Research Square

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Establishment and Optimized Standardization of an Intestinal-Hepatic Organoid Co-Culture System

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Abstract

Background

While organoid technology is capable of mimicking the structure and function of organs, models focused on individual organs fall short in replicating the inter-organ communication seen in conditions like tumor metastasis or hepatic encephalopathy. The objective of this research was to develop a reliable co-culture system for intestinal and hepatic organoids by modifying the durations of their pre-culture phases.

Result

Our findings indicated that initiating co-culture during the early stages of organoid development significantly enhanced the viability of both organoid types. In contrast, co-culturing after they had matured independently often led to organoid mortality. The differences observed could stem from the fact that early-stage organoids are still undergoing the early processes of stem cell differentiation, lacking specific functional traits and exhibiting high adaptability to their surroundings. In contrast, organoids that are in the mid to late stages possess distinct features and find it challenging to adjust to significant alterations in their environment.

Conclusion

Our findings demonstrate that the initial developmental status and seeding timing of both intestinal and hepatic organoids critically determine the structural integrity and physiological stability of the co-culture system. These findings offer a more stable and reliable novel organoid co-culture model that simulates physiological and pathological interactions along the gut-liver axis in vitro.

1. Introduction

Cells serve as the fundamental building blocks of structure and function in living organisms, while multicellular entities consist of tissues, organs, and systems that arise from the specialization and coordination of cells with distinct roles [1, 2]. Presently, the primary techniques for evaluating the potential hazards, therapeutic benefits, and mechanisms of drugs in humans depend on non-clinical experimental approaches, specifically in vitro two-dimensional (2D) cell cultures and in vivo animal studies[3–6]. Although animal models provide a closer approximation of human physiological traits, they are often influenced by confounding factors, interspecies variations, and ethical concerns, which can compromise their reliability and relevance in human health investigations[7, 8]. While 2D cell lines are convenient and economical, they are limited by their simplistic monolayer structure, failing to accurately represent the intricate three-dimensional (3D) microenvironment and cellular interactions found in living organisms. While 2D cell lines are convenient and economical, they are limited by their simplistic

monolayer structure, failing to accurately represent the intricate three-dimensional (3D) microenvironment and cellular interactions found in living organisms[9]. The primary benefit of 3D cell culture lies in its ability to more accurately replicate the in vivo physiological context, restoring the native morphology, polarity, and interactions between cells and their matrices, while maintaining their genuine functional and metabolic properties. This makes it ideal for various research purposes, including disease modeling and drug discovery. 3D organoids represent a sophisticated advancement in 3D cell culture[10]. 3D organoids represent a sophisticated advancement in 3D cell culture. These miniature tissue constructs are created in vitro from stem cells (such as pluripotent or adult stem cells) using 3D culture techniques, allowing for self-organization and the mimicry of real organ structure and function[11]. Organoids typically comprise multiple cell types and replicate essential features of their organ of origin, including cellular arrangement, intercellular interactions, and certain physiological or pathological functions. They find extensive application in developmental biology[12], disease modeling[13], drug screening[14], and regenerative medicine[15]. Despite the promise of organoid technology for constructing disease models, it has inherent limitations. For instance, the commonly used Matrigel contains complex components and exhibits significant batch-to-batch variability, complicating the accurate simulation of the in vivo microenvironment[16], single organ models fail to capture the dynamic interactions between multiple organs, which is particularly critical single organ models fail to capture the dynamic interactions between multiple organs, which is particularly critical[17], exhibiting strong interdependence in substance metabolism and signaling. The development of various diseases, such as non-alcoholic fatty liver disease and alcoholic liver disease, is closely tied to disruptions in gut-liver interactions[18, 19]. A solitary intestinal organoid can only model local absorption and metabolism within the intestine, lacking the ability to replicate the complete enterohepatic circulation of drugs and nutrients that travel to the liver through the portal vein and the return of metabolic products to the intestine. Furthermore, the absence of mature vascular structures and inter-organ signaling pathways hinders material exchange and functional communication between the intestine and liver in vivo[20], limiting the representation of liver regulation by intestinal-derived substances and the feedback of liver metabolism on the intestine. Consequently, developing a co-culture model of intestinal and hepatic organoids that simulates inter-organ substance transport and metabolic cycles is anticipated to provide a more accurate reflection of the true physiological and pathological environment in vivo, limiting the representation of liver regulation by intestinal-derived substances and the feedback of liver metabolism on the intestine. Consequently, developing a co-culture model of intestinal and hepatic organoids that simulates inter-organ substance transport and metabolic cycles is anticipated to provide a more accurate reflection of the true physiological and pathological environment in vivo[21]. Previous research has demonstrated the feasibility of conducting intestine-hepatic organoid co-culture using the Transwell system, which can replicate processes related to the bile acid-FGF19-CYP7A signaling axis[22] and the transmission of viruses from the intestine to the liver[23]. However, these studies utilized fixed pre-culture protocols without systematically investigating how the initial maturation states of different organoids affect co-culture results, and the co-culture methods were not sufficiently optimized for the growth conditions of both organ types. This study aims to establish a novel method for co-culturing intestinal and hepatic organoids, emphasizing the support of normal growth for both types, preserving

their inherent morphological traits, and validating the co-culture system's effectiveness through standardized detection techniques. By employing Transwell, we systematically evaluated various pre-culture durations and unexpectedly discovered that the initial states of the intestinal and hepatic organoids significantly influence their survival in co-culture, a finding not previously reported. Overall, this research provides experimental evidence for creating a stable co-culture system of intestinal and hepatic organoids and presents a new in vitro model for optimizing co-culture parameters and regulating signaling homeostasis in cross-organ studies.

2. Materials and Methods

2.1 Experimental Animals

C57BL/6J mice, 6-week-old male mice, were purchased from SPF (Beijing) Biotechnology Co., Ltd., license number SCXK (Beijing) 2024-0001. All animal experiments described above were approved by the Guidelines of the Institute of Traditional Chinese Medicine Health Industry (China Academy of Chinese Medical Sciences) for Institutional Animal Care.

2.2 Primary Isolation of Mouse Intestinal Crypts

Six-week-old SPF mice were euthanized by cervical dislocation. A continuous 10 cm intestinal segment, located 1 cm distal to the stomach, was excised and cut into 2–3 cm segments. These segments were placed in a 100 mm cell culture dish (Corning, USA) containing ice-cold DPBS (Gibco, USA). Fat and mesentery were carefully removed, and the segments were rinsed three times with fresh DPBS. The cleaned intestinal segments were then transferred to a 50 mL centrifuge tube containing 20 mL of 0.25% EDTA solution (Gibco, USA) and incubated with shaking at 4 ℃ for 45 minutes. After digestion, the segments were transferred to a new 50 mL centrifuge tube containing 25 mL DPBS, vigorously shaken, and the resulting suspension was filtered through a 70 μm cell strainer to isolate mouse small intestinal crypts.

2.3 Mouse Intestinal Organoid Culture

Matrigel (Corning, Cat# 356231, USA) was thawed at 4 ℃ after being removed from − 20 ℃ storage, and all subsequent steps were performed on ice in a biosafety cabinet. The concentration of the isolated intestinal crypt suspension was adjusted to approximately 1 × 10^3 crypts per mL. The crypt suspension and Matrigel were mixed at a volume ratio of 4:6, and gently mixed by pipetting to avoid bubble formation. Aliquots of 50 μL of the mixture were dispensed as droplets into each well of a sterile 24-well plate (Corning, USA) without spreading. The plate was placed in a 37 ℃, 5% CO_2 incubator for 10–15 minutes to allow the Matrigel to solidify completely. After solidification, 600 μL of complete intestinal organoid culture medium (STEMCELL Technologies, Cat# 06005, Canada) was carefully added along the wall of each well to avoid disrupting the Matrigel domes. The plate was then cultured statically in a 37 ℃, 5% CO_2 incubator, with fresh medium replaced every 2–3 days. After 72–96 hours of culture, mature

mouse intestinal organoids with clear crypt-villus structures were formed and used as the control group for subsequent co-culture experiments.

2.4 Liver Tissue Processing and Isolation of Liver Duct Fragments

Six-week-old SPF mice were euthanized by cervical dislocation. Livers were collected within 2 h and kept on ice. The liver tissues were washed twice with cold DMEM/F-12, cut into 3–5 mm pieces, and transferred to a 50 mL centrifuge tube on ice. After sedimentation by gravity for 2 min, the supernatant was discarded. The tissue was then incubated with 10 mL of tissue dissociation cocktail at room temperature in a 37 ℃ water bath for 20 min, followed by vigorous pipetting 7 times using a 10 mL pipette. After sedimentation by gravity for 1 min, the supernatant from the first digestion was discarded. This digestion - pipetting - sedimentation cycle was repeated for a total of six digestion rounds, each lasting 20 min. The supernatants from the second to the sixth digestions were collected into a 50 mL centrifuge tube on ice, yielding a pooled volume of approximately 50 mL. The pooled supernatant was sequentially filtered through a 70 μm cell strainer and a 37 μm cell strainer. The 37 μm strainer was then inverted and rinsed with 10–12 mL of cold Advanced DMEM/F-12 to collect the liver duct fragments into a pre- wetted 50 mL tube. The liver duct suspension was thoroughly mixed and equally divided into four pre- wetted 15 mL tubes. After centrifugation at 290 × g for 5 min, approximately 5–10 μL of supernatant was left in each tube, and the tubes were kept on ice.

2.5 Matrigel Dome Method for Primary Culture

A 24-well plate (Corning, USA), preferably the central 8 wells to ensure a uniform surface for dome formation, was used. A pre-cooled 200 μL pipette tip was placed in a biosafety cabinet. All operations were performed rapidly on ice to prevent premature gelation of Matrigel. Thawed Matrigel (30 μL) was added to the liver ductal pellets in each centrifuge tube, mixed gently, and 50 μL of the mixture was dispensed into the center of each well to form a dome. The culture plate was placed in a 37°C, 5% CO_2 incubator for 15 min to allow the Matrigel to solidify completely. Then, 750 μL of room-temperature liver progenitor organoid growth medium (STEMCELL Technologies, Cat#06030, Canada) was slowly added along the well wall to avoid direct contact with the dome. Unused wells were filled with sterile DPBS. The plate was cultured in a 37°C, 5% CO_2 incubator. The culture medium was completely replaced every 2–3 days, and organoid morphology was monitored daily. Primary culture was maintained for 4–6 days, and the organoids were used as a control group for subsequent co-culture experiments.

2.6 Intestinal-Hepatic Organoid Co-Culture

2.6.1 Co-culture protocol 1

Step 1: Intestinal organoids were pre-cultured alone for 4 days. Intestinal organoids were seeded onto Transwell (pore size 8 μm, Cat#724331, NEST, China) inserts, and Matrigel was applied to cover the porous membrane at the bottom of the insert. The organoids were cultured independently in the insert for 4 days to allow the formation of mature intestinal organoid structures. Step 2: On day 4 of intestinal

organoid culture, hepatic organoids were seeded into a cell culture dish and pre-cultured alone for one day to allow stable growth and establishment of basic structures. Step 3: Co-culture of intestinal and hepatic organoids was performed in Transwell systems for 4 days. The Transwell inserts containing the cultured intestinal organoids were transferred into the culture dish containing the hepatic organoids. At this stage, the intestinal organoids were located in the upper insert, while the hepatic organoids were in the lower compartment, allowing interaction through soluble signaling molecules in the medium. This setup mimicked the physiological connections of the gut-liver axis in vivo, thereby achieving coculture.

2.6.2 Co-culture protocol 2

Intestinal organoids were first seeded into the upper chamber of Transwell inserts and cultured alone for 2 days to allow the formation of mature intestinal organoids with intact crypt structures. On day 2 of culture, the Transwell inserts containing the intestinal organoids were transferred to new culture dishes, and pre-established hepatic organoids (day 0) were simultaneously seeded into the bottom chamber of the same dishes. This approach enabled direct co-culture of the two organoid types within the same culture system.

2.5 Analysis of Intestinal Crypt Domains and hepatic Organoid Circularity

2.5.1 Crypt Domain Counting

The criteria for identifying valid crypt domains were first established. Only finger-like or cystic protrusions that independently emerged from the organoid surface, exhibited clear boundaries, and remained tightly connected to the main body were counted. Fused clusters, damaged or blurry structures, and structures that were too small were excluded. For quantification, 5–10 non-overlapping fields of view were randomly selected from each cultured sample. For intact organoids within each field, the number of valid crypt domains was counted sequentially in a fixed direction. High-resolution images were simultaneously captured for documentation and reference. At least three biological replicates were included per experimental group. The results were verified either by double-blind counting performed by two independent investigators, or by the same investigator counting twice with a two-hour interval, ensuring that the discrepancy between the two counts was less than 10%. For data processing, the number of crypt domains per-organoid was used as the core statistical unit. The average number of crypt domains per-organoid was first calculated for each replicate, and then the mean ± standard deviation was determined for each experimental group. During counting, consistent imaging parameters were maintained. Protrusions with unclear boundaries were classified as invalid structures, and crypt domains in obscured regions of the organoids were excluded from counting to minimize subjective bias.

2.5.2 hepatic Organoid Circularity Analysis

Circularity was calculated using the formula: Circularity = $4\pi A/P^2$, where A represents the effective area of the organoid (with background, debris, Matrigel beads, and other interfering regions removed, retaining only the actual area of the target organoid), P represents the perimeter of the organoid (defined

as the complete contour along the edge of the target without damage or breaks), and π was taken as 3.1416. Circularity values ranged from 0 to 1. The analysis was performed using Image Pro Plus 6.0 software.

2.6 Calcein-AM/Propidium Iodide Double Staining Analysis

Prepare the live/dead dye premixed solution: Take 1 mL of DPBS, add 1.7 μL of Hoechst 33342, 1 μL of Calcein AM, and 0.5 μL of PI stock solution. Mix well to obtain a solution with final concentrations of 5 μg/mL Hoechst 33342, 4 μM Calcein AM, and 8 μM PI. Take out the stock solutions of Hoechst 33342, Calcein AM, and PI and equilibrate at room temperature for 30 min; add 500 μL of DPBS to each culture well to wash the organoids once; discard the wash solution and add 500 μL of the prepared live/dead dye mixture to each well. Incubate at 37 ℃ for 30 min. After staining, discard the dye mixture and wash the organoids twice with 500 μL DPBS to remove residual dye.

Set the inverted fluorescence microscope (Leica Microsystems, DMi8, Germany) according to the excitation/emission wavelengths (Ex/Em) of the three dyes to observe and capture images: Hoechst 33342: Ex/Em = 350/461, labels the nuclei of all cells; Calcein AM: Ex/Em = 495/520, labels live cells; PI: Ex/Em = 530/620, labels dead cells.

Live/dead cell staining was performed using the Live/Dead Cell Staining Kit (Calcein-AM/PI double staining) (Kangji Bio, Cat# KGA9501-1000, Nanjing, China) according to the manufacturer's instructions for cell viability analysis.

2.7 Statistical Analysis

Data were presented as mean and standard deviation (SD). The One-way ANOVA was performed for the statistical analysis using GraphPad Prism software 6.01 (GraphPad, San Diego, CA, USA). The p values represent significant differences (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$) and ns indicates no difference.

3. Results

3.1 Co-culture After Separate Pre-culture Impairs intestinal-hepatic Organoid Viability

During the first four days following the setup of the intestinal-hepatic organoid coculture system (as shown in Fig. 1), we continuously assessed the growth and morphological stability of both intestinal and hepatic organoids within the coculture environment. During the first four days following the setup of the intestinal-hepatic organoid coculture system (as shown in Fig. 1), we continuously assessed the growth and morphological stability of both intestinal and hepatic organoids within the coculture environment. Daily brightfield imaging was conducted using a Leica DMi8 fluorescence microscope (Leica DMi8 FM) with a calibrated 20× objective lens, capturing images at consistent locations of the organoids grown in Transwell inserts. The images collected over time were organized and displayed in Fig. 2(showing morphological changes from days 1 to 2) and Fig. 3(illustrating changes from days 3 to 4), providing a

visual representation of the morphological features, structural integrity, and growth patterns of the two organoid types during the early stages of coculture.

Figure 1 Schematic diagram of co-culture of intestinal organoids and hepatic organoids

(A)(C)(E)(G) Morphology of intestinal organoids and hepatic organoids co-cultured under different medium ratios on day 1 and day 2;

(B)(D)(F)(H) Morphology of intestinal organoids and hepatic organoids cultured in monoculture on day 1 and day 2. Scale bar = 200 μm

In Fig. 2, it can be seen that during the first two days of coculture, there were no significant variations in the overall structure or growth conditions of organoids among the various medium ratio treatment groups when compared to the control group. However, by the third and fourth days of culture (refer to Fig. 3), the intestinal organoids showed significant morphological alterations, including a notable darkening and a blackish hue in contrast to the control group, indicating a rapid onset of apoptosis and cell death. At the same time, hepatic organoids exhibited considerable irregularities compared to those cultured alone in the control group, marked by shrinkage, collapse, and disorganized, unevencellborders.

(A)(C)(E)(G) Morphology of co-cultured intestinal and hepatic organoids on day 3 and day 4.

(B)(D)(F)(H) Morphology of mono-cultured intestinal and hepatic organoids on day 3 and day 4. Scale bar = 200 μm

In order to thoroughly investigate and characterize the viability, growth patterns, and structural consistency of intestinal and liver organoids in a coculture setting, as well as to assess the coculture system's capacity to preserve the physiological traits of both organoid types, a dual staining method using Calcein AM and propidium iodide (Calcein AM/PI) along with roundness evaluation was conducted on the fourth day of the coculture process.

Figure 4A illustrates that the intestinal organoids in the coculture condition demonstrated pronounced red fluorescence when compared to the control group, signifying a high level of cell death among the intestinal organoids grown under various medium ratios. Conversely, the hepatic organoids primarily exhibited green fluorescence, indicating a conducive environment for cell proliferation. Live cell viability was assessed using Calcein-AM staining, with the relative fluorescence intensity normalized to the 100% IOs group, as depicted in Fig. 4B. Co-culture at a 5:5 ratio (HOs: IOs) resulted in the highest fluorescence intensity of live cells (***$p < 0.0001$), indicating optimal cell survival.

Ratios of 6:4 and 8:2 also significantly enhanced the live cell signal, while ratios of 4:6 and 2:8 demonstrated moderate but significant increases ($p < 0.05$). Figure 4C illustrates the dynamic changes in the roundness of liver organoids from day 1 to day 4 across various co-culture ratios (HOs: IOs = 100%, 8:2, 6:4, 5:5, 4:6, 2:8). A roundness value closer to 1 indicates a more spherical organoid morphology, which reflects optimal growth; conversely, a decrease in roundness suggests organoid spreading,

deformation, or cell migration. From day 1 to day 2, the roundness of liver organoids remains consistently high across all ratios (e.g., > 0.99), indicating that organoid morphology is stable during the initial co-culture phase, with no significant deformation observed. Beginning on day 3, a decrease in roundness is noted in certain ratios, specifically in the 6:4, 5:5, and 2:8 ratios, suggesting that an excessive number of intestinal organoids may induce spreading or depolarization of liver organoids. By day 4, the roundness remains relatively high in the 5:5 and 6:4 ratios (e.g., > 0.96), indicating that an appropriate liver-intestine ratio is beneficial for maintaining the spherical structure of liver organoids, while the roundness in other ratios continues to decline.

Figure 4 Organoid viability and morphology under different IOs/HOs ratios.

1. (A) Calcein-AM/PI staining on day 4. Green: live, red: dead. Scale bar = 200 μm.
2. (B) Relative fluorescence intensity of live cells on days 1–4 (higher = greater viability).
3. (C) Hepatocyte circularity on days 1–4 (higher = rounder).

3.2 Early co-culture helps the survival of hepatic-intestine organoids

The second co-culture model was developed as illustrated in Fig. 5. Initially, intestinal organoids were placed in the upper chamber (insert) of Transwell plates and allowed to grow independently for two days, facilitating the development of mature organoids with complete crypt structures. On the third day, the inserts containing the intestinal organoids were moved to new culture dishes, where pre-cultured hepatic organoids were introduced into the lower chamber, allowing for the simultaneous co-culture of both organoid types in a shared environment. From day 0 to day 7 post- co-culture initiation, daily observations and imaging of both organoid types were conducted using a Leica DMi8 fluorescence microscope (Leica DMi8 FM) with a calibrated 20× objective lens. Imaging parameters, including exposure time and focus, were meticulously regulated to maintain consistency across images. The bright-field images captured during this period effectively illustrated the morphological alterations, structural stability, and growth interactions of the intestinal and hepatic organoids within the co-culture setting. The findings were compiled for further morphological analysis, as shown in Fig. 6, offering clear visual evidence to evaluate the effects of this temporal co-culture model on the growth conditions of both organoid types. Throughout days 0 to 5 of co-culture, both intestinal and hepatic organoids thrived under optimal conditions. During this timeframe, the intestinal organoids maintained their budding structures, while the hepatic organoids exhibited normal morphology without significant issues. However, by day 6, notable changes occurred in the growth status of both organoid types: the intestinal organoids' budding structures began to diminish, and the hepatic organoids experienced significant structural degradation.

Figure 7A illustrates that the quantity of crypt domains in intestinal organoids rose from day 0 to day 5 during co-culture, followed by a decline starting on day 6. Statistically, as depicted in Fig. 7B, the median circularity of hepatic organoids held steady at around 0.95 from day 0 through day 5 but dropped to 0.85

by day 6, reinforcing the observation that hepatic organoids experienced shrinkage and a decrease in circularity on that day. Additionally, Fig. 7C shows that hepatic organoids began to show signs of collapse on days 6 and 7 of the co-culture period.

1. (A) Roundness analysis of hepatic organoids.
2. (B) Number of crypt domains per intestinal organoid.
3. (C) Number of non-collapsed hepatic organoids.

4. Discussion

This research successfully developed intestinal and hepatic organoids utilizing organoid culture techniques derived from adult stem cells (ASCs). Building on this foundation, two distinct Transwell-based co-culture methods were implemented to grow the intestinal and hepatic organoids together, differing primarily in their pre-culture durations. In the first method, intestinal organoids were pre-cultured for 4 days, while hepatic organoids were given 1 day of pre-culture, followed by a 4-day co-culture period, during which some organoids died. Conversely, the second method involved a 2-day pre-culture for intestinal organoids alone, with hepatic organoids not undergoing pre-culture, leading to organoid death after 7 days of co-culture. Although the study did not explore the molecular mechanisms behind these observations, we analyzed the phenomena based on the outcomes of both methods and relevant literature regarding organoid culture traits, thus offering experimental support for the creation of a stable co-culture system for intestinal and hepatic organoids. The Transwell co-culture system's key benefit lies in its physical separation through a semipermeable membrane, which facilitates paracrine signaling while preventing interference between the organoids, allowing for signal exchange and functional analysis while preserving their structural independence[24]. This makes it a robust experimental framework for studying interactions between different organ systems.

In the hepatic organoid culture system developed by Huch et al., it has been observed that hepatic organoids (HOs) undergo a phase of adaptation and growth during the initial days post-seeding (days 0–3), characterized by diminished metabolic activity. This early stage necessitates a consistent microenvironment to facilitate their growth and structural development[25]. In the first experimental setup, HOs were introduced to the co-culture system after just one day of being in monoculture, still in a delicate adaptation phase and lacking a stable metabolic and signaling framework. In contrast, the intestinal organoids (IOs) that were co-cultured had already been isolated for four days. As noted by Sato et al., IOs typically experience a rapid growth phase with significant proliferation between days 3 and 5 post-seeding[26]. We propose that the IOs, during this vigorous expansion, could negatively impact the early-stage hepatic organoids (HLOs) by: (1) competing for essential nutrients in the culture medium (such as glucose and glutamine); and (2) generating metabolic byproducts (like lactate and ammonia), which could impose metabolic strain on the still-developing HLOs. In the revised co-culture approach, HOs were paired with IOs that had only been cultured for two days at the time of seeding (day 0). At this stage, the IOs had not yet reached a high metabolic state, leading to a reduced metabolic load and a more nutrient-rich medium, which supported the establishment of a stable growth condition for HOs

during their early phase. This finding supports the assertion made by Lagziel et al. that “cells must adapt metabolically to their environment (particularly the nutritional makeup of the culture medium) during in vitro culture; otherwise, their survival could be compromised by metabolic imbalances, nutrient shortages, or stress responses, resulting in skewed experimental outcomes.” Thus, the adjusted co-culture method may prolong the survival of HOs by allowing them to complete their initial adaptation in a less metabolically demanding environment[27].

Throughout the process of embryonic development, intricate signaling networks govern the formation of both the intestine and liver. Research indicates that pathways such as FGF, BMP, and Wnt are vital for the growth, upkeep, and regeneration of these organs[28]. When hepatic organoids (HLOs) were added to the co-culture system on the fifth day, they were likely subjected to a sudden influx of Wnt signals derived from the intestine. While Wnt signaling is crucial for the proliferation of hepatic organoids, it is equally important to finely tune the intensity of these signals. Perugorria et al. found that excessive Wnt signaling can disrupt the regulation of the cell cycle in hepatocytes, and overly active β-catenin signaling may hinder normal cell differentiation[29]. Nejak-Bowen also observed that Wnt/β-catenin signaling has a dual role in cell survival during liver damage, where moderate activation aids in regeneration, but excessive or prolonged activation can lead to cell death[30]. It is hypothesized that the initial four-day preculture of intestinal organoids led to a buildup of Wnt ligands, resulting in a signaling shock to the hepatic organoids when co-cultured, causing heightened Wnt/β-catenin activation and subsequent cell death. In the second protocol, the preculture duration was reduced to two days, which resulted in lower Wnt secretion and a more gradual establishment of signaling. Therefore, protocol 2 likely enhances the stability of the co-culture by avoiding Wnt signaling shock and fostering a temporary balance in signaling.

5. Conclusions

Our findings demonstrate that the initial developmental status and seeding timing of both intestinal and hepatic organoids critically determine the structural integrity and physiological stability of the co-culture system. These results not only provide a more stable and reliable novel organoid co-culture model for simulating physiological and pathological interactions along the gut-liver axis in vitro, but also establish a methodological foundation for further investigations into gut-liver crosstalk mechanisms and for in vitro pharmacological and mechanistic studies of metabolic liver diseases and intestinal barrier-related disorders.

Declarations

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of the Institute of Traditional Chinese Medicine Health Industry, China Academy of Chinese Medical Sciences [approval number: 2024014], and all animal tests were conducted in strict accordance with animal welfare guidelines.

Competing interests

The author(s) declare no competing interests.

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Author Contribution

Jinlong Tan and Yinju Liu contributed to the conception and design. Jinlong Tan, Yinju Liu, and Ruirui Li contributed to the manuscript writing. Jinlong Tan contributed to the manuscript editing. Huang Zhan, Maolin Wang, Zhixuan Huang, and Dongyue Zhou contributed to the data analysis. Yangwen Ai, Jian Liu, Caifeng Li, and Hui Li contributed to the collection and assembly of data. Qili Yuan and Zuochun Ye contributed to the validation and visualization. All authors approved the final version of the manuscript.

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Data Availability

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

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Figures

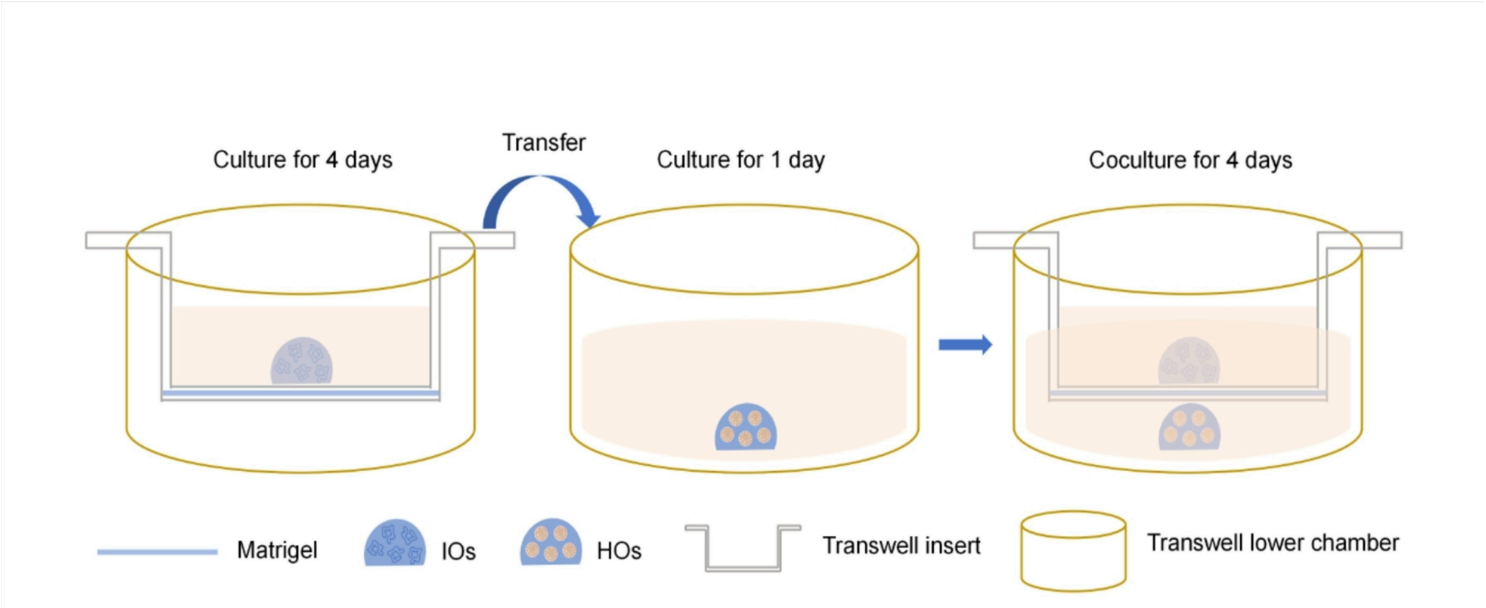


Figure 1

Schematic diagram of co-culture of intestinal organoids and hepatic organoids

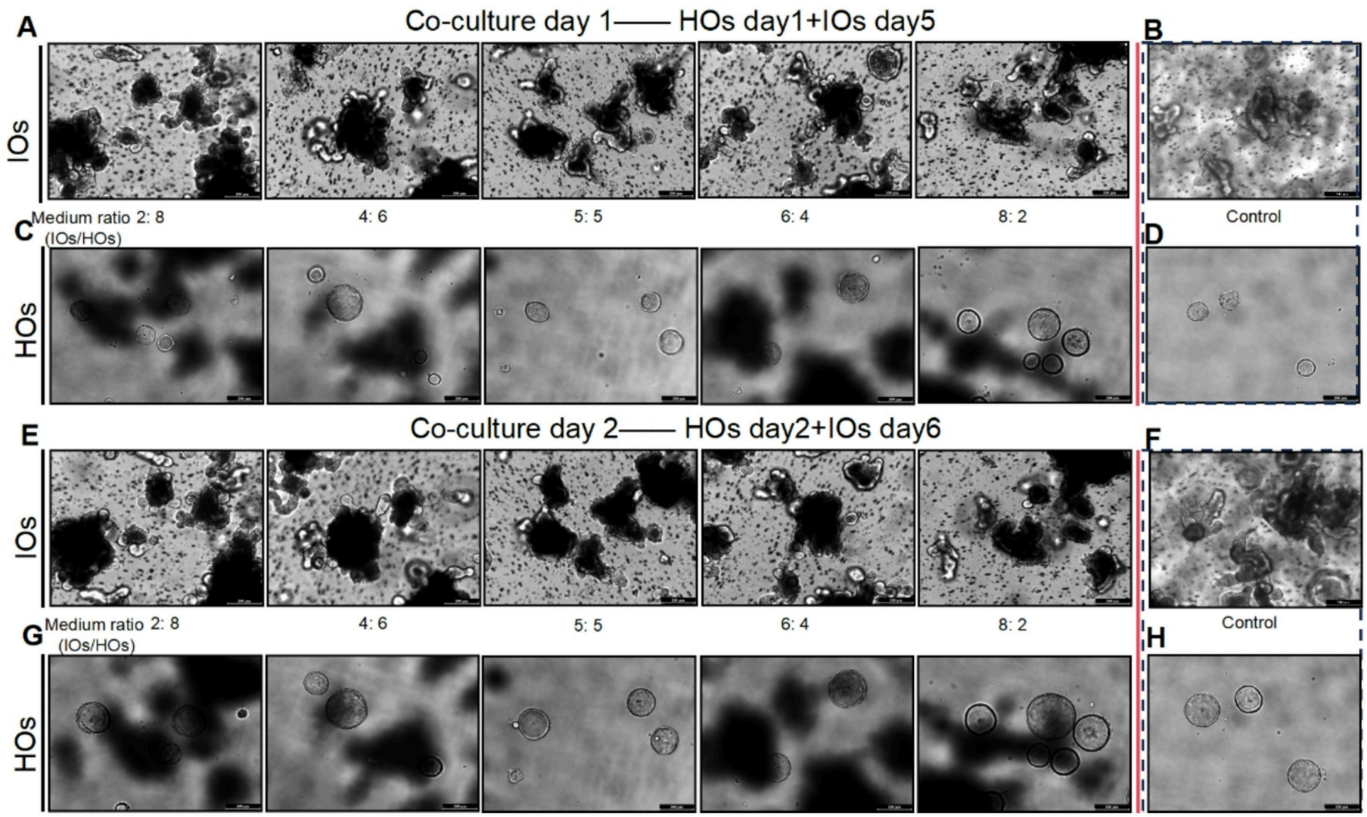


Figure 2

Co-culture of intestinal and hepatic organoids on day 1 and day 2.

(A)(C)(E)(G) Morphology of intestinal organoids and hepatic organoids co-cultured under different medium ratios on day 1 and day 2;

(B)(D)(F)(H) Morphology of intestinal organoids and hepatic organoids cultured in monoculture on day 1 and day 2. Scale bar = 200 μm

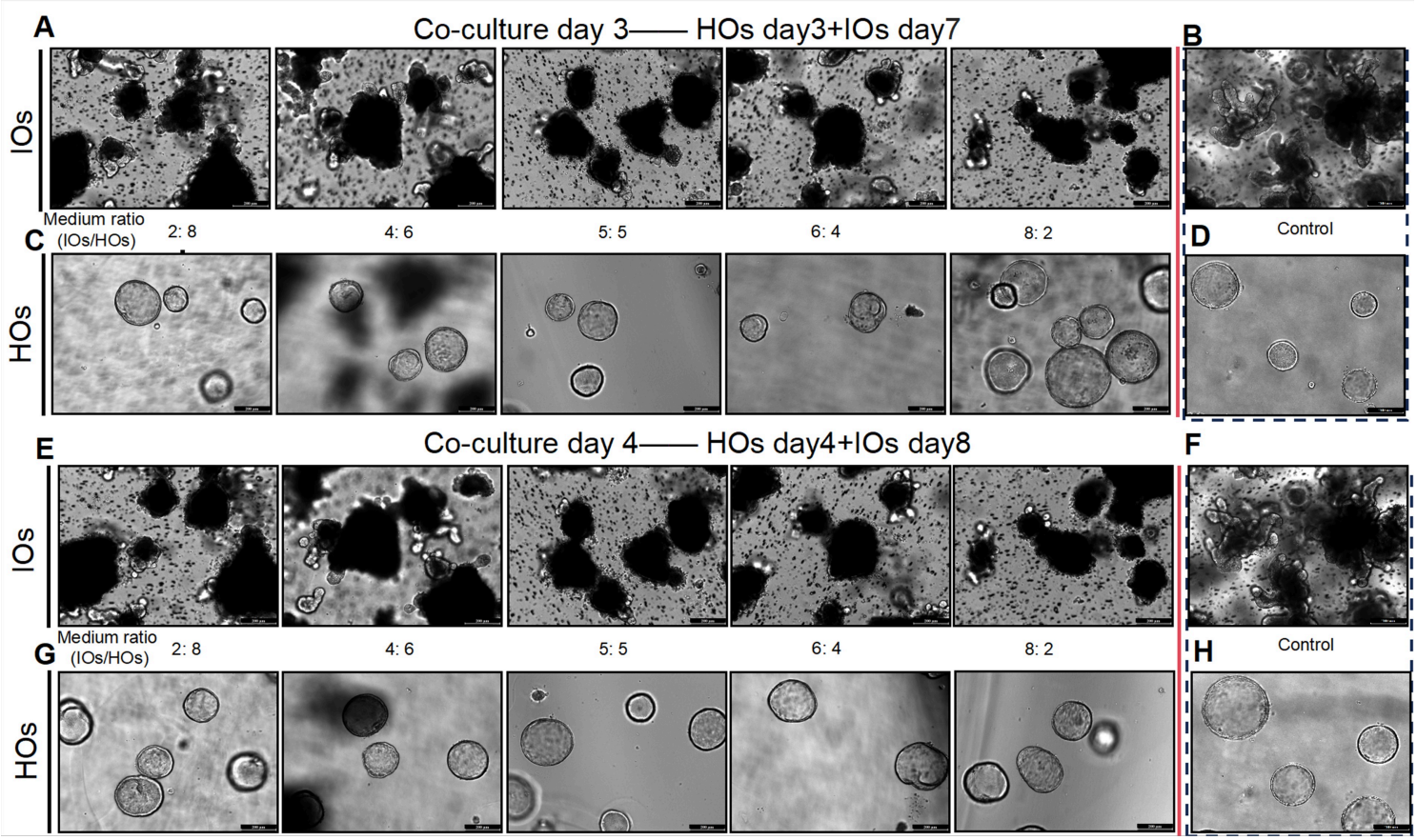


Figure 3

Co-culture of intestinal and hepatic organoids on day 3 and day 4.

(A)(C)(E)(G) Morphology of co-cultured intestinal and hepatic organoids on day 3 and day 4.

(B)(D)(F)(H) Morphology of mono-cultured intestinal and hepatic organoids on day 3 and day 4. Scale bar = 200 μm

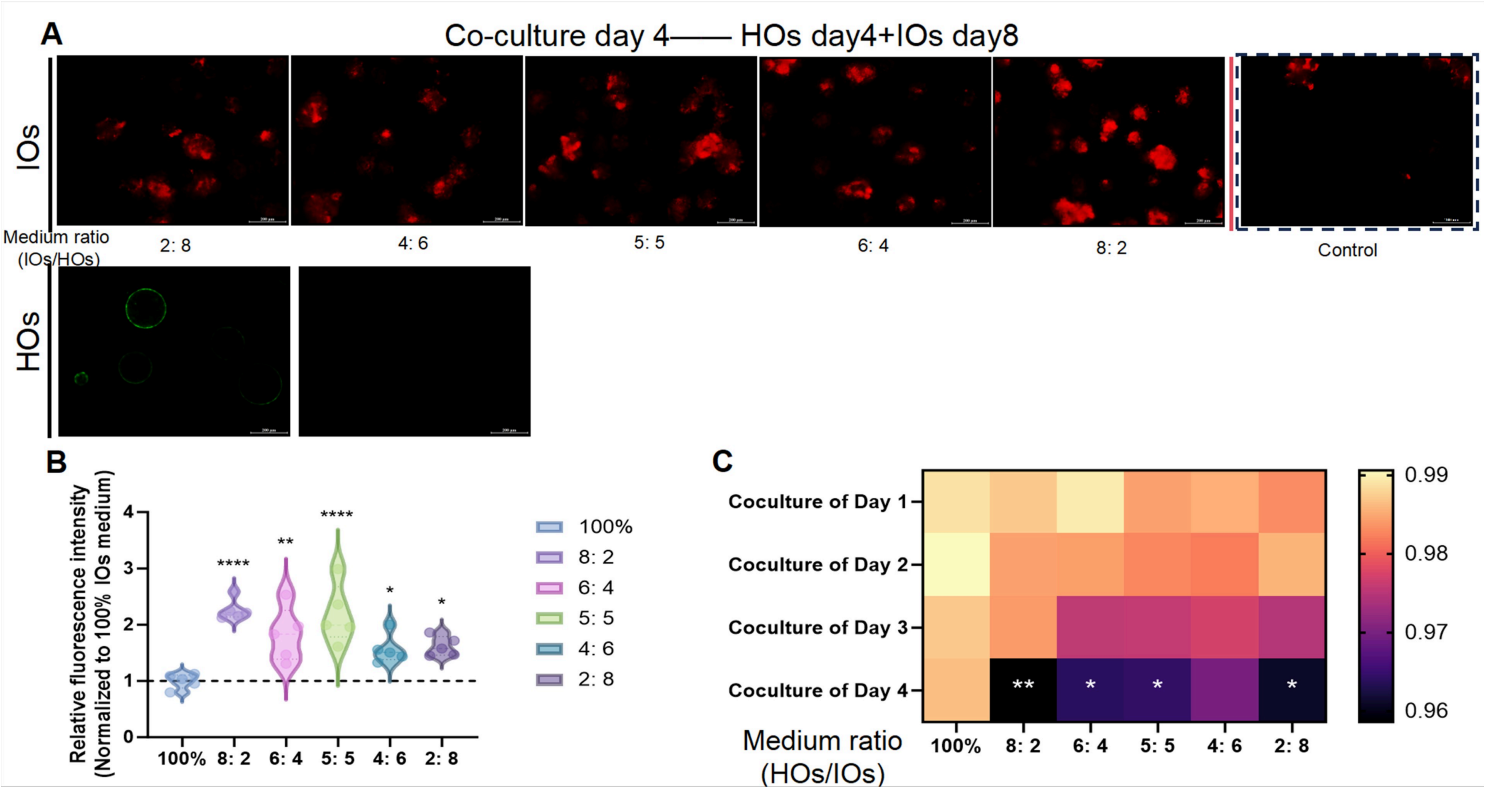


Figure 4

Organoid viability and morphology under different IOs/HOs ratios.

(A) Calcein-AM/PI staining on day 4. Green: live, red: dead. Scale bar = 200 μm.

(B) Relative fluorescence intensity of live cells on days 1-4 (higher = greater viability).

(C) Hepatocyte circularity on days 1-4 (higher = rounder).

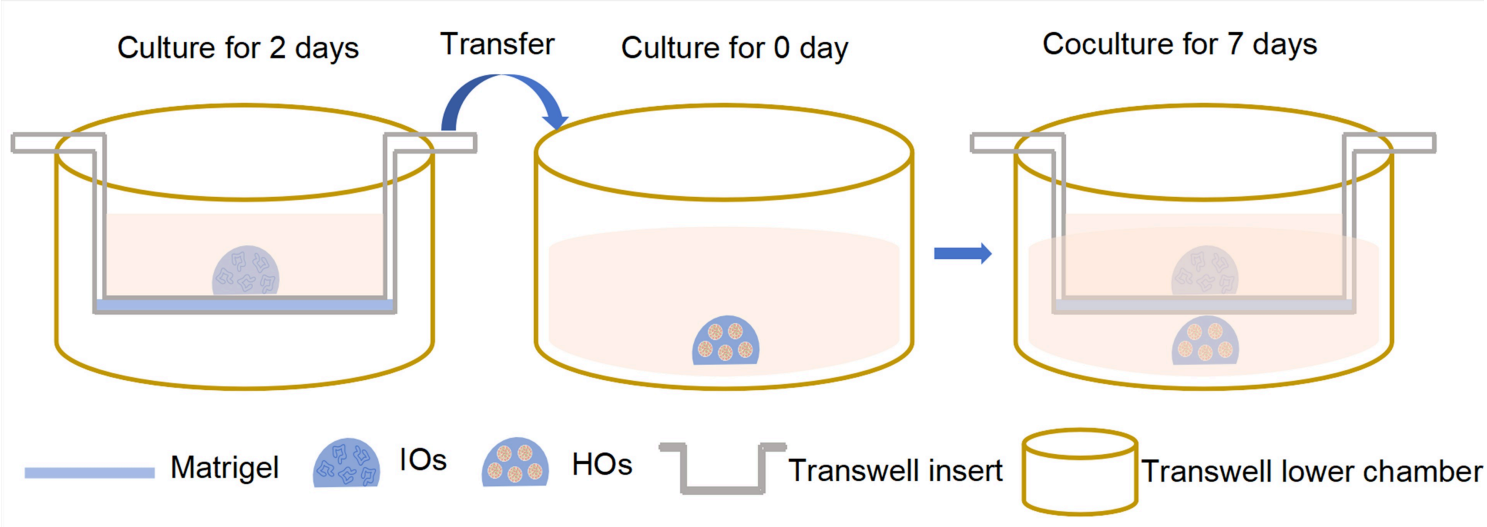


Figure 5

Schematic diagram of the second co-culture model

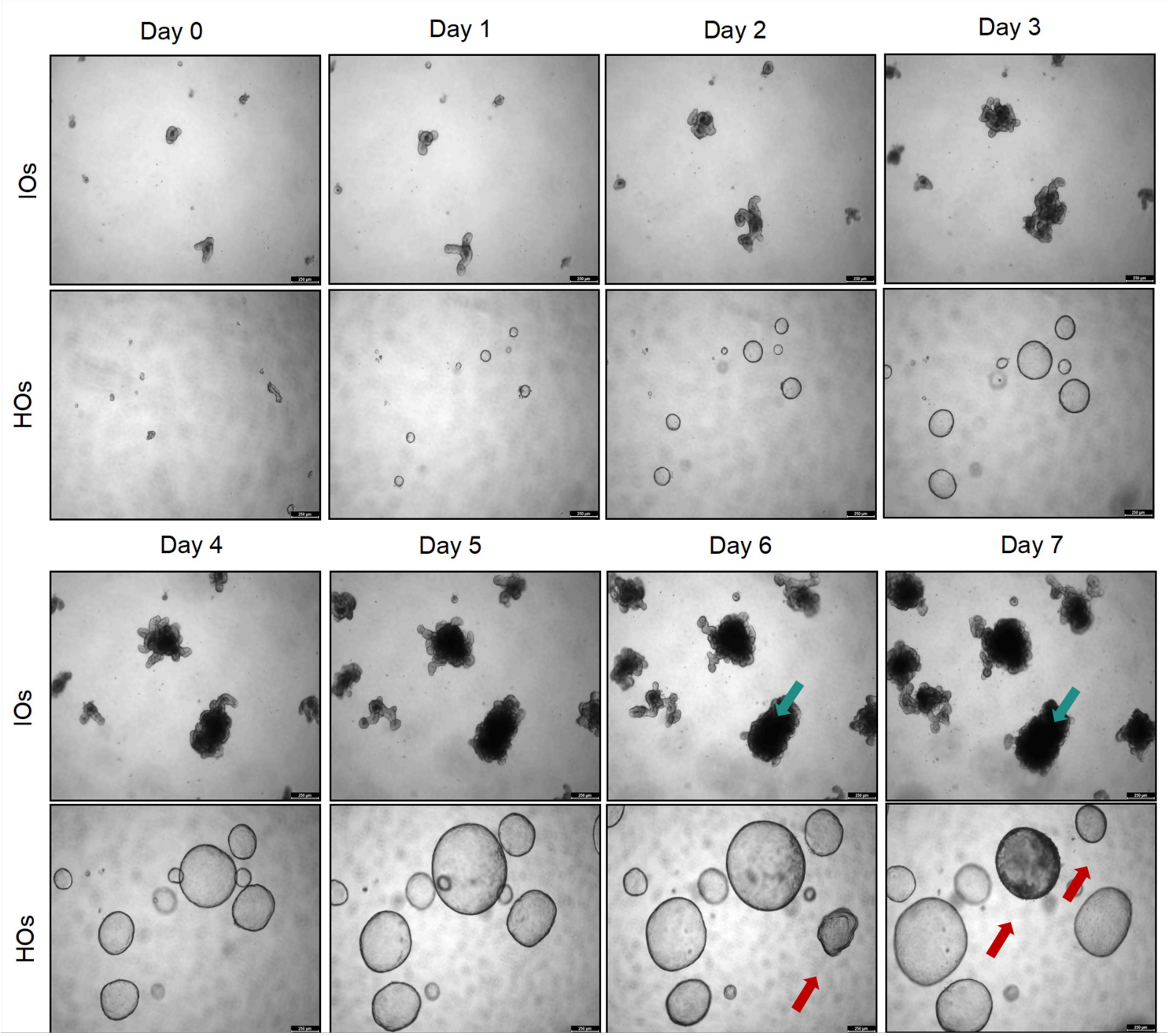


Figure 6

Morphology of intestinal and hepatic organoids in the second co-culture model. Scale bar = 200 μm

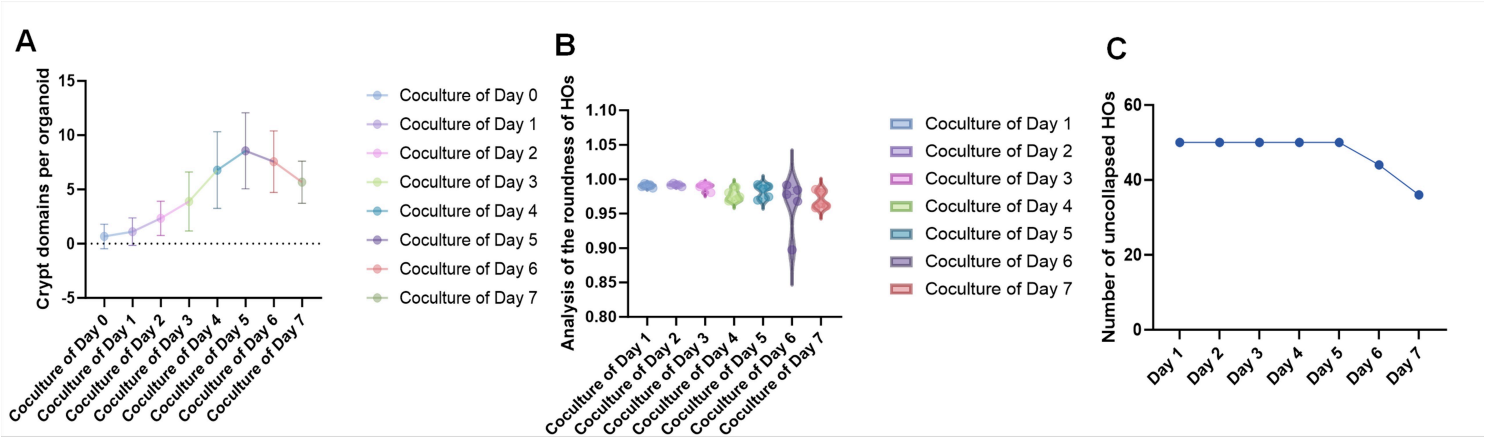


Figure 7

Quantification of hepatic organoid morphology and intestinal organoid crypt formation.

(A) Roundness analysis of hepatic organoids.

(B) Number of crypt domains per intestinal organoid.

(C) Number of non-collapsed hepatic organoids.