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A new, economical, and easy protocol to culture 3D mouse hepatoid and cholangoid

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Abstract

Despite the high efficiency of self-regeneration in liver, liver stem cell organoid culture is way behind other cell types such as intestinal organoid (Enteroid). The contributing factor is the lack of efficient technique to isolate liver cells and to culture liver stem cell organoid. Here we reported a new, economical, and simple method to generate 3D hepatocyte organoid (Hepatoid) and cholangiocyte organoid (Cholangoid). We have isolated liver cells using ice-cold 3mM EDTA PBS medium. The isolated cells were cultured on 50% Conditional Medium. After 1 or 4 days post isolation and culture, Cholangoid and Hepatoid were readily to be detected under light microscope and they continued to grow well after several passages. This method could be used to isolate and culture human liver organoids. This feasible protocol will greatly facilitate more research on liver function and diseases using liver cell organoids.

Keywords: Organoid; Stem cell; 3D culture; Liver

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Introduction

Liver is comprised of 80% hepatocytes in volume and the rest cells are sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells, cholangiocytes (bile duct epithelium), and intrahepatic lymphocytes [1]. Hepatocytes are responsible for many critical biological processes including protein synthesis, sugar metabolism, detoxification, as well as synthesis and secretion of cholesterol,

bile acids, and phospholipids. Because of hepatocyte essential role in liver function, freshly isolating primary hepatocytes is important to research various aspects of liver health and diseases including metabolic disease, toxicology, oncology, and parasitology [2]. The current golden standard method for isolating hepatocytes is so-call “two-step perfusion” method [3]. In this method, water bath, heating table, perfusion pump, and collagenase I or IV are required. The operation is time consuming, complicated, and expensive.

Liver possesses incredible regenerative capacity where it recovers from two third partial hepatectomy in mice [4]. Recently, primary stem cell and 3D organoid culture become more and more popular to be used for biomedical research. Despite its easy regeneration, organoid culture using liver cells is still at infantile stage. Recently, Hu and colleagues reported a method to culture hepatocyte 3D organoid from mouse and human liver with expensive reagents and complicated procedure [5]. Here we reported a new, economy, and simple method to isolate liver cells and successfully generate 3D hepatocyte organoid (hepatoid) and cholangiocyte organoid (Cholangoid). This feasible protocol will greatly facilitate more research on liver function and diseases using liver organoid.

Method and Materials

Reagents and dispensable used: cold PBS

1. Cold shaking buffer (3mM EDTA and 2% Sorbitol in PBS)
2. 15 and 50 ml tubes
3. 70 μ m strainers
4. Matrigel, put on ice to pre-thawed for 2-3 hours or overnight at refrigerator.
5. 50% conditional medium from L-WRN cells was prepared according to protocol reported by Miyoshi et al. [6].
6. The 50% conditional medium was supplemented with 1x antibiotics, 10 μ m Y27632 (ROCK inhibitor), and 10 μ m SB431542 (inhibitor of the TGF- β /Activin/NODAL).

Liver and its cell collection:

1. Immediately after 8-50-week-old BL6 *Il10*^{-/-} mice were sacrificed, the abdominal cavity was opened.
2. The gastric system was moved to the right and the visceral vena cava was exposed.
3. A 22G syringe filled with 6 ml shaking buffer was inserted into the vena cava

through the right atrium. Fix the needle with a string tying the needle and the blood vessel.

4. After making a small cut in the visceral vena cava, the shaking buffer was injected into the liver.
5. The liver was then resected and put into ice-cold PBS.
6. The liver was cut into small pieces and pipetted into 15 ml conical tube.
7. The tube was rocked in 4 °C cold room for 5 min.
8. After settling on ice, the supernatant was decanted (careful not to discard tissue!).
9. Then 5 ml shaking buffer was added into 15ml tube and rocked in cold room for 30 minutes.
10. After settling, the supernatant was discarded, 5 ml PBS was then added.
11. The tube was then shaken gently by hand for 2 minutes to release hepatocytes.
12. To check the release of hepatocyte, 10 μ l of resuspended tissue (right after shaking) onto a slide and check for hepatocyte release.
13. The supernatant was then filtered through 70 μ m strainer.
14. The tube was then centrifuged at 150 g for 10 min at 4 °C and the cells were resuspended in 50% conditional medium on ice.
15. The cells were then mixed at 1:1 with Matrigel on ice and pipet multiple times till homogeneous mixture.
16. 50 μ l of the cell mixture were pipetted into the middle of a 24-well plate.
17. The plate was then put into 37 °C incubator for 15-20 min to harden the Matrigel.
18. 500 μ l 50% conditional medium was then slowly added into the well.
19. The cells were checked daily and pictures were taken using Nikon ST2 microscope. The medium was replaced with fresh 50% conditional medium every 3 days.

20. To pass the organoids, put the plate on ice for 30-45 minutes to thaw Matrigel.
21. Pipet up and down with P-1000 to assist on breaking up Matrigel.
22. Transfer to a 1.7mL tube and pass through needle (26 ½ to 30 ½ -G).
23. Follow procedures 14 to 19.

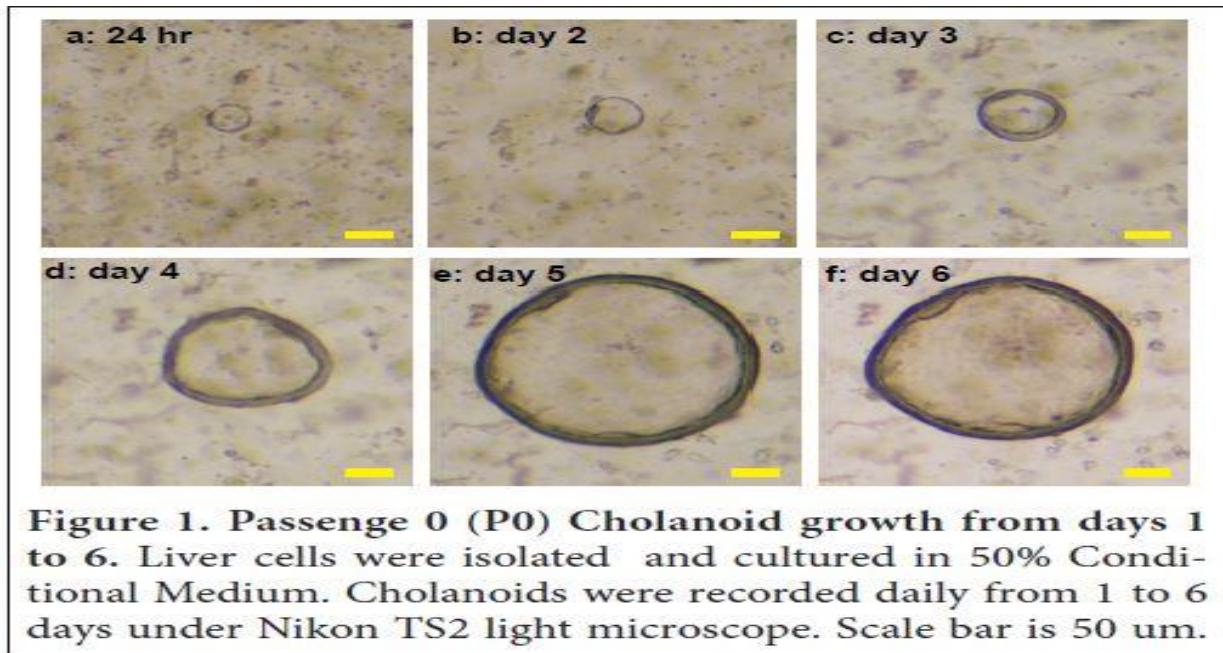
Results and Discussion

Successfully culturing hepatocyte organoid from mouse and human liver tissues was first reported in November 2018 [5]. In the report, the liver cells were isolated using two-step perfusion and a total of 11 growth factors and inhibitors were used, hence the method was expensive and difficult to operate. Here, we showed a new, inexpensive, and simple protocol to isolate liver cells and to successfully culture 3D hepatocyte organoid (Hepatoid) and cholangiocyte organoid (Cholangoid).

To isolate mouse liver cells, we adopted a protocol for isolating and culturing mouse intestinal organoid (Enteroid). Our protocol was based on using EDTA to release cells and on sitting tissue/cells on ice to increase isolated cell viability. In addition, as showed in the method section, our procedure to isolate liver cells was easy to perform. In contrast, the old “two-step perfusion” method requires

anesthesia, complicated surgery procedures, and perfusion pump [3]. Hence, our protocol has obvious advantage over the “two-step perfusion” on liver cell collection.

Furthermore, our protocol is the ease to make liver organoid culture medium. We used L-WRN cells to generate 50% conditional medium [6]. The liver cell grew very well in the medium. Interestingly, the medium supported the growth of both Hepatoid and Cholangoid. Specifically, at 24 hours (1 day) post isolation and culture, ring/circle-shape organoids with size of 50 µm was detected in the Matrigel under light microscope (Figure 1a) and the center of the ring was void of cells, resembling a duct. According to Hu and colleagues’ report, these cells comprised of cholangiocyte (bile duct epithelial cell) organoids (Cholangoid). At days 2, 3, 4, 5, and 6, the Cholangoid size increased to 65, 100, 175, 300, and 325 µm without obvious cell budding (Figure 1b-f). At this time, we decided it is time to separate the Cholangoid with other cells and transferred the individual Cholangoid without disrupting the ring/tube to new wells. After four time passages, the Cholangoid continued to grow well. Interestingly, some Cholangoids developed into Hepatoids after several day culture.



For Hepatoid development, at day 4, ball-shape Hepatoids with diameter of 50 μm were readily detected with cell budding on the edge (Figure 2a). At day 5, the Hepatoid size increased to 80 and it was more visible for more grape/ball-shape buddings at the surface (Figure 2b). Interestingly, the total number of Hepatoid (34) at day 5 was twice of Cholangoid (17). At day 6, Hepatoid size increased to 88 μm and grape/ball shape buddings at the surface were more evident (Figure 2c). At this time, we

decided it is time to separate the Hepatoid with other cells and we then transferred the individual Hepatoid without disrupting the ball to new wells. After four-time passages, the Hepatoids continued to grow well. We plan to nanoinject bacteria (e.g. *Campylobacter jejuni*, *Clostridium perfringens*) or cytokine (e.g. $\text{TNF}\alpha$) into the Hepatoids and host responses (e.g. inflammation, apoptosis) will be assessed.

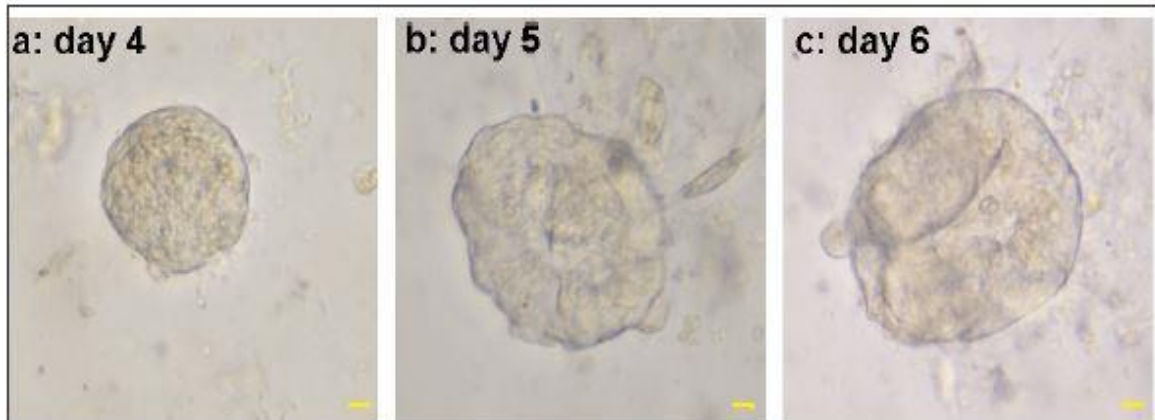


Figure 2. Passenge 0 (P0) Hepatoid growth from days 4 to 6 . Liver cells were isolated and cultured in 50% Conditional Medium. Hepatoids were recorded daily from 4 to 6 days under Nikon TS2 light microscope. Scale bar is 10 um.

In summary, we have developed a new, economical, and simple procedure to isolate and culture mouse Hepatoid and Cholangoid. The culture media for human hepatoid reported by Hu and colleagues is the same formula to culture mouse hepatoid [5]. Hence, this method could be adopted to isolate and culture human Hepatoid and Cholangoid. Our finding would promote the liver research using liver organoids.

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Author Contribution: X.S. and H.W. wrote the manuscript.

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