Long-Term Expansion of Mouse Hepatic Stem Cells in 3D Culture Using HepatiCult™ : A Serum-Free Hepatic Organoid Expansion Medium

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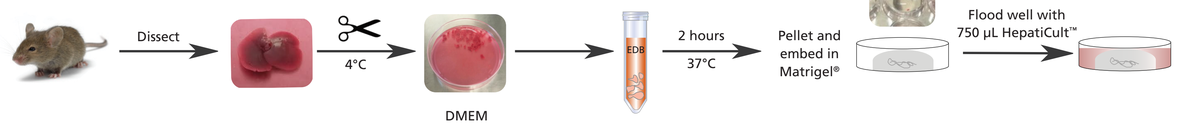
Introduction

Growing hepatocytes as organoids in a three-dimensional (3D) cell culture environment represents a more physiological model system than conventional 2D adherent cell culture for studying many different aspects of liver cell biology. These hepatic organoids are spherical structures composed of a polarized monolayer of epithelial cells that retain many key features of hepatocytes *in vivo* (Huch et al. Nature 2013). We are currently developing HepatiCult™, a serum-free medium that promotes the generation of hepatic progenitor-derived organoids from mouse liver tissue. Mouse livers were enzymatically digested to remove mature hepatocytes, and the resulting hepatic ducts were embedded in Corning® Matrigel® and cultured in HepatiCult™. Liver organoids can be visualized budding from nearly 100% of plated ducts within 24 hours after plating, thereby indicating the presence of a putative population of liver stem and progenitor cells. These hepatic progenitor -derived organoids were then serially passaged every 5 - 7 days at 1:4 to 1:10 split ratios and maintained long-term for > 1 year, thereby indicating the presence of self-renewing hepatic stem cells. Cells within organoids expressed Lgr5, Sox9, Keratin (Krt) 7, Krt19, EpCAM and Hnf4α, but not markers of mature hepatocytes; and can be induced to differentiate into mature functional hepatocytes using published protocols (Huch et al, Nature 2013). These results demonstrate that HepatiCult™ efficiently generates and expands hepatic progenitor-derived organoids from mouse livers, and promotes the long-term maintenance and self-renewal of mouse hepatic stem cells that maintain their differentiation capacity.

Protocol

Figure 1. Protocol for Isolation of Hepatic Ducts from Mouse Liver Tissue



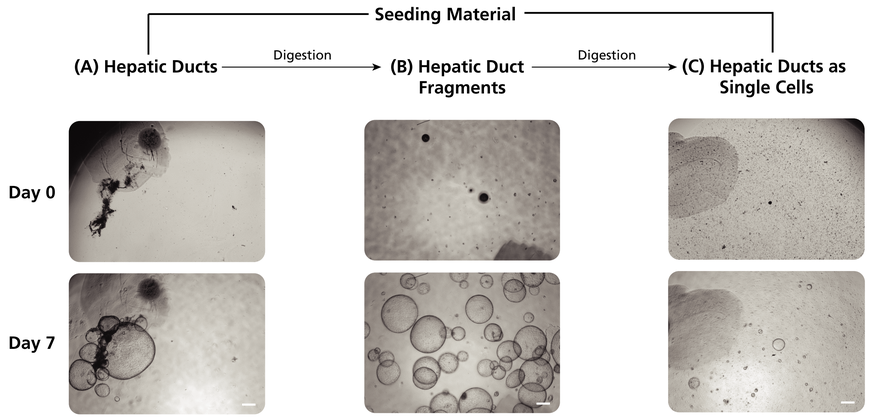
Mouse liver tissue was dissected, minced and digested with enzymatic digestion buffer (EDB) for 2 hours at 37°C. Digested hepatic ducts were pelleted and embedded in Matrigel® by plating 30 - 50 µL domes at the centre of a pre-warmed 24-well plate. The domes were solidified at 37°C for 10 minutes and subsequently flooded with 750 µL HepatiCult™.

Results

Figure 2. Hepatic Ducts Embedded in Matrigel® and Cultured in HepatiCult™ Yield Budding Organoids Over the Course of Seven Days

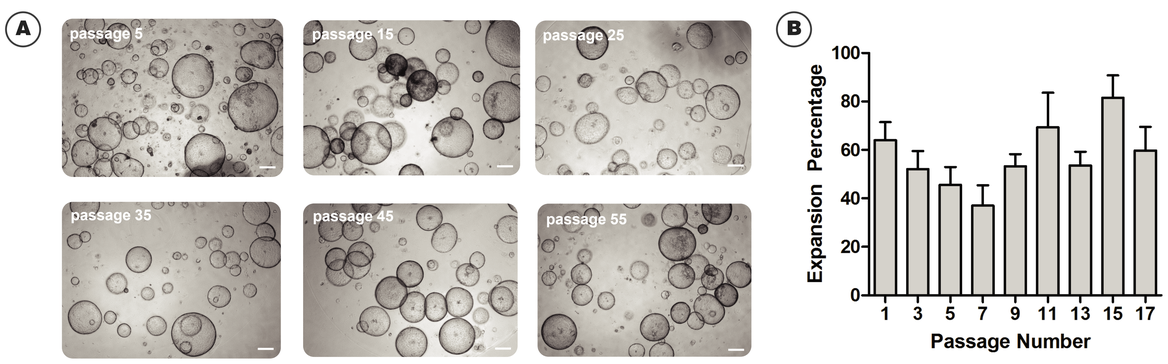
Hepatic ducts first thicken and form organoid buds at the ductal ends, before organoids also grow from the ductal sides. Single organoids may also be generated from small hepatic fragments that have been generated during digestion. Scale bar, 200 µm.

Figure 3. Hepatic Organoids Expand from Hepatic Ducts, Hepatic Duct Fragments, and Single Cells



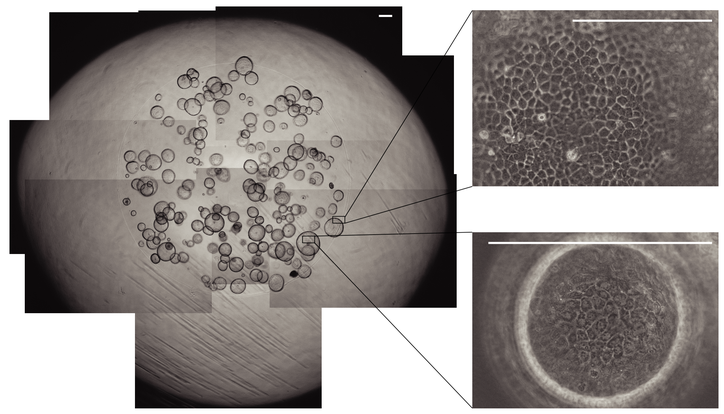
Hepatic ducts isolated from mouse liver tissue may be plated as **(A)** whole ducts or may undergo an additional cell dissociation step, yielding **(B)** hepatic duct fragments or **(C)** quantifiable single cells. The yield of hepatic organoids from whole ducts and duct fragments exceeds that of embedded single cells. Once hepatic organoid cultures have been established from primary mouse tissue after 7 days of culture in HepatiCult™, organoids can be passaged by mechanical tritutation into organoid fragments and plated in desired densities using splitting ratios between 1:4 and 1:10 or fragment counts. Scale bar, 200 µm.

Figure 4. Hepatic Organoids Can be Maintained Long-Term and Scaled-Up When Cultured in HepatiCult™



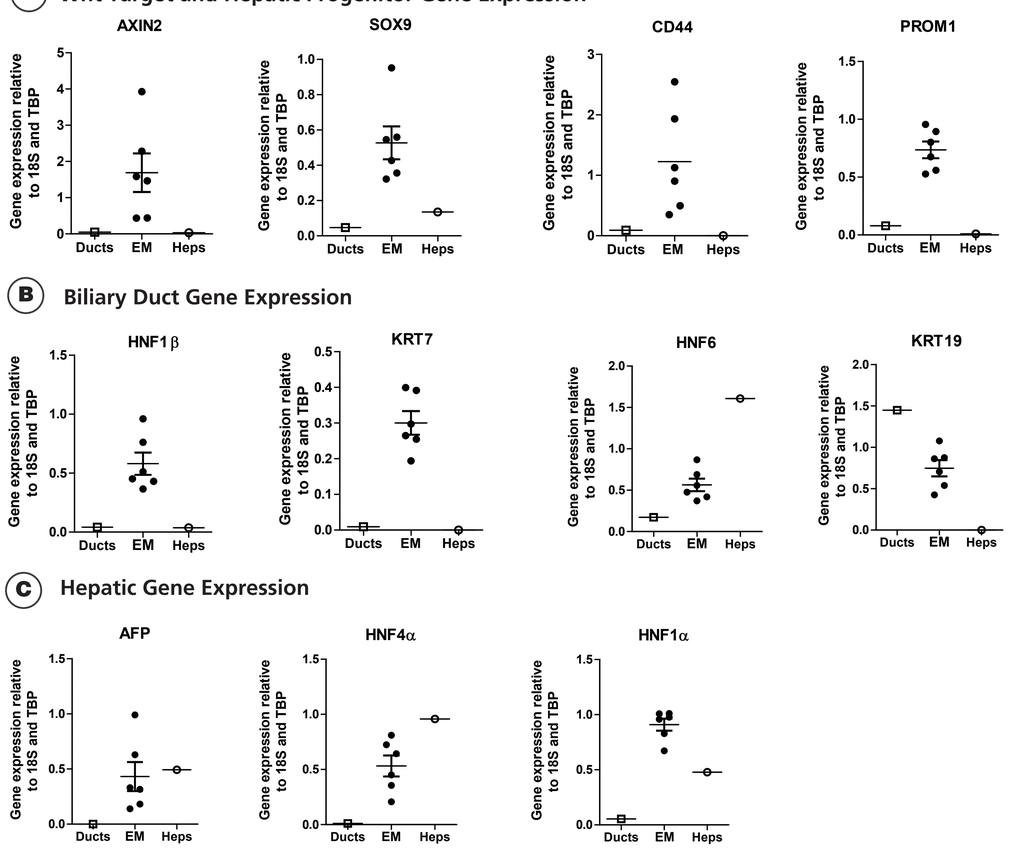
(A) Established hepatic organoids can be maintained for at least 50 passages (>1 year) by passaging every 5 - 7 days at ratios between 1:4 to 1:10. Scale bar, 200 µm. **(B)** Expansion efficiencies across long-term passaging remain stable at above 50% expansion percentages (n=3 - 17, error bars indicate SEM).

Figure 5. Hepatic Progenitor Derived-Organoids Exhibit Characteristic Hepatocyte-Like Morphologies



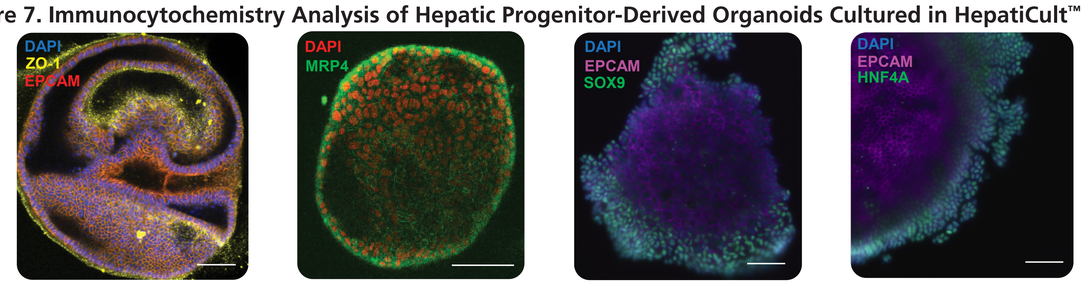
Established organoids display the typical polygonal morphologies of hepatic cells, tight junctions and bi-nucleation. Hepatic progenitor derived organoids are shown at passage 20. Scale bar, 200 µm.

Figure 6. Gene Expression Profile of Hepatic Progenitor-Derived Organoids Cultured in HepatiCult™



The gene expression profile of hepatic progenitors within organoids derived from three different mice grown in HepatiCult™ Expansion Medium (EM) between passage 1 and 20 was compared to hepatic ducts seeded on day 0 (ducts) and primary mouse hepatocytes (heps). Analysis by qPCR shows representative **(A)** Wnt target and hepatic progenitor derived genes, **(B)** biliary duct genes, and **(C)** hepatocyte markers to be strongly expressed in hepatic progenitor-derived organoids compared to seeded ducts on day 0. Horizontal bars indicate mean. Error bars indicate SEM, n=6.

Figure 7. Immunocytochemistry Analysis of Hepatic Progenitor-Derived Organoids Cultured in HepatiCult™



Hepatic organoids emerging from seeded ducts stained positive for epithelial transmembrane protein EPCAM, tight-junction protein ZO-1, multidrug resistance protein MRP4 effluxing glutathione from the hepatic basolateral cell membrane, progenitor marker SOX 9 and hepatic transcription factor HNF4α. Scale bar, 100 µm.

Summary

- Hepatic progenitor-derived organoids can be generated from hepatic ducts, organoid fragments and single cells when cultured in HepatiCult™
- Liver organoids can be expanded and maintained in HepatiCult™ while embedded in Matrigel®
- Hepatic progenitor organoids can be maintained for over 50 passages (> 1 year)
- Hepatic progenitor cells within organoids cultured in HepatiCult™ express ductal and hepatic progenitor markers detected by qPCR and immunocytochemistry

References
(1) Huch M et al. (2013). Nature 494: 247-50 (2) Wang B et al. (2015). Nature 524: 189-185