simultaneously. These practical obstacles have prompted an improved culture protocol for liver biliary organoids. Suspension culture could overcome the disadvantages of static culture. First, suspension culture is superior to static culture in culturing volume. Second, suspension culture promotes the expansion of liver biliary organoids. Third, the operation of suspension culture is relatively facile and could be carried out in series, reducing the time and labor.

Opening the avenue to a new culture method for liver biliary organoids: suspension culture

In this technical note, we would like to introduce a new culture method for liver biliary organoids—suspension culture. The suspension culture in our protocol is established on general laboratory equipment, and the operation process is facile and convenient. Different from the separation of Matrigel and medium in static culture, we mixed the Matrigel and medium together and used an orbital shaker to facilitate contact between the organoids and oxygen in this suspension culture. This new culture method includes the following five main parts (Fig. 1): Matrigel suspension (MS) preparation, digestion of liver biliary organoids, resuspension of the liver biliary organoid pellets, shift to the orbital shaker in incubator, and medium replacement. We examined the feasibility of this protocol by culturing and identifying the characteristics and differentiation potential of liver biliary organoids after suspension culture. In summary, our suspension culture method could be extended to scalable culture to meet the rising demand for liver biliary organoids.

Workflow

Part I: Matrigel suspension (MS) preparation

The first part of suspension culture is to prepare the Matrigel suspension (MS). The MS consists of Matrigel and expansion medium (EM, advanced DMEM/F-12 supplemented with 1% penicillin/streptomycin, 1% GlutaMAX and HEPES 10 mM, 1:50 B27 supplement, 1 mM *N*-acetylcysteine, 1:2000 R-spondin-1, 10 mM nicotinamide, 10 nM recombinant human gastrin I, 50 ng/mL recombinant human EGF, 100 ng/mL recombinant human FGF10, 25 ng/mL recombinant human HGF, 10 μ M Forskolin and 5 μ M A83-01) for liver biliary organoids. Specifically, part I consists of the following three steps:

1. Thaw the Matrigel and precool the EM on ice.
2. Mix 10% (vol/vol) thawed Matrigel with 90% (vol/vol) EM together thoroughly to obtain the MS (i.e., 50 μ L Matrigel and 450 μ L EM for one well of the 24-well plates).
3. Add the MS-1 (90% of MS) to the petri dish, then transfer it to 37 °C for incubation for approximately 15 min; put the MS-2 (10% of MS) for subsequent purpose on ice for precooling (i.e., 450 μ L MS was added to one well of the 24-well plates, with the remaining 50 μ L MS left on ice).

Note: We would better prepare slightly more medium due to the loss of medium during the operation inevitably (i.e., 530 μ L medium was prepared for a 500 μ L culture system).

Part II: Digestion of liver biliary organoids

Since suspension culture starts with static culture [17], we need to collect organoids from static culture. Part II can be subdivided into six steps.

4. Use a 1 mL blue pipette to collect the organoids embedded in the Matrigel in static culture into the sterile tube (i.e., one well of the 24-well plates was collected in one 1.5 mL sterile tube).
5. Add an appropriate volume of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS) to the tube (depending on the volume of collected solution, i.e., 600–800 μ L when one well was collected). The organoid suspension was resuspended gently by pipetting up and down with a cell culture pipette to disperse the Matrigel and organoids.
6. The tube was centrifuged at 250g for 3 min at room temperature, and the supernatant was removed.

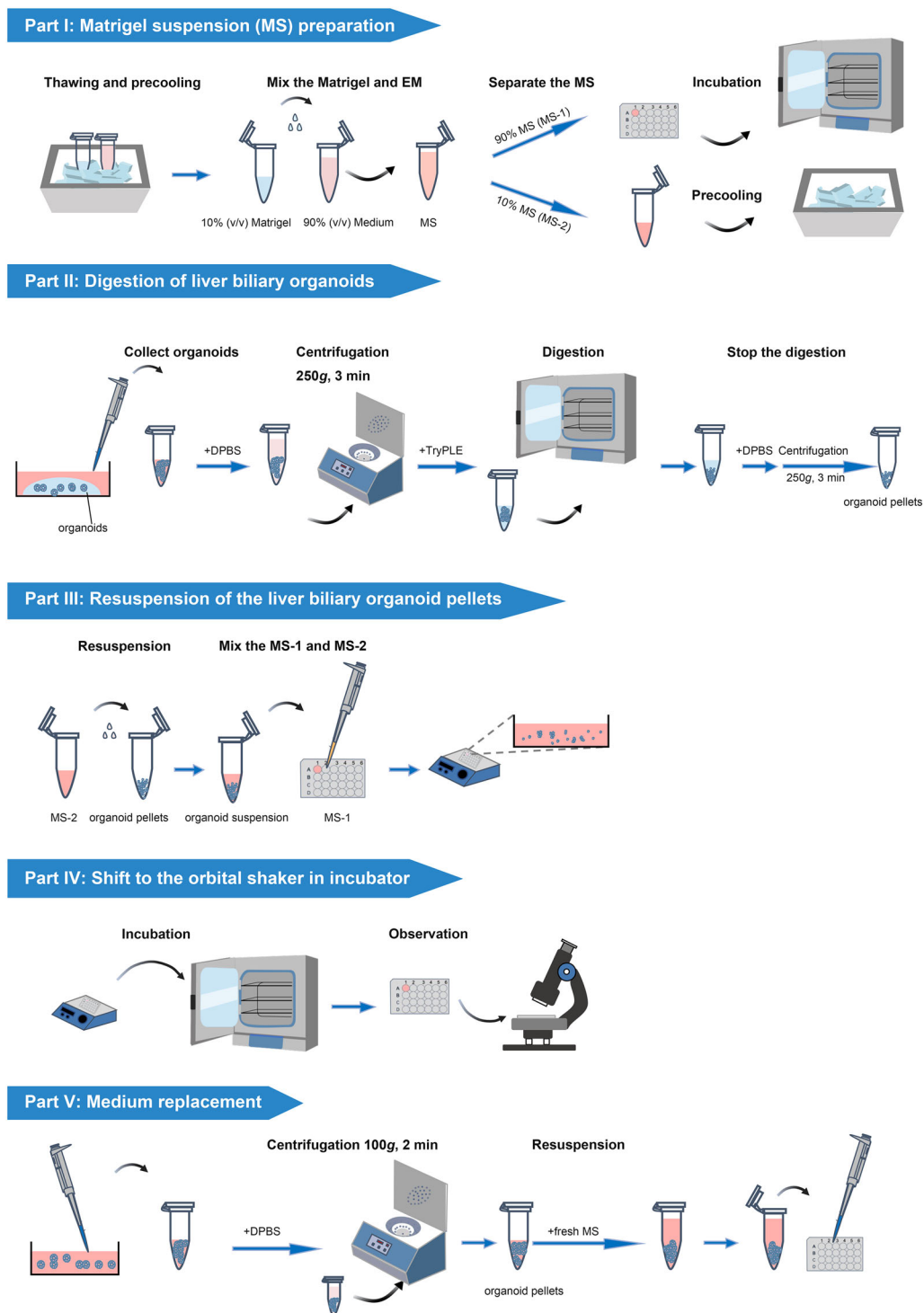


Fig. 1 The workflow sketch of the facile suspension culture method. *MS* Matrigel suspension, *EM* expansion medium

7. Add 200 μL of $1 \times$ TryPLE to a tube with gentle shaking and then transfer it to a 37°C incubator for approximately 10 min for organoid digestion.

Note: The effect of TryPLE-induced digestion was monitored by sampling every 3–4 min under light microscopy.

8. Stop the digestion by adding four to five volumes of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS into the tube and resuspend it by pipetting up and down.
9. Centrifuge the tube at $250g$ for 3 min at room temperature and remove the supernatant.

Part III: Resuspension of the liver biliary organoid pellets

10. Resuspend the liver biliary organoid pellets with MS-2 (Step 3) by pipetting up and down to obtain the organoid suspension.
11. Add the organoid suspension to the petri dish containing MS-1 (Step 3) and mix it thoroughly so that the organoid pellets are as evenly distributed in the medium as possible.

Part IV: Shift to the orbital shaker in incubator

12. Transfer the whole culture system to the orbital shaker (WIGGENS, SRC) in a 37°C incubator as soon as quickly. The shaker was set at 120 r/min which was obtained from the test (Fig. S2 in Supplementary Information).

Note: Although various types of orbital shakers are suitable for suspension culture, their power systems vary in their characteristics. We should determine the optimal speed by testing.

13. Observe the distribution of organoids under light microscopy after 10 min to ensure that most of the organoid pellets are suspended in the medium but not adherent to the bottom of the dish.

Part V: Medium replacement

It generally takes approximately 4–6 days to obtain spherical organoids from single cells for liver biliary organoids. At this point, we need to change to fresh medium or passage to sustain the expansion of liver biliary organoids. We introduce the operation of medium replacement here, and part II of digestion is the passage operation.

14. According to Step 1, prepare the fresh MS (EM + 3% (vol/vol) Matrigel).

Note: We could reduce the concentration of Matrigel in the fresh MS due to the residual Matrigel in the previous medium; generally, approximately 3% (vol/vol) Matrigel is sufficient.

15. According to Step 4, collect the organoids into tubes gently to reduce the damage.
16. The tube was centrifuged at $100g$ for 2 min at room temperature, and the supernatant was removed.
17. Fresh MS was used to gently resuspend the organoids, which were then transferred to the new wells of petri dish.

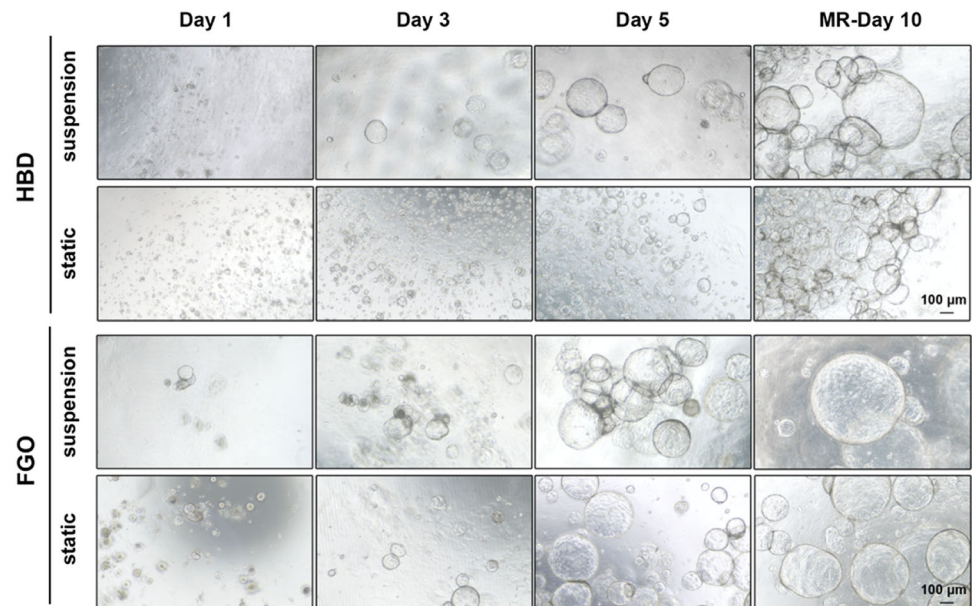
Examples

Experimental methods

First, we established two kinds of liver biliary organoids: the human bile duct organoid (HBD) and the fetal gallbladder organoid (FGO). Both of them were obtained from Huch's method for liver organoid culture and establishment [17]. HBD is a spherical cavity, while FGO is a multiprotruded spherical cavity (Fig. S1a in Supplementary Information). They all displayed a high proliferation capacity. Moreover, quantitative RT-PCR showed that FGO is indeed the source of gallbladder tissue and expresses a comparable amount of *KRT19* but almost no hepatocyte signature genes (Fig. S1b in Supplementary Information). We cultured the two kinds of liver biliary organoids in suspension culture and performed three experiments to verify the feasibility of the suspension culture. First, we identified the characteristics and viability of the liver biliary organoids in suspension culture. Second, we tested the differentiation potential of liver biliary organoids into hepatocytes in suspension culture. Third, we conducted a comparison of the static and suspension cultures in two aspects, that is, culturing volume (the effective maximum volume) and proliferation capacity.

To test the differentiation potential of liver biliary organoids, we referred to and modified Huch's differentiation protocol. Specifically, we cultured the organoid pellets in EM for 4 days and transferred them to differentiation medium-1 (DM-1, EM supplemented with 25 ng/mL BMP7). Until day 7, we changed the medium to differentiation medium-2 (DM-2, advanced DMEM/F-12 supplemented with 1% penicillin/streptomycin, 1% GlutaMAX and HEPES 10 mM, 1:50 B27 supplement, 1 mM *N*-acetylcysteine, 10 nM recombinant human [Leu¹⁵]-gastrin I, 50 ng/mL recombinant human EGF, 25 ng/mL recombinant human HGF, 0.5 μM A83-01, 10 μM DAPT, 3 μM dexamethasone, 25 ng/mL BMP7 and

Fig. 2 Representative bright-field images of human bile duct organoid (HBD) and fetal gallbladder organoid (FGO) on different days. *MR* medium replacement



100 ng/mL recombinant human FGF19). Finally, on day 12, we collected the organoids for analysis.

Experimental results

Liver biliary organoids in suspension culture grow into spherical structures with cavities over time as in static culture. As shown in Fig. 2, the HBD and FGO organoids expanded gradually from day 1 to day 5 and retained growth until day 10 after medium replacement. Moreover, from day 5, the size of organoids in suspension culture was generally larger than that in static culture, which was consistent with the average area of organoids (Fig. S4 in Supplementary Information). Therefore, the duration we suggested for culturing high-quality liver biliary organoids is approximately 5 days in this suspension culture protocol. As shown in Fig. 3, the liver biliary organoids retained their characteristics and viability after suspension culture. Figure 3a shows that FGO and HBD in suspension culture, as in the static culture, preserve the expression of KRT19 and E-cadherin. In Fig. 3b, comparable fluorescein diacetate (green) in the organoids showed that the HBD and FGO retained comparable viability after suspension culture. In Fig. 3c, the upregulated expression of the hepatocyte signature genes in the differentiation medium (DM) group showed that HBD retained the differentiation potential in suspension culture, as confirmed by albumin staining (Fig. 3d), which was in line with the results in static culture (Fig. S4 in Supplementary Information). However, FGO did not show the potential to differentiate into hepatocytes in the test, which was consistent with reports, possibly because FGO is not derived from hepatoblasts [18, 19].

Furthermore, the proposed suspension culture method showed advantages in culturing volume and proliferation

capacity. As Fig. 4a shows, the culture volume of suspension culture was 2.4 times that of static culture in the 24-well plate. The advantage in culturing volume of suspension culture could be extended to the larger system to achieve scalable culture. Moreover, as Fig. 4b shows, given the equal initial cells (1.0×10^4 cells in one well of the 24-well petri dish) and culturing time, we obtained more cells in suspension culture than in static culture on days 5 and 10. Moreover, Fig. S3 (Supplementary Information) shows that the average organoid area of HBD and FGO in suspension culture was larger than that in static culture. This result demonstrates that the suspension culture facilitated the expansion of HBD and FGO.

Discussion

The application of liver biliary organoids has penetrated various fields, including regeneration. The highly efficient cultivation of organoids is a booster for its offhand application, especially for research in regeneration. Although several studies have focused on it, they use bioreactors or spinner flasks to enlarge the scale of viable cell cultures, mainly stem cells [20–22]. In this technical note, we developed a suspension culture method for liver biliary organoids including IHBD (HBD) and EHBD (FGO), making full use of the culture space to realize highly efficient cultivation. The specific high efficiency of the suspension culture method is as follows: (1) It achieves the cultivation goal earlier because of its advantage over static culture in culturing volume; (2) it is superior to static culture in terms of the proliferation capacity of liver biliary organoids; (3) it reduces the time and labor costs because we can carry out several steps in

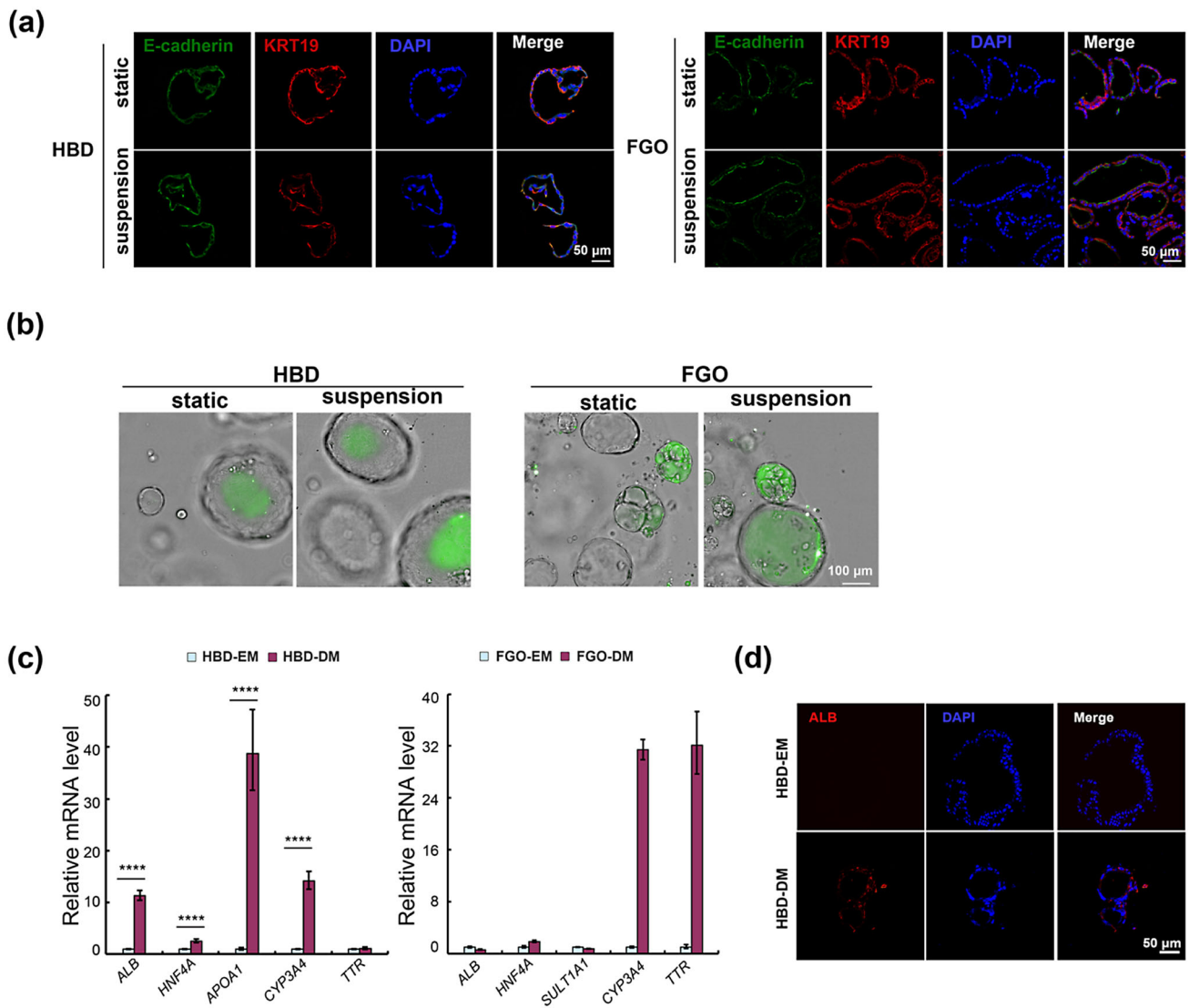


Fig. 3 The characteristics and functional analysis of the liver biliary organoids. **a** Confocal images of E-cadherin and KRT19 staining. **b** Fluorescein diacetate (green) demonstrating the viability of organoids. **c** qRT-PCR showing the expression of the hepatocyte signature genes

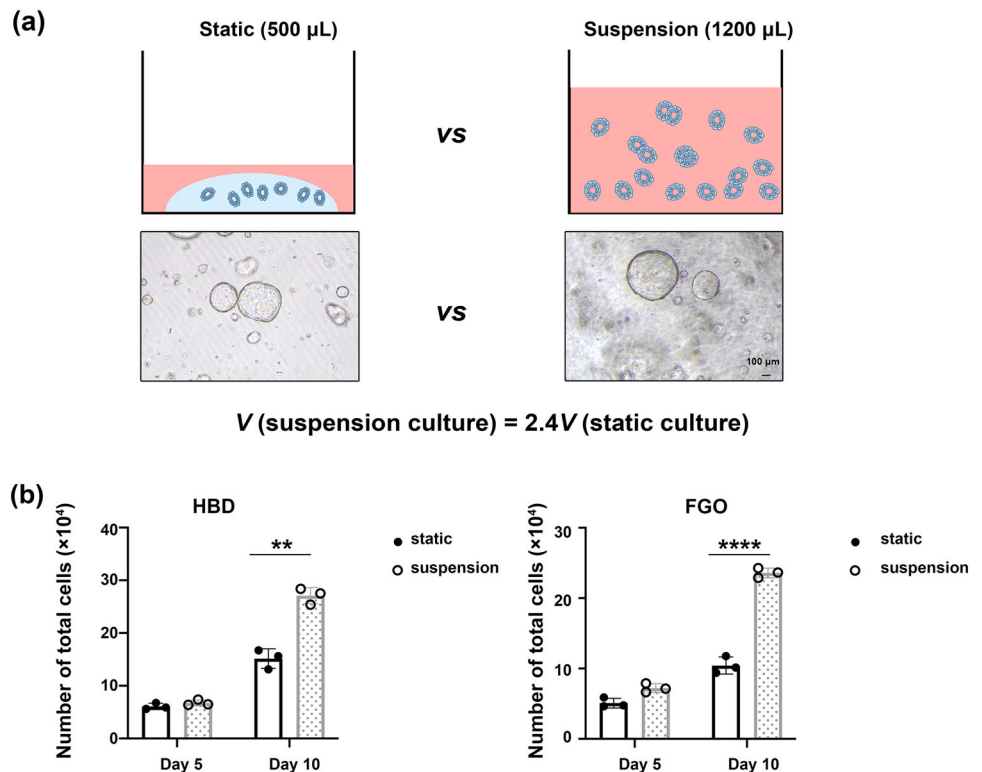
of organoids. Data are presented as mean \pm standard deviation (SD) ($n = 3$). **** $p < 0.0001$. **d** Confocal images of albumin staining in HBD after suspension culture. EM expansion medium, DM differentiation medium

series. The suspension culture method without a threshold for use could cover our daily research and be further extended to larger-scale engineering. In addition, we established that FGO, an organoid derived from human fetal extrahepatic tissue, is promising for application in the regeneration study of EHBD. Second, we focused on the effect of the Matrigel amount and found that although 10% (vol/vol) Matrigel is better, 5% (vol/vol) Matrigel could also be applied in some cases (Fig. S2 in Supplementary Information), suggesting the possibility of Matrigel reduction.

The suspension culture does not change the essence of liver biliary organoids in vitro culture; that is, it retains the

role of Matrigel and medium or is more like a dilution culture of static. Therefore, as a general culture method, this suspension culture method may be suitable for other kinds of organoids. Although the proposed suspension culture protocol optimizes the culture method for liver biliary organoids, it still relies on Matrigel, which is limited in clinical application because of its derivation from the tumors of mice. A way to overcome this limitation is the development of a new cell matrix. Moreover, in our experience, it is not recommended to seed too few cells in this suspension culture, as there is no significant advantage in the overall growth rate at low density compared to static culture. Although it is not convenient to track and record organoids due to the foggy shape of

Fig. 4 Comparison of the static and suspension cultures. **a.** The comparison of the static and suspension culture in culture volume for a 24-well system. **b** The cell counting results of human bile duct organoid (HBD) and fetal gallbladder organoid (FGO) in static and suspension cultures on days 5 and 10. Three independent replicates were performed for each sample. ** $p < 0.01$, **** $p < 0.0001$



Matrigel diluted in the medium, we could use sampling analysis to solve this problem. To some extent, our suspension culture method is a complement to static culture.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s42242-022-00213-3>.

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Author contributions JC and BZ conceived the study; JC, JZ and LY performed the experiments; BZ supervised the work; JC and BZ wrote the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures followed were in accordance with the ethical standards of the Medical Ethical Council of Obstetrics and Gynecology Hospital of Fudan University (the ethical approval number: 2018-77-C1) and with the Helsinki Declaration of 1975, as revised in 2000 (5). The fetal gallbladder tissue was obtained with the informed consent by the patient who had made a legally elective abortion from the Obstetrics and Gynecology Hospital of Fudan University. The HBD from the patient was used for this study also with the informed consent by the patient.

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