**TITLE**

# *In vitro* cultivation techniques for modeling liver organogenesis, building assembloids, and designing synthetic tissues

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**ABBREVIATION LIST:**

**BMP4** Bone morphogenetic protein 4
**FGF-2** Fibroblast growth factor 2
**PROX1** Prospero homeobox 1
**HBs** hepatoblasts
**HEP** hepatic
**HGF** hepatocyte nuclear factor 4 alpha
**hMSC** human mesenchymal stem cells
**HPSC** human pluripotent stem cells
**MES** mesenchymal
**MES-CM** fibroblast-conditioned medium
**TBX3** T-box transcription box factor 3
**TGFβ1** transforming growth factor beta1

**SUMMARY:**

Organoids revolutionize personalized tissue modeling for organ development, drug discovery, and disease research. Organoid engineering advances into creating intricate synthetic tissues. The aim is to integrate morphogenesis, assembloid technology, and biomatrices to advance tissue engineering. The presented methods aid in modeling liver organogenesis and establishing guidelines for synthetic tissue construction.

**ABSTRACT:**
Chronic liver disease has reached epidemic proportions, affecting over 800 million people globally. The current treatment, orthotopic liver transplantation, has several limitations. Promising solutions have emerged in the field of liver regenerative medicine, with liver organogenesis holding significant potential. Early liver organogenesis, occurring between E8.5 and 11.5, involves the formation of epithelial-mesenchymal interactions leading to morphogenesis, hepatic cord formation, and collective migration. However, there is a lack of methods for *in vitro* modeling of this process. In this study, a detailed series of methods are presented enabling the modeling of various stages and aspects of liver organogenesis. In one method series, assembloid technology with hepatic and mesenchymal spheroids is utilized, replicating early structures found in liver organogenesis, modeling early morphogenesis, and demonstrating interstitial cell migration as seen *in vivo*. These innovative assembloid systems help identify factors influencing assembloid formation and migration. Hepatic spheroid cultivation systems were also employed to model collective migration and branching morphogenesis. Fibroblast-conditioned media (MES-CM) plays a significant role in initiating dose-dependent branching migration. Future work will involve high temporal and spatial resolution imaging of hepatic and mesenchymal interactions to determine the cascade of cellular and molecular events involved in tissue formation, morphogenesis, and migration.

**INTRODUCTION:**
Liver cell migration plays a significant role in liver organogenesis, disease, and cell therapy. During liver organogenesis (E8.5-9.0, mouse), the ventral foregut pre-hepatic epithelium begins to express liver genes due to inductive signals emanating from the surrounding mesenchyme and heart. At E9.0, the foregut epithelium thickens as the cells transition from cuboidal to pseudostratified columnar morphology, forming the liver diverticulum [3, 7]. At this critical stage, the liver diverticulum is comprised of only ~1,500 cells. Next, the hepatic endoderm lining the liver diverticulum thickens, delaminates, and forms cords of migrating hepatoblasts that co-migrate with endothelial cells and mesenchymal cells, branching into the surrounding mesenchymal tissue, initiating three-dimensional collective cell migration to form the liver bud [15, 16]. In fact, during this stage, cells collectively undergo: 1) co-migration, or movement together with other cell types, 2) branching morphogenesis, or formation of branching tube-like structures, and 3) interstitial migration, or migration on top of other cells. By E11.5, migration ceases, and the primitive liver has formed, expanding $10^3$-fold [15]. Liver cell migration may also be required in later stages of liver organogenesis, as rat fetal hepatoblasts (HBs) expression has shown evidence of highly upregulated genes associated with 3D collective cell migration, morphogenesis, and extracellular matrix remodeling [18]. In addition to its role in early liver organogenesis, 3D collective migration is intricately linked to the local spread and metastasis of advanced hepatocellular carcinoma (HCC), ultimately leading to worsened prognosis and

increased treatment resistance[25]. Adult and fetal hepatocytes also employ collective migration when moving from the spleen to within the liver during liver repopulation. *In vivo* imaging studies have demonstrated that transplanted hepatocytes enter the portal vein and then the capillaries within hours, migrate across the liver sinusoids, and through the liver tissue[8, 10, 19]. Finally, recent studies demonstrate that migrating hepatoblasts arise during murine and human liver regeneration with some evidence of movement in sheets[11]. Overall, liver collective migration, capable of multiple modes of morphogenesis, plays a significant role in organogenesis, cancer, hepatocyte cell therapy, and liver regeneration.

Numerous genetic studies have investigated the molecular pathways that drive 3D liver collective cell migration. These studies demonstrate that ablation of hepatic cords blocks liver formation and shows that, therefore, the formation of hepatic cords and their ensuing interactions with supporting cells are required for liver formation[3, 12, 21, 22]. These studies also demonstrate that liver growth is initiated by fibroblast growth factor 2 (FGF2) secreted from the cardiac mesoderm, bone morphogenetic protein 4 (BMP4) secreted from the surrounding mesenchyme, hepatocyte growth factor (HGF), endothelial cell interactions, and migration-associated transcription factors including (HEX), PROX1, and TBX3[7, 20]. Overall, genetic studies support the fact that soluble factor signaling leading to transcription factor expression is responsible for driving migration, signaling, and molecular interactions between hepatoblasts and their surrounding mesenchyme.

Although cell migration in early liver organogenesis has been extensively investigated, current *in vitro* hepatic migration studies frequently utilize 2D assays consisting of highly migratory HCC cells combined with *in vivo* tumor models[13]. These studies have provided insight into several factors that play a role in hepatic migration including TGFβ1[6], c-Myc[27], Yes-associate protein (YAP)[5], goosecoid[23], actopaxin[2], and miRNAs[4, 24, 26]. Despite the advancements in understanding the molecular mechanisms in 3D hepatic cell migration, the fundamental mechanisms between 2D and 3D cellular migration are distinct, suggesting 2D assays have their limitations. Furthermore, these models typically do not implement mesenchymal cell types, which are essential to migration/growth. There has been progress in the development of 3D models for liver migration that incorporate the supporting mesenchyme; however, they are solely focused on co-migration rather than the different modes of collective migration.

The ability to form tissues from spheroids through various self-assembly and morphogenetic processes enables the scientific study of synthetic tissues for applications in drug development and screening, disease modeling, therapy, and other biomedical and biotechnological applications. Methodological details for several 3D *in vitro* cultivation systems engineered to exhibit different modes of liver 3D collective migration are presented. These systems include the following: (1) co-spheroid culture with hepatic (HEP) and mesenchymal-derived (MES) spheroids in matrix, (2) spheroid matrix droplet cultured with fibroblast-conditioned medium (MES-CM), and (3) mixed spheroids (HEP and MES cells). These systems enable robust modeling of liver 3D collective migration, improving our molecular and cellular understanding of liver organogenesis, cancer, and therapy.

**PROTOCOL:**

## 1. Preparation of 1% Low EEO Agarose Solution

1.1. Weigh 2.5 g of low EEO agarose powder and transfer it to a beaker.

NOTE: Ensure the beaker is at least twice the size of the desired volume to accommodate bubbling during the solution preparation.

1.2. Use a graduated cylinder to measure 250 mL of distilled (DI) water and pour it into the beaker, aiming for a final concentration (w/v) of 1%.

1.3. Cover the beaker's opening with plastic wrap, leaving a small hole for ventilation. Heat the beaker in the microwave for 30 seconds.

1.4. Carefully remove the beaker and swirl the contents. Repeat this process every 30 seconds until complete dissolution.

CAUTION: Handle microwaved glassware carefully, using proper gloves. Monitor the solution closely to prevent overheating or boiling over.

1.5. Once a homogeneous solution is achieved, remove the beaker from the microwave. Gently swirl the solution before transferring it to a pre-sterilized bottle. Autoclave the solution and store the agarose solution at room temperature until ready to use.

## 2. Coating 96-Well Plate

NOTE: 384-well ultra-low attached spheroid microplates can be utilized in these and further experiments.

2.1. Loosen the cap of the bottle containing the sterile 1% agarose solution. Warm the solution in the microwave in 30-second intervals until it transitions to the liquid phase; then tighten the cap.

CAUTION: Handle microwaved glassware carefully, using proper gloves. Monitor the solution closely to prevent overheating or boiling over.

NOTE: Perform these steps under a sterile tissue-culture laminar flow hood.

2.2. Transfer 55-65 µL of the 1% agarose solution per well to coat the 96-well tissue-cultured plate and immediately rotate the plate.

2.3. Once the 1% agarose solution has been transferred to the desired number of wells, place the plates in a 4°C fridge for 20-30 minutes to allow the agarose to solidify. Before use, bring the plate to room temperature (**Figure 1A**).

## 3. Preparation of HepG2 (HEP) Spheroids

3.1. Cultivate HepG2 cells in a T-75 flask using completed growth medium (cDMEM) comprising of Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep). Maintain the cell culture at 37°C and 5% $CO_2$, changing the medium daily.

3.2. Upon reaching 80% confluency, perform a rinse of the cells with 1X Phosphate Buffered Saline (PBS) and discard the rinse. Add 5 mL of 0.05% of Trypsin-EDTA to the flask and incubate at 37°C and 5% $CO_2$ for 5-10 minutes.

3.3. Add equal amounts of cDMEM and wash the cells off the flask. Once cells detach, transfer the mixture to a 15 mL sterile conical centrifuge tube and centrifuge the cell suspension at 300 *x g* for 5 minutes.

NOTE: All centrifugation steps outlined in this protocol were performed at room temperature. However, these centrifugation procedures can alternatively be carried out at cold temperatures.

3.4. Re-suspend the cell suspension in fresh cDMEM and count the cells to determine the final concentration in cells/mL (**Figure 1B**).

3.5. Dye-labeling of cells

NOTE: This is an optional step.

3.5.1. Transfer the desired amount of cell suspension to a 15 mL sterile conical centrifuge tube and centrifuge the cell suspension at 300 *x g* for 5 minutes.

3.5.2. Re-suspend the cell pellet in a serum-free growth medium to achieve a final concentration of 1.0 x $10^6$ cells/mL. Add 5 µL of Vybrant Cell-Labeling Solution per mL of cell suspension and incubate the cell suspension at 37°C and 5% $CO_2$ on rotation for 20 minutes.

NOTE: Serum-free growth medium is DMEM only supplemented with 1% Pen-strep. Adjust the incubation time for uniform staining based on the cell suspension density.

3.5.3. Once the incubation is completed, centrifuge the cell suspension at 450 *x g* for 5 minutes and re-suspend the cell pellet in fresh cDMEM. Repeat this wash process two more times (**Figure 1C**).

3.6. Spheroid formation

3.6.1. Suspend the cells in fresh cDMEM to achieve a final concentration of 5.0 x $10^4$ cells/mL based on the cell count. Mix the cell suspension well and transfer 100 µL of cell suspension per well to the agarose-coated 96-well plate.

NOTE: The cell suspension density is set to achieve 5,000 cells per well in the agarose-coated 96-well plate.

3.6.2. Centrifuge the plate at 340 *x g* for 10 minutes and incubate at 37°C and 5% CO2 for 5-9 days (**Figure 1D**).

NOTE: Change medium every other day after plating, gently removing 50% of cDMEM and replacing it.

NOTE: HepG2 spheroids are suitable for the HepG2 and HFF/MRC-5 (HEP-MES) assembloid model or MES-CM model.

**4. Preparation of HFF/MRC-5 (MES) Spheroids**

4.1. Cultivate HFF/MRC-5 cells in a T-175 flask using completed growth medium (cDMEM) comprising of Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep). Maintain the cell culture at 37°C and 5% $CO_2$, changing the medium every other day.

4.2. Upon reaching 80% confluency, perform a rinse of the cells with 1X Phosphate Buffered Saline (PBS) and discard the rinse. Add 5 mL of 0.25% of Trypsin-EDTA to the flask and incubate at 37°C and 5% CO2 for 5-10 minutes.

4.3. Add equal amounts of cDMEM and wash the cells off the flask. Once cells detach, transfer the mixture to a 15 mL sterile conical centrifuge tube and centrifuge the cell suspension at *300 x g* for 5 minutes.

4.4. Re-suspend the cell suspension in fresh cDMEM and count the cells to determine the final concentration in cells/mL (**Figure 1B**).

4.5. Dye-labeling of Cells

NOTE: This is an optional step.

4.5.1. Transfer the desired amount of cell suspension to a 15 mL sterile conical centrifuge tube and centrifuge the cell suspension at 300 *x g* for 5 minutes.

4.5.2. Re-suspend the cell pellet in a serum-free growth medium to achieve a final concentration of 1.0 x $10^6$ cells/mL. Add 5 µL of Vybrant Cell-Labeling Solution per mL of cell suspension and incubate the cell suspension at 37°C and 5% CO2 on rotation for 20 minutes.

NOTE: Serum-free growth medium is DMEM only supplemented with 1% Pen-strep. Adjust the incubation time for uniform staining based on the cell suspension density.

4.5.3. Once the incubation is completed, centrifuge the cell suspension at 450 *x g* for 5 minutes and re-suspend the cell pellet in fresh cDMEM. Repeat this wash process two more times (**Figure 1C**).

4.6. Spheroid Formation

4.6.1. Suspend the cells in fresh cDMEM to achieve a final concentration of 1.0 x $10^5$ cells/mL based on the cell count. Mix the cell suspension well and transfer 100 µL of cell suspension per well to the agarose-coated 96-well plate.

NOTE: The cell suspension density is set to achieve a density of 10,000 cells per well in the agarose-coated 96-well plate.

4.6.2. Centrifuge the plate at 340 *x g* for 10 minutes and incubate at 37°C and 5% CO2 for 5-9 days (**Figure 1D**).

NOTE: Change medium every other day after plating, gently removing 50% of cDMEM and replacing it.

NOTE: HFF/MRC-5 spheroids are suitable for the HEP-MES assembloid model.

## 5. HepG2 and HFF/MRC-5 (HEP-MES) Assembloid Formation

NOTE: Refer to Sections 3 and 4 for formation of HepG2 (**Figure 2A)** and HFF/MRC-5 spheroids (**Figure 2B**).

5.1. Collect HFF/MRC-5 spheroids individually using a pipette from the 96-well plate. Transfer them to a 15 mL sterile conical centrifuge tube containing fresh cDMEM. Allow the spheroids to settle in the tube for 5 minutes and then gently rinse with warm cDMEM.

NOTE: Perform the rinsing process gently and with care.

5.2. Gently transfer a single HFF/MRC-5 spheroid using a pipette to a well containing a HepG2 spheroid. Add ice-cold growth factor reduced (GFR) Matrigel (MG)/ Collagen (CG) in a 1:1 and 1:5 dilution and incubate at 37°C at 5% $CO_2$ for 3 hours (**Figure 2C**).

308 NOTE: Thaw MG overnight on ice in a 2°C to 8°C fridge. Ensure the use of pre-cooled pipettes,
309 tips, and tubes when handling MG, as it forms a gel above 10°C.
310
311 5.3. After incubation, add 75 µL of fresh cDMEM to each well and incubate at 37°C at 5% $CO_2$
312 for 2-3 days.
313
314 NOTE: Change medium every other day after plating, gently removing 50% of cDMEM and
315 replacing it. Assembloids will still form without media changes for up to 3-4 days.
316
317 NOTE: Assembloid formation can occur without the use of matrix.
318
319 **6. HEP Spheroid Droplet Formation**
320
321 NOTE: Refer to Section 3 for formation of HepG2 spheroids (**Figure 3A**).
322
323 NOTE: Two different materials can be used for suspending the HepG2 spheroids in droplets. The
324 two methods are provided below.
325
326 6.1. Matrigel (MG) droplets
327
328 6.1.1. Collect HepG2 spheroids individually using a pipette from the 96-well plate, allowing
329 them to settle in a 15 mL sterile conical centrifuge on ice.
330
331 6.1.2. In a separate 15 mL sterile conical centrifuge tube, mix 1 mL of ice-cold GFR MG and ice-
332 cold control growth medium at a 1:1 dilution. Aspirate the medium from the 15 mL tube
333 containing the HepG2 spheroids and place it on ice.
334
335 NOTE: Thaw MG overnight on ice in a 2°C to 8°C fridge. Ensure pre-cooled pipettes, tips, and
336 tubes when handling MG, as it forms a gel above 10°C.
337
338 6.1.3. Add the MG solution to HepG2 spheroids, ensuring even spheroid distribution inside the
339 MG solution.
340
341 6.1.4. Using a 200 µL pipette, collect a 15 µL volume of a single spheroid in the MG solution and
342 slowly seed it onto a 60 mm petri dish (**Figure 3B**).
343
344 NOTE: If more than one spheroid is seeded per droplet, remove, and reseed properly. Spheroids
345 are repositioned if needed to ensure they are at the center of the droplet.
346
347 NOTE: For optimal spheroid seeding, consider using reverse pipetting to minimize air bubble
348 formation.
349
350 6.2. Collagen (CG) Droplets
351

NOTE: All CG preparation should be done on ice.

6.2.1. Collect HepG2 spheroids individually using a pipette from the 96-well plate, allowing them to settle in a 15 mL sterile conical centrifuge tube on ice (**Figure 3B**).

6.2.2. In a microcentrifuge tube, add 358.8 µL of de-ionized water, 100 µL of 10X PBS, 12.1 µL of 1 N NaOH, and 529.1 µL of stock rat tail CG for a total volume of 1 mL.

NOTE: Stock rat tail CG should always be added at the end.

6.2.3. Add the CG solution to HepG2 spheroids, ensuring even spheroid distribution inside the CG solution.

6.2.4. Using a 200 µL pipette, collect a 15 µL volume of a single spheroid in the CG solution and slowly seed it onto a 60 mm petri dish.

NOTE: If more than one spheroid is seeded per droplet, remove, and reseed properly. Spheroids are repositioned to ensure they are at the center of the droplet.

NOTE: For optimal spheroid seeding, consider using reverse pipetting to minimize air bubble formation.

6.3 Incubate the droplets at 37°C and 5% CO2 for 60 minutes before the addition of growth medium.

6.4 Slowly add 5 mL of the desired growth medium to the petri dish and incubate at 37°C and 5% $CO_2$ with medium changes every three days (**Figure 3C**).

NOTE: HFF/MRC-5 conditioned-media was used in the droplet formation assay.

## 7. Preparation of HFF/MRC-5 Conditioned Media (MES-CM)

7.1. Harvest HFF/MRC-5 cells and seed them into a T-75 tissue culture-treated flask at a seeding density of 5,000 cells/$cm^2$ and incubate the flask at 37°C and 5% CO2 for 72 hours in 15 mL of cDMEM.

NOTE: Monitor the flask during this period to ensure optimal cell health.

7.2. After the 72-hour incubation, collect the fibroblast-conditioned growth medium in a 15 mL sterile conical centrifuge tube. Centrifuge the fibroblast-conditioned growth medium at 290 *x g* for 5 minutes and filter using a 0.2 µm filter (**Figure 4A**).

7.3. Dilute the fibroblast-conditioned growth medium with complete growth medium at a 1:1 to 1:7 dilution ratio and add to the desired experiment (**Figure 4B**).

NOTE: Utilize HFF/MRC-5 conditioned-media in the droplet formation assay.

**8. HepG2 and HFF/MRC-5 Mixed Spheroid Formation**

NOTE: Refer to steps 3.1 – 3.5. for HepG2 cell suspension preparation and steps 4.1. – 4.5. for HFF/MRC-5 cell suspension preparation.

8.1. Suspend the HepG2 cells in fresh cDMEM to achieve a final concentration of 1.0 x 105 cells/mL for each cell suspension.

8.2. Suspend the HFF/MRC-5 cells in fresh cDMEM to achieve a final concentration of 1.0 x 105 cells/mL for each cell suspension.

8.3. Transfer HepG2 and HFF/MRC-5 cell suspensions to a 15 mL sterile conical centrifuge tube at a 1:1 ratio to obtain a final concentration of 2.0 x $10^5$ cells/mL. Thoroughly mix the cell suspension and transfer 100 µL of cell suspension per well to the agarose-coated 96-well plate.

NOTE: The cell suspension density is set to obtain 20,000 cells per well in the agarose-coated 96-well-plate.

8.4. Centrifuge the plate at 340 *x g* for 10 minutes and incubate at 37°C and 5% CO2 for 1-2 days (**Figure 5C**).

8.5. Addition of Fibrin Hydrogels

NOTE: This is an optional step that is done in 72 hours after plating HEP-MES mixed spheroids.

8.5.1. In a 15 mL sterile conical centrifuge tube, mix fibrinogen (3.25 mg/mL) and thrombin (12.5 units/mL) at a 4:1 ratio for the formation of fibrin and put the tube on ice.

8.6. Transfer the fibrin to each well with a pre-existing mixed spheroid at a 1:1 dilution with current cDMEM in the well. Incubate the plate at 37°C and 5% $CO_2$ for 30 minutes.

8.7. After incubation, add 75 µL of fresh cDMEM to each well and incubate at 37°C at 5% $CO_2$ for 1-2 days.

NOTE: Change medium every other day after plating, gently removing 50% of cDMEM and replacing it.

**9. High Density HFF/MRC-5 Migratory Model**

NOTE: Refer to section 3 for HepG2 spheroid preparation (**Figure 6A**) and steps 4.1. – 4.5. for HFF/MRC-5 cell suspension preparation (**Figure 6B**).

9.1. Suspend the HFF/MRC-5 cells in fresh cDMEM to achieve a final concentration of 6.0 x $10^5$ cells/mL and put on ice.

9.2. Mix the HFF/MRC-5 cell suspension and ice-cold MG/CG at a 1:1 dilution (**Figure 6C**).

NOTE: The cell suspension density is set to obtain 30,000 cells per well in the agarose-coated 96-well-plate.

NOTE: Thaw MG overnight on ice in a 2°C to 8°C fridge. Ensure pre-cooled pipettes, tips, and tubes when handling MG, as it forms a gel above 10°C.

9.3. Transfer 100 µL of HFF/MRC-5 and MG solution using a pipette to a well containing a HepG2 spheroid. Incubate the plate at 37°C and 5% $CO_2$ for 30 minutes.

9.4. After incubation, add 75 µL of fresh cDMEM to each well and incubate at 37°C at 5% $CO_2$ for up to 12 days.

**REPRESENTATIVE RESULTS:**

Currently, increased interest exists in synthetic tissues for various biomedical applications, including disease modeling, drug discovery, and tissue engineering (**Figure 7**). In this field, human pluripotent stem cell (hPSC)-derived organoids, spheroids, and cells can be transformed into more synthetic, complex, and larger tissues. Achieving this involves applying principles of morphogenesis, utilizing tools like microfabrication, and incorporating biomatrices to engineer synthetic tissues more precisely (**Figure 7**). Several methods are presented here with this theme in mind.

***Effects of clustering on spheroid formation***

The methods developed for this study were contingent upon successful 3D spheroid formation. Successful spheroid formation is indicated by cells fusing within five to nine days, forming a full spheroid. An early indication of success is cell clustering in the center of the well post-centrifugation of the cultivation plate. While spheroid formation occurred less frequently when cells were initially scattered, it still demonstrated that successful spheroid formation could occur. Unsuccessful spheroid formation is characterized by cells not spreading and fusing within nine days post-plating.

Spheroids of two different sizes were used to perform this experiment. Small spheroids (S) were plated at a concentration of 1,500 cells per well, and large spheroids (L) were plated at a concentration of 3,000 cells per well. HEP spheroids compacted and fully cultured by day five, irrespective of the spheroid size. The cell density per well did not affect the rate at which the spheroid formed but had a significant impact on spheroid size (**Figure 8A**). In this instance, it is crucial to observe an increase in opacity, indicating thickening of the initial disc-shaped tissue,

482 which transitions from being more translucent to a spheroid configuration with increased
483 opacity. HEP spheroids are uniformly less round and increase in optical density (increasing
484 opacity) over time. If these HEP spheroids are still translucent, the spheroids should be either
485 discarded or the experiment should be re-done. The same experiment was performed for
486 HFF/MRC-5 spheroids, in which, the mesenchymal-derived spheroids compacted and fully
487 cultured within 24 hours, irrespective of spheroid size. The cell density per well did not affect
488 the rate at which the spheroid had formed (**Figure 8B**). Unlike hepatic spheroids, mesodermal-
489 derived spheroids compact within 24 hours, irrespective of cell seeding density. Due to their
490 contractile properties, MES spheroids are uniformly round and optically dense spheroids.
491 Notably, HFF/MRC-5 cells compact much tighter than HepG2 cells, likely resulting in little
492 impact of cell density on spheroid size (**Figure 8C**).
493
494 ***Factors that affect hepatic and mesenchymal (mesodermal-derived) assembloid formation***
495 Hepatic and mesodermal-derived spheroids of varying sizes underwent cultivation to
496 investigate the impact of size on the compaction time of spheroid formation. Hepatic and
497 mesodermal-derived spheroids were co-cultured in GFR Matrigel (MG) or Collagen (CG) gel at a
498 1:5 dilution with complete growth medium (cDMEM) or fibroblast-conditioned medium (MES-
499 CM) to explore the potential influence of matrix and conditioned medium on assembloid
500 formation. Both cell types were dye-labeled before spheroid formation to illustrate the
501 interaction between spheroids. In all experiments in which spheroids were dye-labeled, they
502 were visualized using standard filters for green (488 nm), blue (450 nm), and red (594 nm).
503 Images of the cells were captured using a Zeiss Axio fluorescent microscope equipped with
504 Axiovision software to zero the background noise and images were analyzed using Image J.
505 Phase-contrast, individual, and overlay images were obtained as .zip files, which were
506 formatted into .tiff files using Image J.
507 It was observed that assembloid formation occurs regardless of matrix and medium
508 (**Figure 9**). The effects of CG, MG, and MES-CM were studied, and assembloid formation was
509 observed in all cases, although morphological details varied slightly (**Figure 9**). Details regarding
510 these images will be re-used in later figures. To assess the impact of inter-spheroid distance on
511 assembloid formation, distance measurements were taken along with the success of
512 assembloid formation in the same experimental system (MG and DMEM). It was noted that the
513 compaction time of an assembloid is directly proportional to the initial distance between
514 hepatic and mesodermal-derived spheroids (**Table 1**).
515
516 ***Building more complex assembloids with arm-like structures***
517 Methods were developed to construct assembloids with branching cords (**Figure 10A**).
518 To perform this experiment, hepatic spheroids were combined with biomatrix (GFR MG) in 384-
519 well plate and surrounded by single fibroblasts at high density. These fibroblasts clustered,
520 providing guides toward which hepatic cells migrated, thickening over time and forming cords.
521 Hepatic spheroids were cultured in the MG droplet system containing a high density of MRC-5
522 cells (30,000 cells) in 100 µL of cDMEM and matrix in 384-wells, displaying small clusters of
523 MRC-5 cells in the MG (**Figure 10B, days 3-4**). Subsequently, liver spheroids formed thick
524 migrating strands protruding towards the fibroblast clusters, creating thick strands containing
525 both cell lines (**Figure 10B, days 9-12**). This approach resulted in longer arms or cords, likely

526 containing a mix of hepatic and fibroblast cells. Another approach involved constructing bridges
527 or small interconnections (arms) between spheroids. To build small armed-structures, a larger
528 HEP spheroid could be co-cultured in 384-well plate with a smaller mixed spheroid (**Figure 10C**),
529 leading to small, knob-like arms. In summary, two approaches for forming additional arms to
530 spheroids are presented.
531
532 ***Spot-welding (fused edges) of complex assembloids***
533 Methods were employed to construct assembloids with fused edges. The methodology
534 to perform these experiments was HEP-MES assembloid formation assay, however, the MES
535 spheroid had a lower density than previous experiments. Hepatic, and mesodermal-derived
536 spheroids were positioned in a rigid CG environment (2 mg/mL) **(Figure 10D)**. In this rigid
537 setting, instead of cupping, spheroids were observed to fuse at the edges, creating assembloids
538 with indications of short arms or spot-welding (**Figure 10E**). On the other hand, described
539 below, a cupping mechanism was also observed using the same culture system, in which the
540 HEP spheroid surrounds the MES spheroid to form a partially fused assembloid. Regarding the
541 spot-welding mechanism, similar data were observed with MRC-5 fibroblasts in CG (**Figure 10F**).
542
543 ***Infiltrating and layering of complex assembloids***
544 During liver organogenesis, in the developing liver diverticulum, HEP cells are
545 surrounded by mesenchyme. Ultimately, they migrate or infiltrate into the mesenchyme (**Figure**
546 **10G**). In MES-CM and CG conditions, the addition of MES and HEP spheroids results in a
547 different type of fusion, where an infiltrative pattern is observed (**Figure 10H**). Furthermore, a
548 layering pattern can be achieved by placing single human mesenchymal stem cells (hMSC) in
549 GFR MG at high density. They migrate towards a HEP spheroid and layer on the surface without
550 infiltration, as shown with dye-labeling of the hMSC (**Figure 10I**). It is noteworthy that this is a
551 different phenotype than when HFF was employed. The latter arrangement is observed in the
552 liver diverticulum stage and many other endoderm-derived tissues. Two approaches are
553 presented here, relevant for modeling the developing liver diverticulum and creating aspects of
554 synthetic tissues.
555
556 ***Fused hepatic and mesenchymal (mesodermal-derived) assembloid formation***
557 The fusion of two spheroids to form assembloids is crucial for modeling the liver bud.
558 This is because when two spheroids fuse, the cells likely migrate on top or between other cells,
559 a phenomenon referred to as interstitial migration. Interstitial migration occurs in the liver bud
560 when early migrating hepatoblasts move through the mesenchyme, potentially on top of other
561 cells. Therefore, the fusion of two spheroids serves as a model for interstitial migration during
562 liver organogenesis. Methods were developed to construct assembloids that fuse completely
563 with separate layers (**Figure 11A**). HEP and MES (MRC-5) spheroids in MG form a fused
564 assembloid by day 9 (**Figure 11B**). Dye-labeling analysis demonstrated that MES tissue
565 remained inside, while the HEP tissue remained outside (**Figure 11C**). Importantly, the final
566 spheroid is approximately the same size as the original spheroids, suggesting that the cells are
567 packed at high density. Notably, the same phenomena occur in the absence of MG in 384-well
568 plates when multiple MES spheroids are placed with a single HEP spheroid (**Figure 11D**). HEP-
569 MES assembloids, in the absence of matrix, also form under low serum (2% FBS) and extremely

570 low serum (0.2% FBS) conditions (**Figure 11E**). We then determined that at large distances
571 (approximately 3 diameters of the spheroid), assembloids did not form (**Figure 11F**). To
572 determine how spheroid composition determines spheroid fusion, it was demonstrated that
573 mixed spheroids (containing MES and HEP cells) can fuse with MES spheroids by day 5,
574 accompanied by an increased packing density, as expected (**Figure 11G**).
575
576 ***Partially fused or cupping in complex assembloid formation***
577 Successful assembloid formation in MG demonstrates several phenotypes, including the
578 observation that the HEP spheroid undergoes "cupping" of the MES spheroid to form a partially
579 fused assembloid (**Figure 11H**). This also establishes a visual model of interstitial migration and
580 points toward the current mechanism of fused assembloid formation. Time-series studies of
581 HEP and MES spheroids demonstrate that in CG, fusion occurs via a cup-like mechanism (**Figure**
582 **11I**). This is further demonstrated using dye-labeling (**Figure 11J**). Finally, the same phenomena
583 are clearly illustrated when multiple HEP spheroids are used with a large, single MES spheroid
584 (**Figure 11K**). The significantly smaller HEP spheroid cannot completely encompass the larger
585 MES spheroid; therefore, it results in the same cupping mechanism. The data suggest that
586 cupping without fusion can occur in MG or in cases where the MES spheroid is much larger than
587 the HEP spheroids.
588
589 ***Induced hepatic branching and linear migration via mesenchymal (mesodermal-derived)***
590 ***conditioned growth medium***
591 Collective migration is a pivotal morphogenetic process during liver organogenesis.
592 Tools for inducing collective migration *in vitro* are described here. Successful droplet formation
593 of HEP spheroids and the use of MES-CM demonstrate outgrowth branching from the HEP
594 spheroids (**Figure 12A-B**). HEP spheroids cultivated in the MG droplet system show that MES-
595 CM induces collective migration, and the data demonstrate a concentration-dependent effect
596 (**Figure 12B, right**). Cellular strands protruding from the hepatic spheroid are present with small
597 branching, thick strands, and multiple levels of branching (**Figure 12B, left**). On day 11, the
598 protrusions increased with interconnections and sheet formation (**Figure 12B, right**). MES-CM is
599 potent, as a 1:7 dilution or MES-CM present for one day still leads to migration (**Figure 12B,**
600 **right**). It was hypothesized that the MES-CM induced cell migration via TGFβ signaling pathway.
601 A83-01, a TGFβ pathway inhibitor, was incorporated into the MES-CM at varying
602 concentrations, and migration was significantly inhibited in a dose-dependent manner (**Figure**
603 **12C**). To determine the effects of extracellular matrix on collective migration, HEP spheroids
604 cultivated in the CG droplet system demonstrate that MES-CM induces cell migration. However,
605 the protrusions were thin linear strands and less branching by day 7 compared to the migration
606 observed in MG (**Figure 12D**). Fibrin hydrogels were also tested, and thin, hair-like, narrow,
607 radial protrusions of both HEP and MES cells were observed (**Figure 12E**).
608
609 ***Inducing hepatic co-migration via MES conditioned medium***
610 Mixed spheroids (HEP-MES spheroids) were utilized for collective migration, as a model
611 of co-migration which occurs during early liver organogenesis (**Figure 12F**). These mixed
612 spheroids in MG result in migration, and when TGFβ1 growth factor was added, it resulted in
613 significantly increased collective migration (**Figure 12G**). Thus, co-migration can also be

614 modeled with mixed spheroids.
615
616 **FIGURE AND TABLE LEGENDS:**
617 **Figure 1. Schematic of spheroid formation assay. (A)** Coating a 96-well plate with 1% agarose
618 solution. Agarose solution is melted in the microwave, 55-65 µL is transferred to each well and
619 the plate is cooled for 20-30 minutes. **(B)** Passaging of cell culture at 80% confluency. **(C)** Dye-
620 labeling of cell suspension. 5 µL of cell-labeling solution is added per mL After incubation and
621 centrifugation, the cell suspension is washed with warm cDMEM before plating the cells. **(D)**
622 Plating of cell suspension for spheroid formation. 100 µL of cell suspension is added to each
623 well and the plate is centrifuged. Mesodermal-derived spheroids form in 1-3 days, while hepatic
624 spheroids form in 5-9 days.
625
626 **Figure 2. Schematic of assembloid formation assay with hepatic and mesodermal-derived**
627 **spheroids. (A)** Hepatic spheroid formation. HepG2 cells are dye-labeled and plated for spheroid
628 formation. **(B)** Mesodermal-derived spheroid formation. HFF/MRC-5 cells are dye-labeled and
629 plated for spheroid formation. **(C)** HEP-MES assembloid formation. Transfer mesodermal-
630 derived spheroid to hepatic spheroid for assembloid formation. Matrix (MG or CG) is added
631 (1:1-1:5 dilution), incubated for 3 hours, followed by the addition of 75 µL cDMEM. Incubated
632 for 2-3 days until assembloid formation is complete.
633
634 **Figure 3. Schematic of Droplet Formation Assay. (A)** Hepatic spheroid formation. HepG2 cells
635 are dye-labeled. **(B)** Spheroid collection and matrix suspension. Collect and gently rinse hepatic
636 spheroids with cDMEM. Spheroids are suspended in MG or CG solution (1:1 – 1:5 dilution). **(C)**
637 Droplet seeding and addition of growth medium. Hepatic spheroids are seeded onto a 60mm
638 petri dish and incubated for 60 minutes, and 5 mL of MES-CM is added. Petri dish is incubated
639 with media changes every three days.
640
641 **Figure 4. Schematic of Preparation of MES-CM. (A)** Cell seeding and MES-CM collection.
642 HFF/MRC-5 cells are seeded at 5,000 cells/cm$^2$ in a T-75 flask and incubated for 72 hours. The
643 media is collected and centrifuged for debris removal. **(B)** MES-CM sterilization and addition.
644 The media is sterilized with a 0.2 µm filter and combined with cDMEM (1:1-1:7 dilution). Media
645 is added to a hepatic spheroid and incubated with media changes every three days.
646
647 **Figure 5. Schematic of Hepatic and Mesodermal-derived Mixed Spheroid Formation Assay. (A)**
648 Preparation of hepatic cell suspension. HepG2 cells are harvested and dye-labeled. **(B)**
649 Preparation of Mesodermal-derived cell suspension. HFF/MRC-5 cells are harvested and dye-
650 labeled. **(C)** HEP-MES mixed spheroid formation. HepG2 and HFF/MRC-5 cells are mixed at a 1:1
651 ratio at a final concentration of 2.0 x $10^5$ cells/mL and plated. The plate is incubated for 1-2 days
652 until mixed spheroid formation is complete.
653
654 **Figure 6. Schematic of High Density HFF/MRC-5 Migratory Model. (A)** Hepatic spheroid
655 formation. HepG2 cells are dye-labeled and plated for spheroid formation. **(B)** Preparation of
656 Mesodermal-derived cell suspension. HFF/MRC-5 cells are harvested and dye-labeled. **(C)**
657 HFF/MRC-5 cell suspension is pre-mixed with MG solution and added to pre-existing HepG2

658 well. The plate is incubated for 30 minutes and 75 µL of cDMEM is added. The plate is
659 incubated for up to 12 days.
660
661 **Figure 7. Overview.** Based in the science of morphogenesis (top), stem cells will be
662 implemented to build assembloids. The toolbox (middle), through the use of cells, tools, and
663 biomatrix, and together with aspects of morphogenesis, can together be used to generate new
664 synthetic tissues and assembloids.
665
666 **Figure 8. Compaction time of varying spheroid size. (A)** Phase-contrast images of small (1,500
667 cells) and large (3,000 cells) hepatic spheroids on days 1, 3, and 5. Bar = 300 µm. **(B)** Same study
668 as **(A)** except HFF cells. Bar = 100 µm. **(C)** Bar graph comparing HepG2 and HFF spheroid
669 compaction. (n = 5). Right: bar graph plotting area of HepG2 and HFF spheroids over time. (n =
670 5).
671
672 **Figure 9. The effect of media and matrix on assembloid formation.** Phase contrast and
673 fluorescent images of fused HepG2/MRC-5 spheroids exposed to varying matrices (1:5 dilution)
674 and growth medium: DMEM in CG, MES-CM in CG, DMEM in MG, and MES-CM in MG. Left:
675 phase-contrast (day 2) images of fused HepG2/MRC-5 assembloid. Bar = 100 µm. Middle:
676 fluorescent (day 2) of fused HepG2/MRC-5 assembloid. Bar = 200 µm. Left: phase-contrast of
677 fused HepG2/MRC-5 assembloid. Bar = 200 µm. (green: HepG2; orange: MRC-5; arrows
678 indicating spheroid types).
679
680 **Figure 10. Engineering assembloids arms, junctions, and layers. (A)** Schematic of building of
681 large and small arms onto hepatic spheroids. **(B)** Phase-contrast images of HepG2 spheroids
682 suspended in the MG droplet system, bearing high density (30,000 cells) of MRC-5 cells. Top:
683 days 3, 4, 9, and 12. Day 3: MRC-5 cells initially after seeding (arrow). Bar = 1,000 µm. Day 4:
684 MRC-5 cells spreading and interconnecting (arrows). Bar = 1,000 µm. Day 9: Thick hepatic cord
685 (arrows). Bar = 400 µm. Day 12: multiple, thick hepatic cords. Bar = 400 µm. Bottom: images of
686 a separate experimental replicate on day 12 demonstrating thick hepatic cord formation
687 (arrows). Bar = 400 µm. Subfigures obtained with permission from Frontiers Journal[17]. **(C)**
688 Fluorescent images of hepatic spheroid co-cultured with a HEP-MES mixed spheroid on days 0
689 and 5. Day 0: HepG2 and mixed spheroid initially after plating. Bar = 100 µm. Day 5: mixed
690 spheroid has fused to the edge of HepG2 spheroid. Bar = 100 µm. (need to add dye-labeling
691 information) **(D)** Schematic of building HEP-MES assembloids with fused edges (spot-welding).
692 (arrows indicating spheroid type). **(E)** Phase-contrast images of HEP and MES spheroids co-
693 cultivated in the CG droplet system exposed to MES-CM. Day 0: HEP and MES spheroids initially
694 after plating. Bar = 100 µm. Day 1: HEP and MES migrating towards one another, but no fusion
695 has occurred. Bar = 100 µm. Day 4: HEP and MES spheroids have fused at the edges of the
696 spheroids. Bar = 100 µm. **(F)** Phase-contrast and fluorescent images of HEP-MES assembloid
697 cultivated in CG droplet system (2 mg/mL) on day 3 demonstrating fused edges. (green: HepG2;
698 orange: MRC-5; arrow indicating where fusion occurred). Bar = 100 µm. **(G)** Schematic of
699 building HEP-MES assembloids with infiltration or a surface-MES layering. **(H)** Phase-contrast
700 and fluorescent images of HEP-MES assembloid cultivated in CG droplet system (2 mg/mL)
701 suspended in MES-CM on day 3 demonstrating infiltration of spheroids. (green: HepG2; orange:

MRC-5; arrow indicating where fusion occurred). Bar = 100 µm. **(I)** Phase-contrast and fluorescent images on day 6 of HepG2 spheroid and MSC in MG droplet system. The right columns on the right are replicates 1 and 2 with fluorescent images on day 6. (green: HepG2; red: MSC). Bar = 100 µm. Subfigures obtained with permission from Frontiers Journal[17].

**Figure 11. Partially and completely fused HEP-MES assembloids. (A)** Schematic of building fused HEP-MES assembloids with increased packing density consisting of separate layers. **(B)** Phase-contrast images of HepG2 and MRC-5 spheroids co-cultivated in MG droplet system on days 3, 5, 7, 9. Days 3-5: HepG2 and MRC-5 spheroids interact. Day 7: migration at the spheroids edge and fusion occurred. Day 9: spheroid fusion and assembloid formation; arrows demonstrate protrusions. Bar = 200 µm. Subfigures obtained with permission from Frontiers Journal[17]. **(C)** Fluorescent images of HEP-MES assembloid, the same study as **(B)** on day 13. Day 9: spheroid fusion and assembloid formation; arrows demonstrate protrusions. (green: HepG2; red: MRC-5). Bar = 200 µm. Subfigures obtained with permission from Frontiers Journal[17]. **(D)** Phase-contrast images of HepG2 spheroid co-cultured with multiple mesodermal-derived spheroids on days 0, 1, and 5. Day 5: HEP-MES assembloid formation occurred but overall volume did not increase. **(E)** Phase-contrast images of HepG2 and MRC-5 spheroids co-cultivated in MG under low serum conditions (0.2% and 2% FBS) on days 0-2. Top: HEP-MES assembloid fusion occurs in 2% FBS by day 2. Bar = 100 µm. Bottom: HEP-MES assembloid fusion occurs in 0.2% FBS by day 2. Bar = 100 µm. **(F)** Phase-contrast images of HepG2 and MRC-5 spheroids co-cultured in the CG droplet system exposed to MES-CM. Day 0: HEP and MES spheroids initially after plating. Bar = 100 µm. Day 5: HEP and MES spheroids have not fused. Bar = 100 µm. **(G)** Fluorescent images of MES spheroid co-cultured with a HEP-MES mixed spheroid on days 0 and 5. Day 0: HFF and HEP-MES mixed spheroid initially after plating. Bar = 100 µm. Day 5: HFF and HEP-MES mixed spheroid fuse. Bar = 100 µm. **(H)** Schematic of building partially fused HEP-MES assembloid with cupping. **(I)** Phase-contrast images of HEP and MES spheroids co-cultured in the CG droplet system on days 0, 1, 2, and 4. Day 0: HEP and MES spheroids initially after plating. Days 1-2: HEP and MES spheroids interact. Day 4: HEP-MES assembloid formation occurs in a cup-like mechanism. Arrow indicating spheroid position during fusion. Bar = 100 µm. **(J)** Phase-contrast and fluorescent images of HEP-MES assembloid cultivated in MG droplet system on days 2 and 4. Top: HEP and MES spheroid interaction occurs. Bottom: partial fusion of HEP-MES assembloid. (green: HepG2; orange: MRC-5; arrow indicating where fusion occurred). Bar = 100 µm. **(K)** Phase-contrast images of MES spheroid co-cultured with multiple HEP spheroids on days 0, 1, and 5. Day 0: MES and HepG2 spheroids initially after platting. Day 1: HEP and MES spheroid interaction occurs. 5: HEP spheroid forms a cup around MES spheroid.

**Figure 12. Methods for modulating collective migration from spheroids. (A)** Schematic of branching and linear migration in HEP spheroids in the presence of MES-CM. **(B)** Phase-contrast images of HEP spheroid in the MG droplet system cultivated in varying dilutions of MES-CM on day 7 (low and high magnification) and 11. Day 7: HEP spheroid demonstrates various modes of 3D collective migration. Bar = 300 µm. Day 11: HEP spheroid outgrowth demonstrates interaction between branches. Bar = 500 µm. Plot comparing fold-increase in area across different dilution ratios of MES-CM (1:1, 1:7, MES-CM 1 day, and MES-CM).Comparison of MES-

CM with 1:1 condition (P = 0.15, n = 3 for both conditions), MES-CM with 1:7 condition (P = 0.0056, n = 3 for both conditions), and MES-CM with MES-CM 1-day-only condition (P = 0.019, n = 3 for both conditions). Subfigures obtained with permission from Frontiers Journal[17]. **(C)** Phase-contrast images of HEP spheroid cultivated MG droplet system exposed to MES-CM alone, MES-CM with A83-01 (10 nM), and A83-01 (20 nM) on day 7. Bar = 300 µm. Bar graph analysis comparing day 7 MES-CM and MES-CM + A83-01 (20 nM) (P = 0.047, n = 3). Plotted means ± SD. Significance is defined as P ≤ 0.05. Subfigures obtained with permission from Frontiers Journal[17]. **(D)** Phase-contrast images of HEP spheroid cultured in the CG droplet system cultivated in MES-CM on days 4 and 7. Arrows specify thin filopodia-like extensions into the CG. Bar = 500 µm. Bar graph comparing protrusion length of HEP spheroids in cDMEM and MES-CM conditions (P = 0.012, n = 3). Subfigures obtained with permission from Frontiers Journal[17]. **(E)** Phase-contrast and fluorescent images of HEP-MES mixed spheroid cultivated in fibrin gels exposed to MES-CM on days 4 and 5. (green: HepG2; red: HFF; arrows indicated protrusions). Bar = 500 µm. Subfigures obtained with permission from Frontiers Journal[17]. **(F)** Schematic of star-shaped migration of HEP-MES mixed spheroids. **(G)** Fluorescent images of HEPG2-GFP (HepG2 cells expressing green fluorescent protein) and HEP-MES mixed spheroids co-cultivated in the MG droplet system after treatment with TGFβ1 (20 ng/ml) on day 4. Left: HepG2 cells in HEP-MES mixed spheroid exposed to TGFβ1 on day 4. Right: HEP-MES mixed spheroid exposed to TGFβ1 on day 4. Replicates 1 (below) and 2 (above) are shown. (green: HepG2; red: MRC-5; yellow: combined; arrows show HepG2 and MRC-5 migration). Bar = 500 µm. Bar graph comparing the area of fibroblast migration when exposed to TGFβ1 (HepG2-GFP/MRC-5 and HepG2-GFP/MRC-5 + TGFβ1, 20 ng/ml), P = 0.012, n = 3. Plotted means ± SD. Significance is defined as P ≤ 0.05. ∗ is used to denote the significance of experimental data. Subfigures obtained with permission from Frontiers Journal[17].

**Table 1. The effect of distance on assembloid formation.** Hepatic spheroids and mesodermal-derived spheroids were transferred to an agarose-coated well. The cells were dye-labeled prior to spheroid formation to demonstrate the interaction between spheroids. The spheroids were suspended in a 1:5 solution of the MG matrix suspended in cDMEM. The initial distance between the spheroids was measured using ImageJ and the corresponding compaction time of the assembloid formation was observed. The compaction time of assembloid formation is directly proportional to the initial distance between the spheroids. (n = 3).

**DISCUSSION:**

In this protocol, multiple methods are presented for cultivating both simple and complex assembloids, as well as techniques for inducing 3D collective cell migration in early liver organogenesis[14, 17]. Several critical steps are outlined in the presented protocols, with spheroid formation being a key aspect across all methods. Achieving spheroid formation involves using microwells (96- or 384-wells) with either non-adherent or agarose-coated plates. Spheroids are abundantly produced for subsequent experimentation, accounting for those that may not reach the expected size or have irregular shapes. Spheroids that do not meet the desired criteria are not utilized in downstream experiments. The initial efficiency depends on various factors, including the user. Considerable expertise is required to handle organoids, involving aspects such as spheroid (or organoid) formation, transfer between wells, addition of

790 biomatrices upon spheroids, and placement of multiple spheroids per well.
791
792 Formation of the spheroids demands meticulous attention to repeatable cell counting
793 and seeding, agarose coating (or non-adherent), regular medium changes, dye-labeling
794 methods, and gentle handling of spheroids and plates, along with microscopy. Dye-labeling
795 specifics, including cell number and type, as well as, the labeling duration, should be
796 experimentally determined. Typically, concentration of MG averaged 9.02 ± 0.63 mg/mL.
797 Furthermore, experience suggests that minor differences do not significantly impact
798 phenotype, it could be highly beneficial to track protein concentrations for each MG lot in initial
799 experiments. In terms of the CG, the concentration was prepared to be 2 mg/mL.
800
801 For instance, preparing the agarose solution necessitates strict adherence to
802 maintaining appropriate concentrations, seeding wells with proper volumes to allow for the
803 development of a meniscus (curvature), and enable cell collection in the center. Careful
804 consideration of agarose concentration, volume, and gel solidification is crucial. Selecting the
805 correct micropipette tip size and employing proper transferring techniques are essential for
806 handling spheroids effectively. The use of glass wells can enhance visualization. As observed in
807 the paper, spheroids initially appear as translucent discs, becoming more opaque over time,
808 providing a means to monitor spheroid formation. Several factors can influence this process.
809 Lastly, for assembloid formation[9], precise transfer of spheroids is vital, placing them within a
810 couple of spheroid diameters or less to effectively observe changes.
811
812 Here, a toolbox of methods has been established using cell lines to develop complex
813 assembloids and migrating spheroids. It is noteworthy that cell lines were employed for method
814 focus, but using primary cells or hPSC-derived cells would be advantageous for human
815 personalized tissues, and our assembloid techniques have been validated with hPSC-derived
816 HEP cells[14,15]. However, a significant limitation of these systems is the use of GFR MG, a mouse
817 tumor extracellular matrix protein mixture primarily used in these cultivation models. However,
818 it is a major limitation due to its tumor-derived origin and high cost. Additionally, the droplet
819 formation system degrades significantly after two weeks, suggesting the implementation of
820 alternative gels such as CG mixtures or sodium alginate. Furthermore, the current systems can
821 only grow to the dimensions of the spheroids, but not beyond, emphasizing the need to identify
822 and address limitations to growth.
823
824 The lack of methods to specifically study early events in early liver organogenesis[14] and,
825 more generally, methods to generate synthetic tissues[1]. These methods offer a toolbox for
826 building assembloids and assembling larger, more complex tissues for enhanced *ex vivo* organ
827 modeling. This holds potential for *in vivo* therapeutic approaches, in addition to modeling
828 structures during organogenesis (liver bud) and applications in biopharma.
829
830 The protocols presented enable models of different modes of liver cell migration,
831 including co-migration, interstitial migration, and branching morphogenesis, along with
832 assembloids that collectively can be used to construct more complex tissues for various
833 biomedical and biopharma applications.

834
835 **FIGURE PERMISSIONS:**
836 Permission to reproduce images from Frontiers Biotechnology and Bioengineering Journal were
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838
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844
845 **DISCLOSURES:**
846 NP is the founder of Khufu Therapeutics, an organoid engineering company that develops
847 treatments for acute and chronic liver disease.
848
849 **REFERENCES:**
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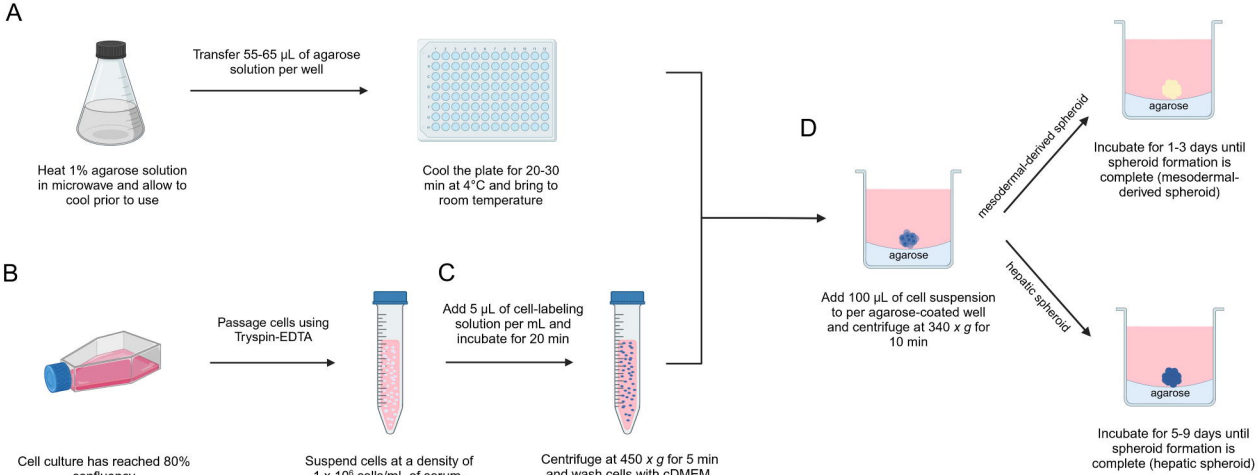
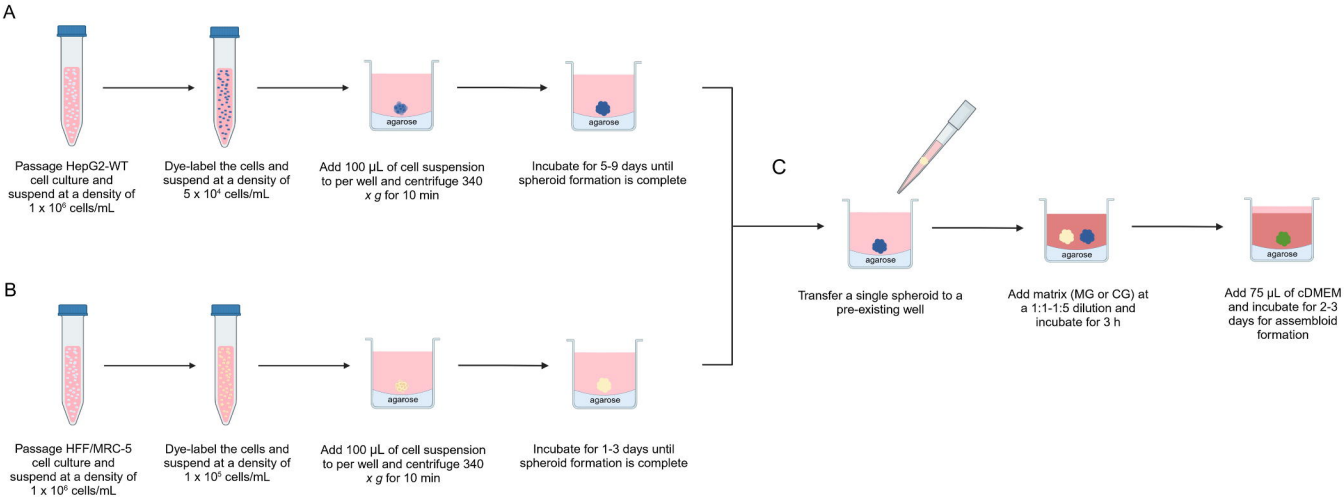
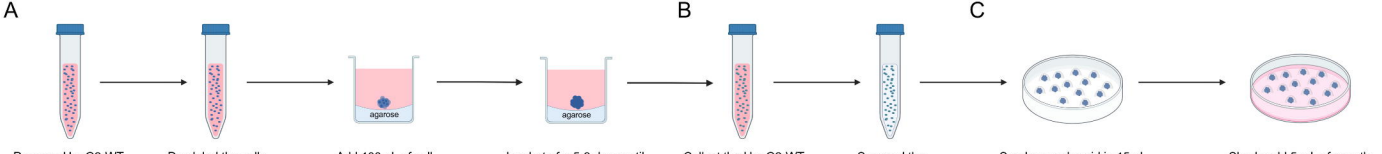
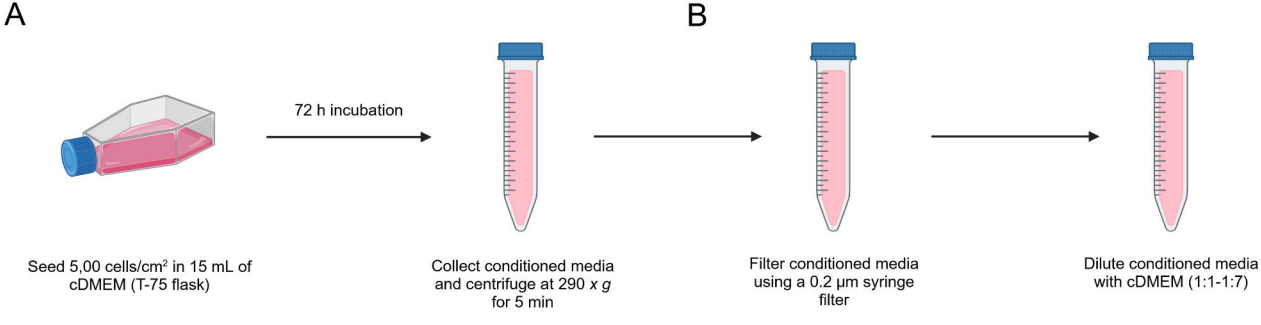
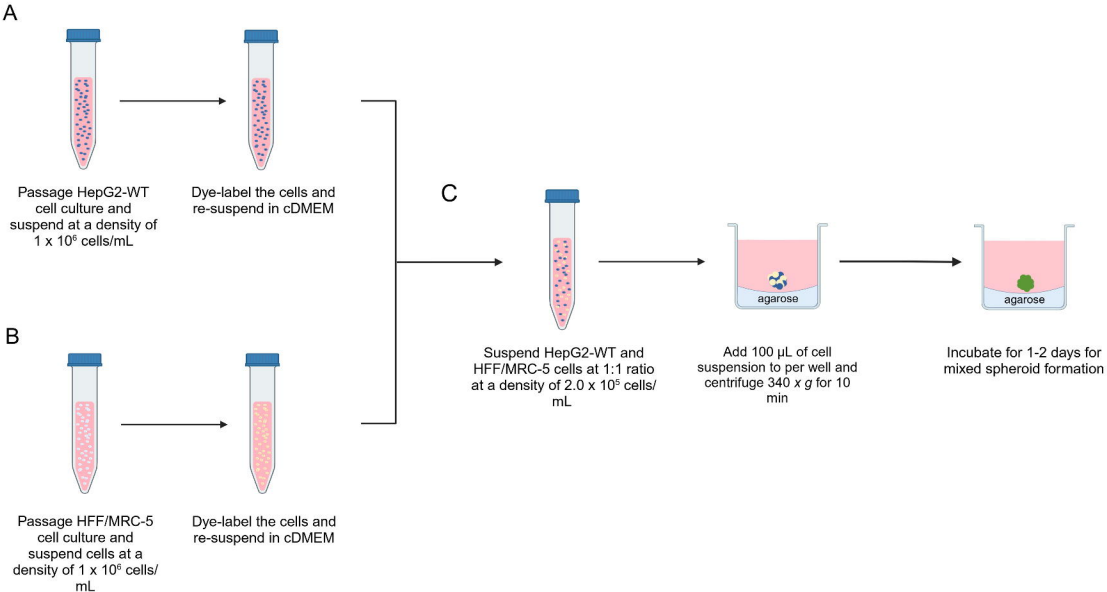
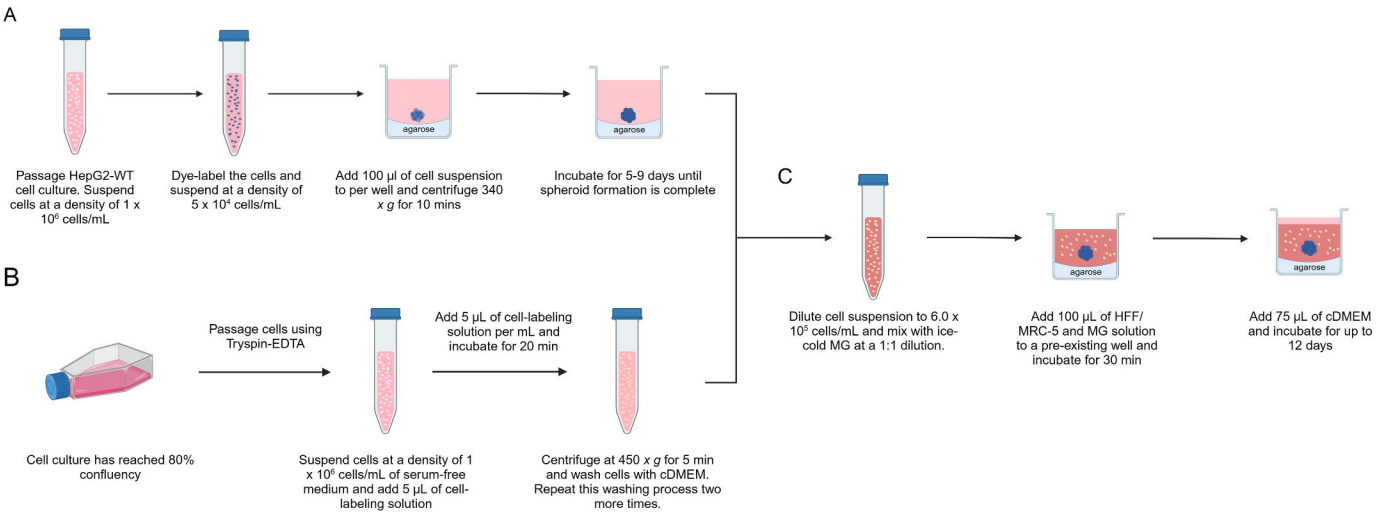

A
Transfer 55-65 µL of agarose solution per well
Heat 1% agarose solution in microwave and allow to cool prior to use
Cool the plate for 20-30 min at 4°C and bring to room temperature
B
Passage cells using Tryspin-EDTA
Cell culture has reached 80% confluency
Suspend cells at a density of 1 x 10^6 cells/mL of serum-free medium and add 5 µL of cell-labeling solution
C
Add 5 µL of cell-labeling solution per mL and incubate for 20 min
Centrifuge at 450 x g for 5 min and wash cells with cDMEM. Repeat this washing process two more times.
D
agarose
Add 100 µL of cell suspension to per agarose-coated well and centrifuge at 340 x g for 10 min
mesodermal-derived spheroid
hepatic spheroid
agarose
Incubate for 1-3 days until spheroid formation is complete (mesodermal-derived spheroid)
agarose
Incubate for 5-9 days until spheroid formation is complete (hepatic spheroid)

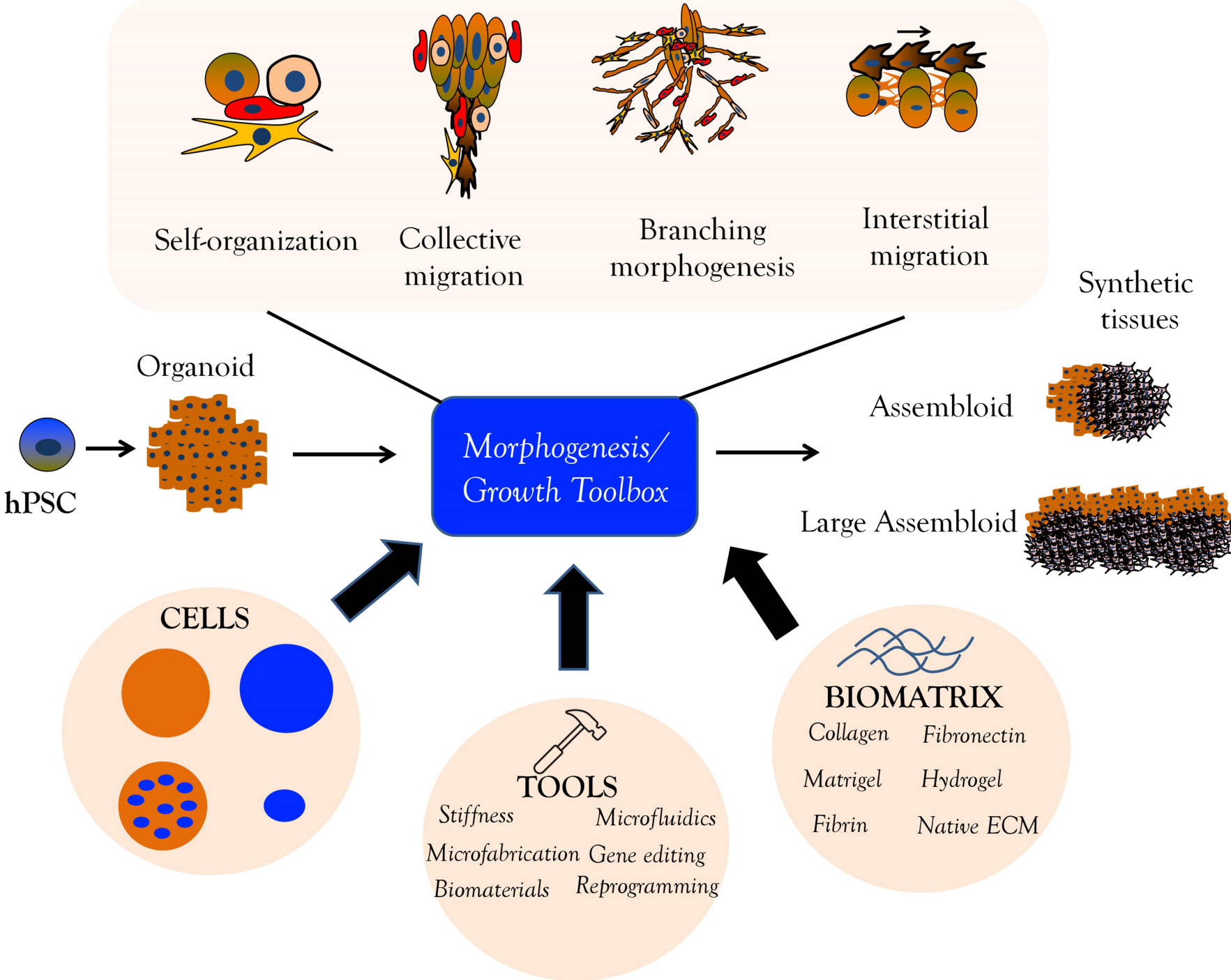

A
Passage HepG2-WT cell culture and suspend at a density of 1 x 10⁶ cells/mL
Dye-label the cells and suspend at a density of 5 x 10⁴ cells/mL
agarose
Add 100 µL of cell suspension to per well and centrifuge 340 x g for 10 min
agarose
Incubate for 5-9 days until spheroid formation is complete
B
Passage HFF/MRC-5 cell culture and suspend at a density of 1 x 10⁶ cells/mL
Dye-label the cells and suspend at a density of 1 x 10⁵ cells/mL
agarose
Add 100 µL of cell suspension to per well and centrifuge 340 x g for 10 min
agarose
Incubate for 1-3 days until spheroid formation is complete
C
agarose
Transfer a single spheroid to a pre-existing well
agarose
Add matrix (MG or CG) at a 1:1-1:5 dilution and incubate for 3 h
agarose
Add 75 µL of cDMEM and incubate for 2-3 days for assembloid formation

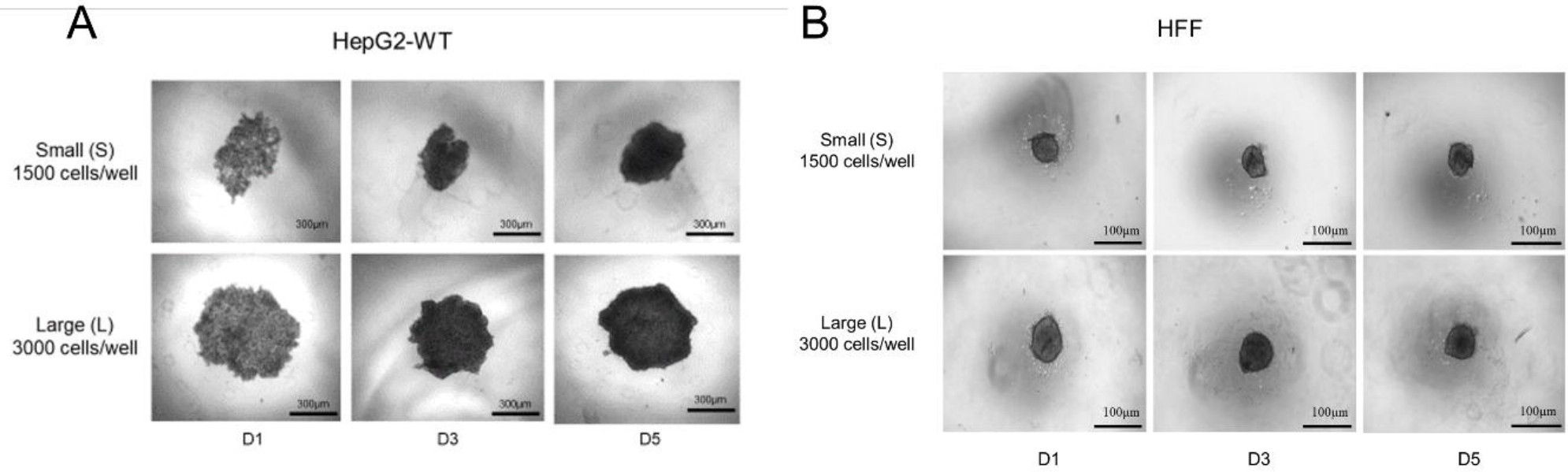

A
B
C
agarose
agarose
Passage HepG2-WT cell culture and suspend at a density of 1 x 10^6 cells/mL
Dye-label the cells and suspend at a density of 5 x 10^4 cells/mL
Add 100 µL of cell suspension to per well and centrifuge 340 x g for 10 min
Incubate for 5-9 days until spheroid formation is complete
Collect the HepG2-WT spheroids and gently rinse with cDMEM
Suspend the spheroids in matrix (MG or CG)
Seed one spheroid in 15 µL of matrix (MG or CG) onto 60mm petri dish and incubate at 37°C for 60 min
Slowly add 5 mL of growth medium and incubate at 37°C


A
B
72 h incubation
Seed 5,00 cells/cm² in 15 mL of cDMEM (T-75 flask)
Collect conditioned media and centrifuge at 290 x g for 5 min
Filter conditioned media using a 0.2 µm syringe filter
Dilute conditioned media with cDMEM (1:1-1:7)

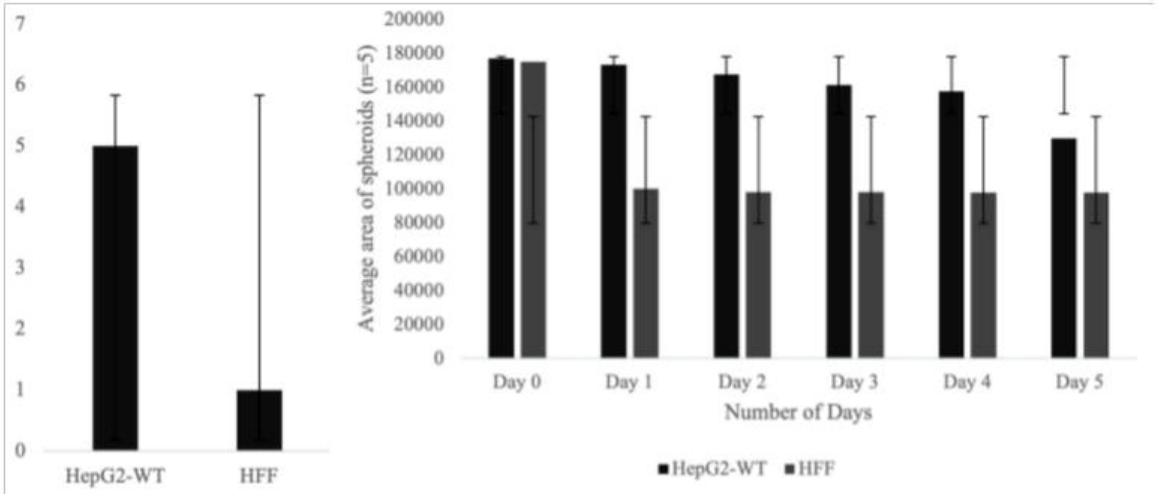

A
Passage HepG2-WT cell culture and suspend at a density of 1 x 10⁶ cells/mL
Dye-label the cells and re-suspend in cDMEM
B
Passage HFF/MRC-5 cell culture and suspend cells at a density of 1 x 10⁶ cells/mL
Dye-label the cells and re-suspend in cDMEM
C
Suspend HepG2-WT and HFF/MRC-5 cells at 1:1 ratio at a density of 2.0 x 10⁵ cells/mL
agarose
Add 100 µL of cell suspension to per well and centrifuge 340 x g for 10 min
agarose
Incubate for 1-2 days for mixed spheroid formation

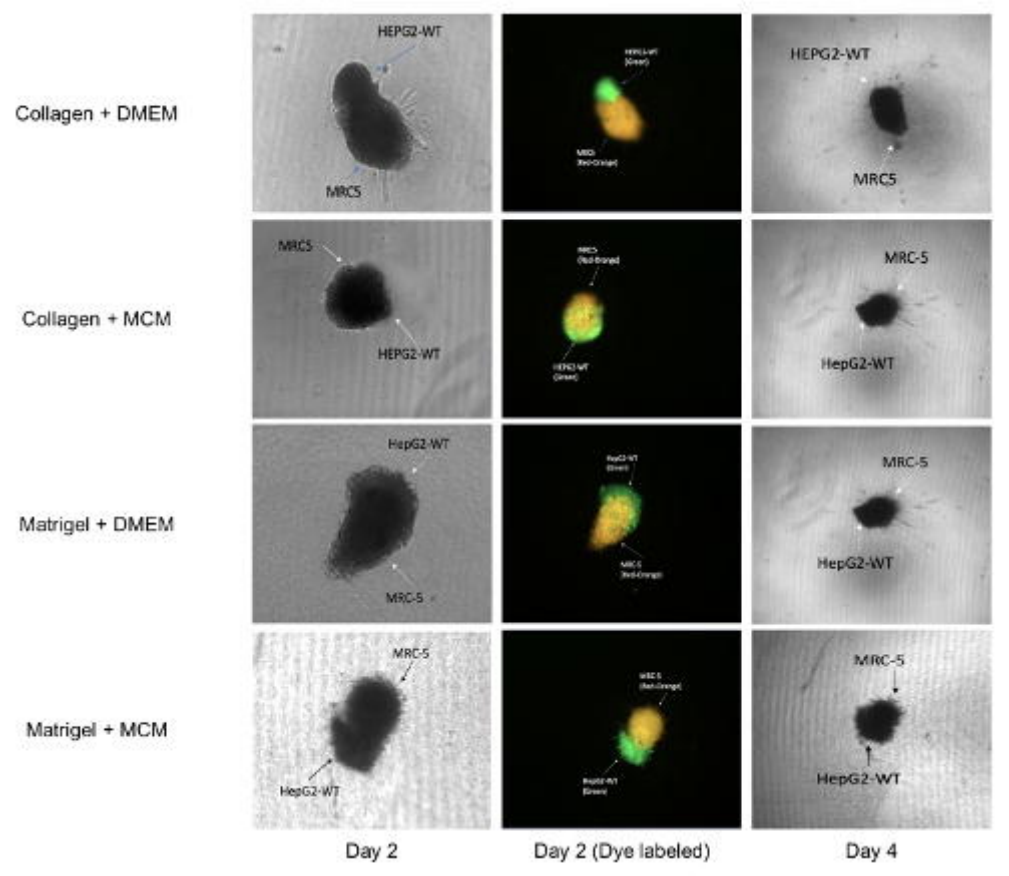

A
Passage HepG2-WT cell culture. Suspend cells at a density of 1 x 10^6 cells/mL
Dye-label the cells and suspend at a density of 5 x 10^4 cells/mL
agarose
Add 100 µl of cell suspension to per well and centrifuge 340 x g for 10 mins
agarose
Incubate for 5-9 days until spheroid formation is complete
B
Cell culture has reached 80% confluency
Passage cells using Tryspin-EDTA
Suspend cells at a density of 1 x 10^6 cells/mL of serum-free medium and add 5 µL of cell-labeling solution
Add 5 µL of cell-labeling solution per mL and incubate for 20 min
Centrifuge at 450 x g for 5 min and wash cells with cDMEM. Repeat this washing process two more times.
C
Dilute cell suspension to 6.0 x 10^5 cells/mL and mix with ice-cold MG at a 1:1 dilution.
agarose
Add 100 µL of HFF/ MRC-5 and MG solution to a pre-existing well and incubate for 30 min
agarose
Add 75 µL of cDMEM and incubate for up to 12 days

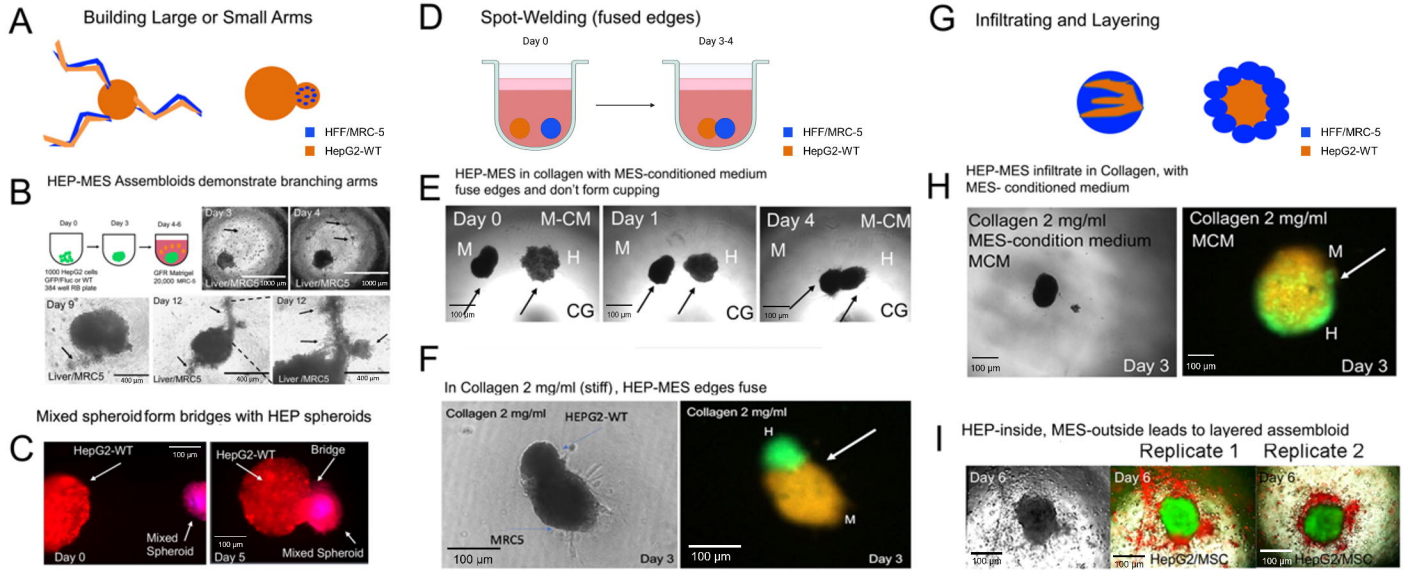

Self-organization
Collective migration
Branching morphogenesis
Interstitial migration
Synthetic tissues
Organoid
hPSC
Morphogenesis/ Growth Toolbox
Assembloid
Large Assembloid
CELLS
TOOLS
Stiffness
Microfluidics
Microfabrication
Gene editing
Biomaterials
Reprogramming
BIOMATRIX
Collagen
Fibronectin
Matrigel
Hydrogel
Fibrin
Native ECM


A
HepG2-WT
Small (S)
1500 cells/well
Large (L)
3000 cells/well
300µm
D1
D3
D5
B
HFF
Small (S)
1500 cells/well
Large (L)
3000 cells/well
100µm
D1
D3
D5

C


7
6
5
4
3
2
1
0
HepG2-WT
HFF
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Average area of spheroids (n=5)
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Number of Days
HepG2-WT
HFF


Collagen + DMEM
Collagen + MCM
Matrigel + DMEM
Matrigel + MCM
HEPG2-WT
MRC5
HEPG2-WT
MRC5
MRC-5
HepG2-WT
HepG2-WT
MRC-5
MRC-5
HepG2-WT
MRC-5
HepG2-WT
MRC-5
HepG2-WT
Day 2
Day 2 (Dye labeled)
Day 4


A
Building Large or Small Arms
HFF/MRC-5
HepG2-WT
B
HEP-MES Assembloids demonstrate branching arms
Day 0
Day 3
Day 4-6
1000 HepG2 cells
GFP/Fluor or WT
384 well RB plate
GFR Matrigel
20,000 MRC-5
Day 3
Day 4
Liver/MRC5
1000 µm
Day 9
Day 12
Day 12
Liver/MRC5
400 µm
Mixed spheroid form bridges with HEP spheroids
C
HepG2-WT
100 µm
Mixed
Spheroid
Day 0
HepG2-WT
Bridge
100 µm
Day 5
Mixed Spheroid
D
Spot-Welding (fused edges)
Day 0
Day 3-4
HFF/MRC-5
HepG2-WT
E
HEP-MES in collagen with MES-conditioned medium
fuse edges and don't form cupping
Day 0
M-CM
M
H
CG
100 µm
Day 1
M
H
CG
100 µm
Day 4
M-CM
M
H
CG
100 µm
F
In Collagen 2 mg/ml (stiff), HEP-MES edges fuse
Collagen 2 mg/ml
HEPG2-WT
MRC5
100 µm
Day 3
Collagen 2 mg/ml
H
M
100 µm
Day 3
G
Infiltrating and Layering
HFF/MRC-5
HepG2-WT
H
HEP-MES infiltrate in Collagen, with
MES- conditioned medium
Collagen 2 mg/ml
MES-condition medium
MCM
100 µm
Day 3
Collagen 2 mg/ml
MCM
M
H
100 µm
Day 3
I
HEP-inside, MES-outside leads to layered assembloid
Replicate 1
Replicate 2
Day 6
100 µm
Day 6
HepG2/MSC
100 µm
Day 6
HepG2/MSC
100 µm

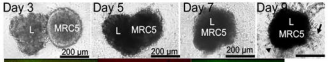
A

Fused HEP-MES assembloids (increased packing density, separate layers)

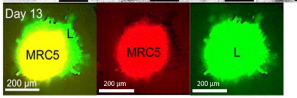
HFF/MRC-5
HepG2-WT

B

HEP and MES spheroids fuse in matrigel

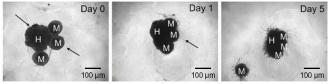



C



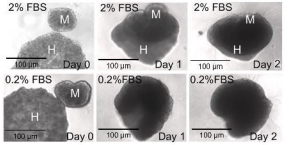

D

HEP-MES assembloids with multiple MES spheroids fuse but overall volume does not increase



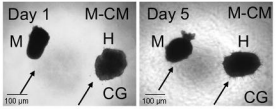

E

HEP-MES assembloids form under low serum



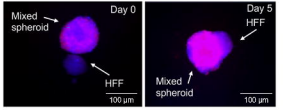

F

HEP-MES don't form at large distances in collagen with MES-conditioned medium




G

Mixed spheroids fuse with MES spheroids



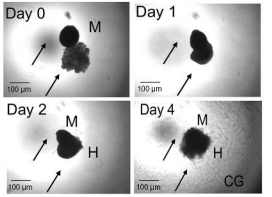

H

Cupping, partially fused HEP-MES assembloids (increased packing density, separate layers)

HFF/MRC-5
HepG2-WT

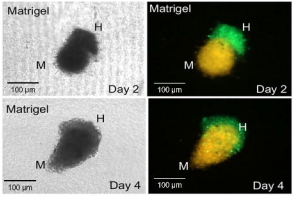
I

In collagen, HEP-MES form assembloids via cup-like mechanism



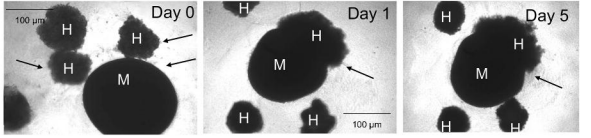

J

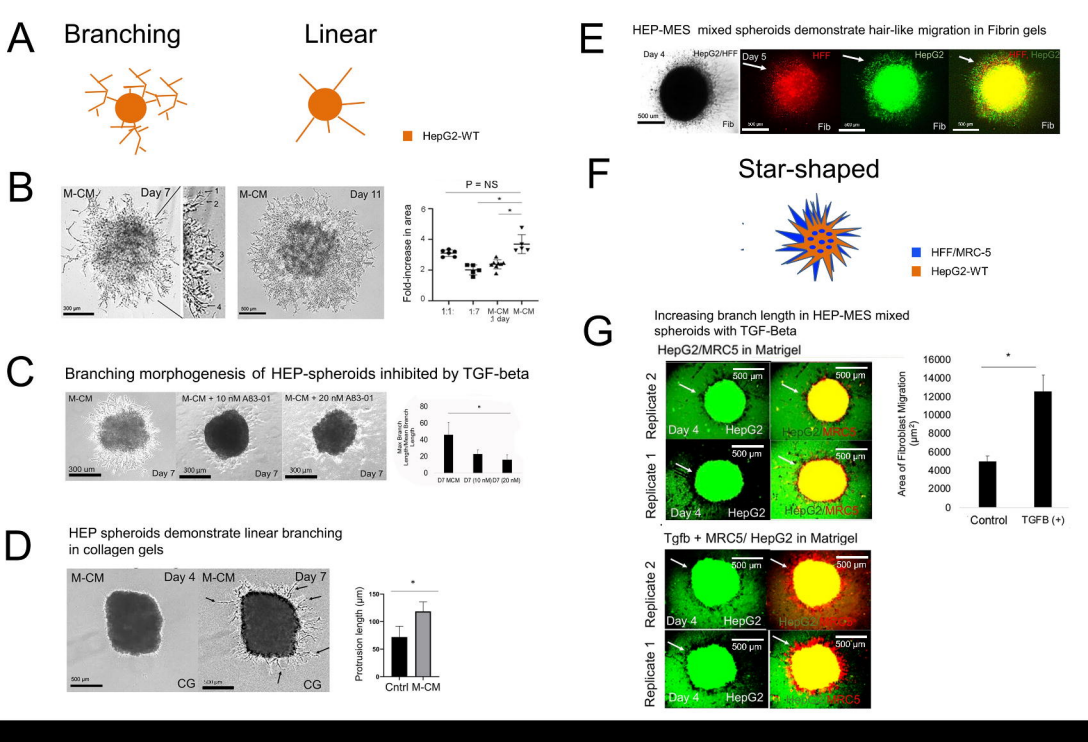
HEP-MES cupping prior to fusion in MG




K

HEP-MES assembloids with multiple HEP spheroids combine with cupping mechanism




A
Branching
Linear
HepG2-WT
B
M-CM
Day 7
M-CM
Day 11
P = NS
Fold-increase in area
1:1
1:7
M-CM 5 day
M-CM 11 day
C
Branching morphogenesis of HEP-spheroids inhibited by TGF-beta
M-CM
M-CM + 10 nM A83-01
M-CM + 20 nM A83-01
Day 7
Max Branch Length/Mean Branch Length
D7 MCM
D7 (10 nM)
D7 (20 nM)
D
HEP spheroids demonstrate linear branching in collagen gels
M-CM
Day 4
M-CM
Day 7
CG
Protrusion length (µm)
Cntrl
M-CM
E
HEP-MES mixed spheroids demonstrate hair-like migration in Fibrin gels
Day 4
HepG2/HFF
Day 5
HFF
HepG2
HFF
HepG2
Fib
F
Star-shaped
HFF/MRC-5
HepG2-WT
G
Increasing branch length in HEP-MES mixed spheroids with TGF-Beta
HepG2/MRC5 in Matrigel
Replicate 2
Replicate 1
Day 4
HepG2
HepG2/MRC5
Area of Fibroblast Migration (µm²)
Control
TGFB (+)
Tgfb + MRC5/ HepG2 in Matrigel
Replicate 2
Replicate 1
Day 4
HepG2
HepG2/MRC5

| Growth Medium & Matrix | Initial Distances (μm) | Days to Merge |
|---|---|---|
| Matrigel + DMEM | 492.742 | 6 |
| Matrigel + DMEM | 580.142 | 8 |
| Matrigel + DMEM | 974.051 | 12 |