### Title

Protocol to generate human liver spheroids to study liver fibrosis induced by metabolic stress




### Journal

STAR Protocols, 5(2)

### ISSN

2666-1667



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### Publication Date

2024-06-01

### DOI

10.1016/j.xpro.2024.103111

Peer reviewed

STAR Protocols

CellPress
OPEN ACCESS

Protocol

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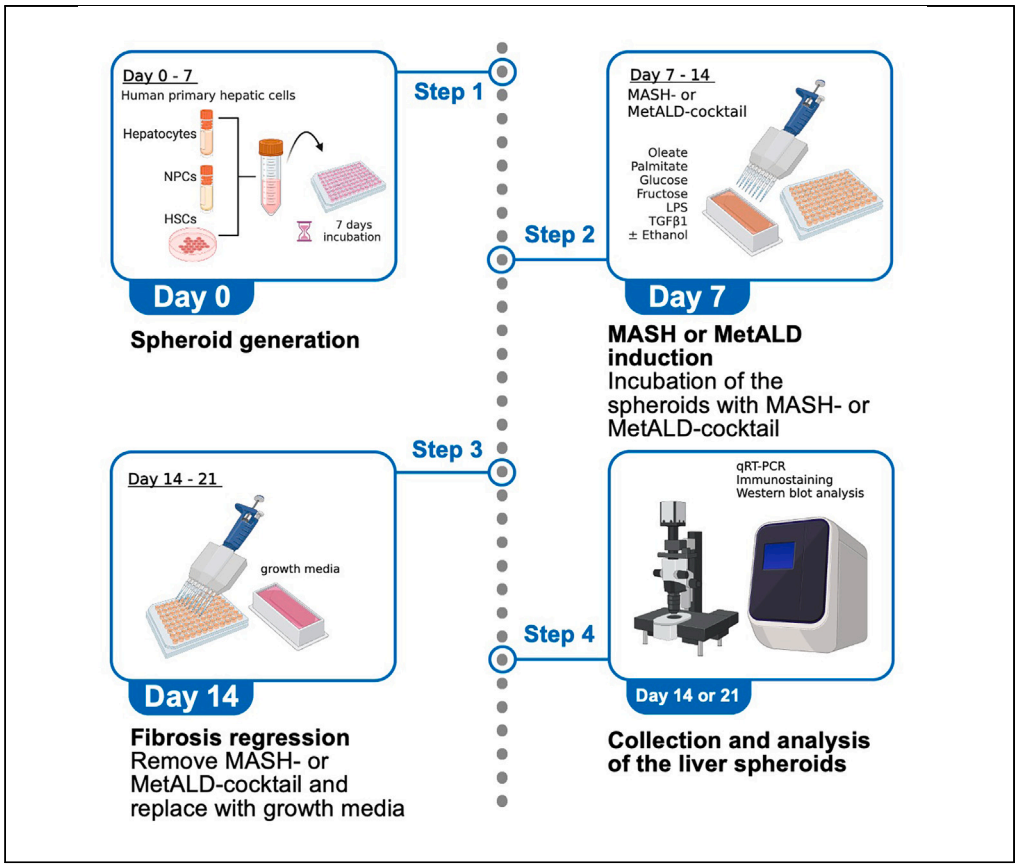


Currently, there is no effective treatment for obesity and alcohol-associated liver diseases, partially due to the lack of translational human models. Here, we present a protocol to generate 3D human liver spheroids that contain all the liver cell types and mimic "livers in a dish." We describe strategies to induce metabolic and alcohol-associated hepatic steatosis, inflammation, and fibrosis. We outline potential applications, including using human liver spheroids for experimental and translational research and drug screening to identify potential anti-fibrotic therapies.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Highlights

Generation of human liver spheroids that are composed of all liver cell types

Induction of MASH or MetALD in human liver spheroids

Regression of MASH-induced fibrosis in human liver spheroids

Use of the human liver spheroids for translational research

Kim et al., STAR Protocols 5, 103111
June 21, 2024 
https://doi.org/10.1016/j.xpro.2024.103111

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# Protocol to generate human liver spheroids to study liver fibrosis induced by metabolic stress

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## SUMMARY

**Currently, there is no effective treatment for obesity and alcohol-associated liver diseases, partially due to the lack of translational human models. Here, we present a protocol to generate 3D human liver spheroids that contain all the liver cell types and mimic "livers in a dish." We describe strategies to induce metabolic and alcohol-associated hepatic steatosis, inflammation, and fibrosis. We outline potential applications, including using human liver spheroids for experimental and translational research and drug screening to identify potential anti-fibrotic therapies.**

## BEFORE YOU BEGIN

### Institutional permissions

This study utilizes primary human liver cells isolated from livers of deidentified donors, whose livers were declined for transplantation for various reasons. Donor livers were obtained through Lifesharing organ procurement organization (OPO). This project (171883XX) was reviewed by the Director of the UCSD HRPP, IRB Chair, or IRB Chair's designee and the patient consent was obtained by www.lifesharing.org. It is certified as "no human subjects" according to the Code of Federal Regulations, title 45, part 46 and UCSD Standard Operating Policies and Procedures, and therefore does not require IRB review.

### Isolation of human primary hepatic cells

Human liver spheroids are generated using a total 5,300 primary human liver cells containing hepatocytes (57%), non-parenchymal cells (NPCs, 28%, NPCs: myeloid cells, endothelial cells, T and B cells, NKT cells, and others) and Hepatic Stellate Cells (HSCs, 15%) at physiological concentrations observed in human MASH livers.[1,2] Human liver spheroids are prepared using primary human liver cells, obtained as previously described.[3] Human liver cells are isolated using enzymatic perfusion and gradient centrifugation. Human hepatocytes, NPCs, and HSCs are cryopreserved. All freshly isolated hepatocytes are suitable for spheroid generation. Meanwhile, only some cryopreserved hepatocytes form human liver spheroids (due to cryopreservation injury). Usually, hepatocytes from a healthy donor can be cryopreserved and used for spheroid generation after thawing. However, we do not recommend using cryopreserved steatotic hepatocytes, as the fragility of steatotic

hepatocytes makes them susceptible to freezing/thawing artifacts, and the resulting poor condition of hepatocytes leads to failure to form spheroids.

### Experimental design

Metabolic dysfunction-associated steatohepatitis (MASH) and metabolic and alcohol-associated liver disease (MetALD) are the major causes of chronic liver injury that leads to steatosis, inflammation, and liver fibrosis. We describe a method to study the development and regression of MASH or MetALD using human liver spheroids. The generation of human liver spheroids include the following steps: (i) formation of human liver spheroids in growth medium for 7 days using round bottom ultra-low attachment (ULA) microplates; (ii) induction of metabolic liver injury in human liver spheroids using MASH- or MetALD-cocktail for 7–14 days, and (iii) cessation of metabolic injury in human liver spheroids upon removal of MASH- or MetALD-cocktail and exchanging to growth medium for day 14–21. Growth medium contains dexamethasone and insulin transferrin selenium (ITS). MASH-cocktail contains fatty acids (FAs), glucose, fructose, lipopolysaccharides (LPS), and transforming growth factor (TGF)-β1. MetALD-cocktail contains ethanol in addition to the MASH-cocktail (Figure 1A).

### Preparation of the reagents for MASH-cocktail

**Timing: 3 h**

**Timing: 1.5 h (for step 2)**

This step describes the preparation of reagents for MASH-cocktail.

*Note:* Prepare stocks for the following reagents using sterile conditions in a tissue culture hood.

1. Dexamethasone (20 μg/mL).
   a. Reconstitute dexamethasone in ethanol to obtain 1 mg/mL dexamethasone solution.
   b. Aliquot the stock solution (1 mg/mL) and store at −20°C for up to 5 years.
   c. For generation of human liver spheroids, dilute 1 mg/mL of dexamethasone in DMEM to obtain 20 μg/mL dexamethasone solution.
   d. Aliquot the 20 μg/mL stock solution and store at −20°C for up to one year.
   e. Once thawed, keep at 4°C for up to one week.
2. Mixture of oleate and palmitate fatty acids (FAs).
   a. Set one water bath to 37°C and another to 70°C. A heat block can be used as an alternative for the water bath. Position the two water baths adjacent to each other, with a vortex mixer in close proximity. This setup is optimal for handling temperature-sensitive solutions during formulation.
   b. Place 10% BSA solution in 37°C water bath to warm while preparing the FAs.
   c. Reconstitute oleate (OA) in 0.1N NaOH in 10 mL glass vial to obtain a 150 mM solution.[4]
      i. For the 10 mL solution, add 422.16 mg of sodium OA to 0.1 N NaOH and bring up volume to 10 mL.
      ii. Place the solution at 70°C for 1 h, until the solution is clear. Mix one or two times by vortex during incubation.
   d. Reconstitute palmitate (PA) in 0.1N NaOH in 10 mL glass vial to obtain a 100 mM solution.[4]
      i. Add 256.42 mg of PA to 0.1 N NaOH and bring volume to 10 mL.
      ii. Place the solution at 70°C for 1 h, until the solution is clear. Mix one or two times by vortex during incubation.

*Note:* OA and PA in 0.1 N NaOH can be stored at −80°C for up to one year.

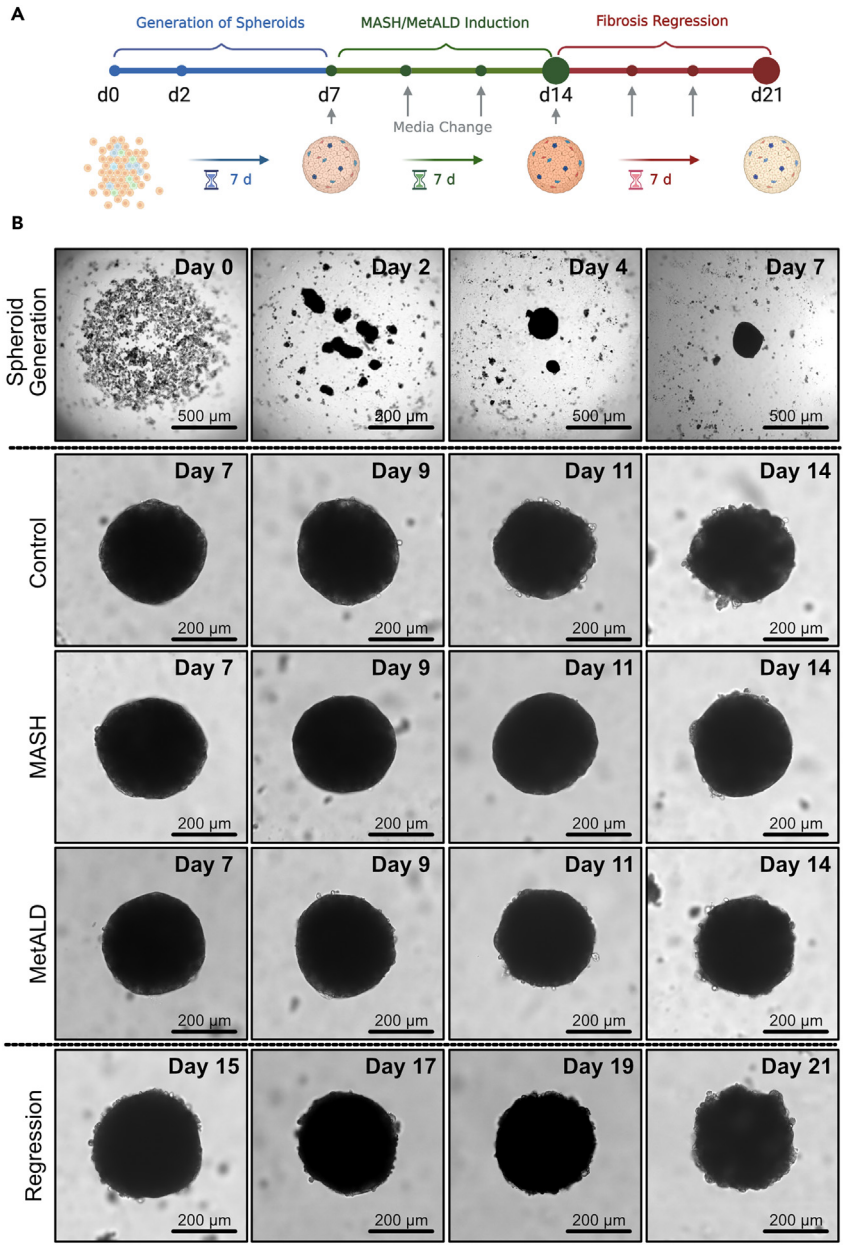


**Figure 1. Generation of human liver spheroids**

(A) Experimental timeline for generation of human liver spheroids.

(B) Bright field images of human liver spheroids (scale bar = 200 or 500 µm).

e. Add FAs dissolved in 70°C directly to 10% BSA at 37°C and mix by inverting several times.[4]
   i. For example, to make 10 mL of a 1:1 molar ratio of OA:PA, add 0.2 mL of 150 mM OA and 0.3 mL of 100 mM PA to 9.5 mL of 10% BSA solution.

*Note:* Albumin has multiple binding sites for FAs, with variable affinity. In this example, the molar ratio of FA:BSA is 2:1 (e.g. 3 mM FAs: 1.5 mM BSA).

f. Incubate FAs in BSA solution at 37°C for an additional 10 min.
g. Filter the solution using 0.2 µm syringe filter.

⚠ CRITICAL: If the solution is not clear, continue the incubation until the solution becomes clear before filtering.

h. FAs in BSA solution can be used immediately or aliquoted (1 mL) and stored at −20°C for up to one year. A freeze-thaw cycle for FAs in BSA solution is not recommended.

3. Fructose (1 M).
   a. Reconstitute fructose (MW = 180.16) in DMEM to obtain 1 M fructose solution.
   b. Aliquot (1 mL) of fructose solution and store at −20°C for up to one year.
   c. Once thawed, keep at 4°C for up to one week.
4. Lipopolysaccharides (LPS, 5 mg/mL).
   a. Add 1 mL of endotoxin-free water to obtain 5 mg/mL LPS solution.
   b. Vortex until completely dissolved.
   c. Aliquot (50 μL) and store at −20°C for up to one year.
   d. Once thawed, keep at 4°C for up to one week.
5. Human TGF-β1 (25 μg/mL).
   a. Centrifuge the vial prior to opening.
   b. Reconstitute TGF-β1 in 10 mM citric acid (pH 3.0) to a concentration of 25 μg/mL.[5]
   c. Aliquot (10 μL) and store −80°C for up to one year.

⚠ CRITICAL: A Freeze-thaw cycle for TGF-β1 is not recommended to ensure its maximal activity. We recommend storing the working stock solution in small aliquots.

### Preparation of hepatic stellate cells

Timing: ~30 min

This step describes the preparation of HSCs.

*Note:* Human HSCs are cultured in 100 mm tissue culture dishes prior to the generation of human liver spheroids.

6. Preparation of HSCs.
   a. Thaw a frozen stock of HSCs.
   b. Resuspend the HSCs ($2.5 \times 10^5$–$3.0 \times 10^5$ cells) in 10 mL DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic reagent.
   c. Plate the HSCs in 100 mm culture dish.
   d. Incubate at 37°C, 5% $CO_2$ for at least 2 days before making spheroids.

*Note:* $2.5 \times 10^5$–$3.0 \times 10^5$ cells per 100 mm culture dish can lead to approximately 80% confluency after 2 days of incubation.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| HEPES | VWR | 45000-690 |
| 10× DPBS | VWR | 45000-426 |
| Percoll density gradient media | Sigma-Aldrich | GE17-0891-09 |
| DMEM, high glucose | Fisher | 11965118 |
| Antibiotic-Antimycotic 100× | Gibco | 15240062 |
| Penicillin-Streptomycin-Glutamine 100× | Gibco | 10378016 |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| Trypsin-EDTA (0.25%), phenol red | Gibco | 25200056 |
| Fetal bovine serum | Gemini Bio-Products | 100-106 |
| 1× PBS, without calcium and magnesium | Corning | 21-040-CV |
| Trypan blue solution | Invitrogen | 15250-061 |
| Insulin-Transferrin-Selenium (ITS -G) (100×) | Thermo Fisher Scientific | 41400045 |
| Dexamethasone | MP Biomedicals | 0219456180 |
| Palmitate | Sigma | P0500 |
| Oleate | Sigma | O1257 |
| Fructose | VWR | 97061-236 |
| Lipopolysaccharides (LPS) | InvivoGen | tlrl-3pelps |
| Ethanol | Koptec | V1016 |
| Bovine serum albumin solution | Sigma | A1595 |
| Sodium hydroxide | Sigma | S5881 |
| Citric acid | Thermo Fisher Scientific | 036664.22 |
| D-(+)-glucose solution | Sigma | G8644 |
| Recombinant human TGF-β1 | Proteintech | HZ-1131 |
| RIPA lysis buffer | Teknova | R3792 |
| Protease and phosphatase inhibitor cocktail | Thermo Fisher Scientific | 78443 |
| 4% Paraformaldehyde solution in PBS | Thermo Fisher Scientific | J19943.K2 |
| Buffer RLT Plus | QIAGEN | 1053393 |
| Software and algorithms | | |
| GraphPad Prism | GraphPad Prism | GraphPad Prism |
| Leica Application Suite X (LAS X) | Leica Application Suite X (LAS X) | Leica Application Suite X (LAS X) |
| Others | | |
| Refrigerated benchtop centrifuge with swinging bucket rotor | Thermo Fisher Scientific | ST4R plus |
| Biosafety cabinet | Thermo Fisher Scientific | 10445733 |
| Vortex mixer | Thermo Fisher Scientific | 02215365 |
| Water bath | Thermo Fisher Scientific | GP 10 |
| Inverted microscope | Olympus | CKX53 |
| Cell culture incubators with 37°C, 5% $CO_2$ | Eppendorf | CellXpert C170 |
| Glass door refrigerator | Thermo Fisher Scientific | FYC-335 |
| Precision balance | Sartorius | SQP |
| Plate shaker | BenchMate | S4P-D |
| Spheroid ultra-low attachment (ULA) surface microplates | Corning | CLS4515 |
| Cell counter/Hemocytometer | Marienfeld | 065-0010 |
| Pipet-aids | Drummond | XP2 |
| Disposable serological pipettes (10 mL, 25 mL) | Biopioneer | GEX0100-S01, GEX250-S01 |
| 100 mm TC-treated cell culture dish | Falcon | 353003 |
| Conical tubes: 15 and 50 mL | Corning | 430790 430828 |
| Multichannel pipette | VWR | 89079-948 |
| 50 mL Reservoir | Biopioneer | RSV-2S |
| Syringe Filters | Fisher | 09-719C |

## MATERIALS AND EQUIPMENT

| **Basal medium** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| DMEM | N/A | 482.5 mL |
| HEPES | 25 mM | 12.5 mL |
| Penicillin/Streptomycin | 1% | 5 mL |
| **Total** | **N/A** | **500 mL** |

*Note:* Store Basal medium at 4°C for maximum one month.

| **Complete medium** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| DMEM | N/A | 42.65 mL |
| HEPES | 25 mM | 1.25 mL |
| Penicillin/Streptomycin | 1% | 0.5 mL |
| FBS | 10% | 5 mL |
| Dexamethasone | 40 ng/mL | 0.1 mL |
| ITS-G | 1% | 0.5 mL |
| **Total** | **N/A** | **50 mL** |

*Note:* Complete medium is always prepared fresh before use. Add FBS, dexamethasone, and ITS-G to the basal medium after warming up the basal medium to 37°C.

| **Growth medium** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| DMEM | N/A | 47.65 mL |
| HEPES | 25 mM | 1.25 mL |
| Penicillin/Streptomycin | 1% | 0.5 mL |
| Dexamethasone | 40 ng/mL | 0.1 mL |
| ITS-G | 1% | 0.5 mL |
| **Total** | **N/A** | **50 mL** |

*Note:* Growth medium is always prepared fresh before use. Add dexamethasone, and ITS-G to the basal medium after warming up the basal medium to 37°C.

## STEP-BY-STEP METHOD DETAILS

### Preparation of hepatic cells for human liver spheroids

Timing: 2 h (Day 0)

This step describes the preparation of a hepatic cell suspension (hepatocytes, HSCs, and NPCs) for the generation of human liver spheroids.

1. Prepare a suspension of viable hepatocytes.
   a. Prepare solution for gradient centrifugation (13.5 mL Percoll + 1.5 mL 10× DPBS + 35 mL complete medium) in a 50 mL conical tube.

   *Note:* Gradient centrifugation of the hepatocyte suspension is required to remove dead cells from live cells. It increases the hepatocyte viability, which is necessary for spheroid generation.

   ⚠ CRITICAL: Mix the solution by inverting several times to make a homogeneous solution.

   b. Defrost cryopreserved hepatocyte stock (1 mL) in a 37°C water bath.
   c. Mix the hepatocytes with the solution for gradient centrifugation by inverting the tube several times.
   d. Centrifuge the tube at 100 *g* for 10 min at room temperature.

⚠ CRITICAL: Set deceleration rate to 0 to make a clear pellet. It is important to proceed with the brake off to avoid contamination of live cells (pellet) with dead cells (supernatant).

e. Gently aspirate off the supernatant containing dead hepatocytes.
f. Resuspend the pellet with 50 mL of complete medium.

*Note:* Resuspend the pellet by tapping or gently inverting the tube.

g. Centrifuge the cells for 5 min at 100 *g* at room temperature.
h. Remove the supernatant by gentle aspiration.
i. Resuspend the hepatocytes in 2–5 mL of fresh complete medium.

2. Prepare NPC suspension.
   a. Thaw NPC stock in a 37°C water bath.
   b. Dilute 1 mL of NPC stock solution in 14 mL complete medium.
3. Prepare HSC suspension.
   a. Remove the medium from the culture dish by gentle aspiration.
   b. Wash the HSCs with sterile pre-warmed (37°C) 1× PBS.
   c. Add 2 mL of 0.25% trypsin-EDTA and ensure that the cells are completely covered.
   d. Place the plates in 37°C, 5% $CO_2$ incubator for 5 min.
   e. Use the microscope to confirm that the cells are detached from the plate.
   f. Add 5 mL of complete medium to stop the trypsin-EDTA reaction.
   g. Collect all the cells from the plate in a 15 mL conical tube.
   h. Centrifuge the tube for 5 min at 300 *g* at room temperature.
   i. Remove the trypsin-EDTA solution by aspiration.
   j. Resuspend the cell pellet in 1–2 mL of fresh complete medium by gently pipetting up and down.

### Generation of human liver spheroids

**Timing: 1 h (Day 0) (for steps 4–9)**

**Timing: 40 min (Day 1) (for steps 10–12)**

**Timing: 1 h (Day 2) (for steps 13–16)**

**Timing: 30 min (Day 7, 9, 11, 13) (for steps 17 and 18)**

In this step, human liver spheroids are formed by coculturing human hepatocytes, NPCs, and HSCs (57% hepatocytes, 28% NPCs containing myeloid, T and B cells, natural killer cells, epithelial cells, and 15% enriched HSCs). Addition of human HSCs is needed, since the number of isolated NPCs lacked physiological concentrations of HSCs (due to perfusion conditions used, especially perfusion of fibrotic livers). Co-cultured hepatocytes, NPCs, and HSCs spontaneously form 3D liver spheroids within 7 days (Figure 1B). Normal human liver spheroids are generated by culturing in growth medium. MASH human liver spheroids are generated by culturing in MASH-cocktail. MetALD-induced human liver spheroids are generated by culturing in MetALD-cocktail. Regression of metabolic liver injury in spheroids is achieved by exchanging MASH- or MetALD- cocktail to growth medium.

4. Count the number of cells in each hepatocyte, NPC, and HSC suspension.
   a. Mix each suspension of hepatocytes, NPCs, and HSCs until homogenous.
   b. To count the number of cells, take 10 μL from each homogenous cell suspension. Use trypan blue and a hemocytometer for the cell count.

**Table 1. Composition of hepatic cells for the spheroids**

| Cell type | Cell viability (%) | Viable cell number per spheroid | Cell number for ULA spheroid plate |
|---|---|---|---|
| Hepatocyte | 80–100 | 3,000 | $3\times10^5$ |
| NPC | 90–100 | 1,500 | $1.5\times10^5$ |
| HSC | 90–100 | 800 | $8\times10^4$ |
| Total Volume | | 100 μL | 10 mL |

*Note:* The cell suspension can be kept at room temperature while you count the cells. Take 10 μL from the cell suspension immediately after mixing.

⚠ CRITICAL: For an optimal spheroid generation, the cell viability at this step should be above 80%.

5. Immediately after counting the cells, transfer the necessary amount of each cell type into a new 50 mL falcon tube containing complete medium following the cell composition below (Table 1).
6. Mix the cell suspension by gently swirling the conical tube.

*Note:* Swirl very slowly to avoid the formation of air bubbles.

7. Add 100 μL of cell suspension to each well of 96-well ULA plate using a pipetting reservoir and multichannel pipette.

⚠ CRITICAL: Gently mix the cell suspension in the pipetting reservoir. It is important to generate a homogenous cell suspension to ensure the formation of spheroids consistent in size.

8. Centrifuge the plates for 5 min at 100 *g* at room temperature.
9. Incubate at 37°C, 5% $CO_2$ incubator.
10. Shake the plates at 450 rpm for 25 min in a 37°C, 5% $CO_2$ incubator.

⚠ CRITICAL: Cell suspension in the microplates should be mixed thoroughly using an orbital shaker. Cell-cell contact is expected during this step.

11. Centrifuge the plates for 5 min at 100 *g* at room temperature.
12. Incubate at 37°C, 5% $CO_2$ incubator.
13. Using a pipetting reservoir and multichannel pipette, add 100 μL of growth medium to the 96-well ULA plate, bringing the volume to a total of 200 μL per well.
14. Shake the plates for at 450 rpm for 25 min in a 37°C, 5% $CO_2$ incubator.
15. Centrifuge the plates for 5 min at 100 *g* at room temperature.
16. Incubate at 37°C, 5% $CO_2$ incubator for 5 days.

*Note:* Monitor the condition of spheroids using a bright field microscope every day to confirm the spheroids are well-formed.

17. Carefully remove 100 μL of medium from the 96-well ULA plate using a multichannel pipette without disturbing the spheroids.

⚠ CRITICAL: Do not touch the bottom of the 96-well ULA plate, as the spheroids are located at the bottom of each well.

18. Add 100 μL of warm (37°C) growth medium to the 96-well ULA plate using a pipetting reservoir and multichannel pipette, bringing the total volume to 200 μL.

**Table 2. Composition of 2× MASH-cocktail**

| Reagent | Stock concentration | 2× MASH-cocktail concentration |
|---|---|---|
| OA : PA[6] | 3 mM : 3 mM | 320 μM : 320 μM |
| Fructose[7] | 1 M | 20 mM |
| Glucose | 0.55 M | 11 mM |
| LPS[7] | 5 mg/mL | 4 μg/mL |
| TGF-β1 | 25 μg/mL | 1 ng/mL |

*Note:* Monitor the condition of spheroid using a bright field microscope every day to confirm the spheroids are well-formed.

### MASH-induced human liver spheroids

**Timing: 1 h (Day 7) (for steps 19–21)**

**Timing: 1 h (Day 9, 11, and 13) (for steps 22–24)**

This step describes the experimental conditions to induce MASH in human liver spheroids. Human liver spheroids are cultured in 1× MASH-cocktail. MASH-cocktail contains final concentration of OA and PA (1:1 ratio, 160 μM each), 5.5 mM glucose, 10 mM fructose, 2 μg/mL LPS, and 1 ng/mL TGF-β1. MASH-cocktail is exchanged every 2 days, starting from day 7 of spheroid formation.

⚠ CRITICAL: Do not touch the bottom of the 96-well ULA plate, as the spheroids are located at the bottom of each well.

*Note:* Spheroids are kept in a 37°C, 5% $CO_2$ incubator while you prepare the cocktails.

19. Carefully remove 100 μL of medium from the 96-well ULA plate using a multichannel pipette without disturbing the spheroids, leaving 100 μL in the well.

*Note:* To bring the final concentration of MASH-cocktail to 1×, 100 μL of 2× MASH is added to the remaining 100 μL growth medium in each well.

20. Prepare 2× MASH-cocktail using reagents listed below (Table 2). Add the reagents to the growth medium.
21. Add 100 μL of 2× MASH-cocktail to the 96-well ULA plate using a pipetting reservoir and multichannel pipette.

*Note:* Mixing of the media in the plate is not required.

22. Carefully remove 100 μL of 1× MASH-cocktail from the 96-well ULA plate using a multichannel pipette without disturbing the spheroids.

*Note:* Spheroids are kept in a 37°C, 5% $CO_2$ incubator while you prepare the 1× MASH-cocktail.

23. Prepare 1× MASH-cocktail following the composition below (Table 3). Add the reagents to growth medium.
24. Add 100 μL of 1× MASH-cocktail to the 96-well ULA plate using a pipetting reservoir and multichannel pipette, bringing the total volume to 200 μL.

**Table 3. Composition of 1× MASH-cocktail**

| Reagent | Stock concentration | 1× MASH-cocktail concentration |
|---|---|---|
| OA : PA | 3 mM : 3 mM | 160 μM : 160 μM |
| Fructose | 1 M | 10 mM |
| Glucose | 0.55 M | 5.5 mM |
| LPS | 5 mg/mL | 2 μg/mL |
| TGF-β1 | 25 μg/mL | 0.5 ng/mL |

### MetALD-induced human liver spheroids

Timing: 1 h (Day 7) (for steps 25–27)

Timing: 30 min (Day 8, 10, 12) (for step 28)

Timing: 1 h (Day 9, 11, and 13) (for steps 29–31)

This step is designed to induce MetALD injury in human liver spheroids. For this purpose, human liver spheroids are cultured in MetALD-cocktail (containing MASH-cocktail + 100 mM ethanol) to recapitulate the metabolic injury complicated by chronic exposure to alcohol. Excessive exposure to alcohol amplifies the metabolic damage of hepatocytes, activation of inflammatory and fibrogenic responses in NPCs and HSCs. To prepare MetALD-cocktail, ethanol (10 M stock) is added to the MASH-cocktail for final concentration of 100 mM. Ethanol (2 μl of 10 M ethanol stock per well) is added every day in MetALD-cocktail (to account for alcohol evaporation), while MetALD-cocktail is exchanged every second day.

⚠ CRITICAL: Do not touch the bottom of the 96-well ULA plate, as the spheroids are located at the bottom of each well.

25. Carefully remove 100 μL of medium from the 96-well ULA plate using a multichannel pipette without disturbing the spheroids, leaving 100 μL in the well.
26. Prepare 2× MetALD-cocktail following the composition below (Table 4). Add the reagents to the growth medium.

*Note:* To bring the final concentration of MetALD-cocktail to 1×, 100 μl of 2× MetALD-cocktail is added to the remaining 100 μl growth medium in each well.

*Note:* MASH-cocktail can be used to make MetALD-cocktail by the addition of ethanol.

27. Add 100 μL of 2× MetALD-cocktail to the 96-well ULA plate using a pipetting reservoir and multichannel pipette.
28. Add ethanol to a final concentration of 100 mM.

⚠ CRITICAL: A significant amount of ethanol evaporates within 24 h.[8] Ethanol is replenished daily.

29. Carefully remove 100 μL of the medium from the 96-well ULA plate using a multichannel pipette without disturbing the spheroids, leaving 100 μL in the well.

*Note:* Spheroids are kept in a 37°C, 5% $CO_2$ incubator while you prepare the 1× MetALD-cocktail.

30. Prepare 1× MetALD-cocktail following the composition below (Table 5). Add the reagents to the basal medium.

**Table 4. Composition of 2× MetALD-cocktail**

| Reagent | Stock concentration | 2× MetALD-cocktail concentration |
|---|---|---|
| OA : PA[6] | 3 mM : 3 mM | 320 μM : 320 μM |
| Fructose[7] | 1 M | 20 mM |
| Glucose | 0.55 M | 11 mM |
| LPS[7] | 5 mg/mL | 4 μg/mL |
| TGF-β1 | 25 μg/mL | 2 ng/mL |
| Ethanol | 10 M | 200 mM |

31. Add 100 μL of 1× MetALD-cocktail to the 96-well ULA plate using a pipetting reservoir and multichannel pipette.

### Fibrosis regression in human liver spheroids

Timing: 1 h (Day 15, 17, and 19)

This step is designed to mimic regression of liver fibrosis. Removal of the etiological cause of injury often results in regression of liver fibrosis in patients and experimental models of liver fibrosis.[9] Regression of liver injury, steatosis, inflammation, and fibrosis can be achieved in human liver spheroids upon exchanging MASH- (or MetALD-) cocktail to growth medium for 7 days (between days 14–21). The growth medium has to be changed every 2 days (specifically, days 15, 17, and 19 of spheroid formation).

32. Remove 100 μL of medium from the 96-well ULA plate using a multichannel pipette, leaving 100 μL in the well.

⚠ CRITICAL: Do not touch the bottom of the 96-well ULA plate, as the spheroids are located at the bottom of each well.

*Note:* Spheroids are kept in a 37°C, 5% $CO_2$ incubator while you prepare the growth medium.

33. Add 100 μL of growth medium to the 96-well ULA plate using a pipetting reservoir and multichannel pipette.

*Note:* Regression is achieved by the gradual dilution of MASH- (or MetALD-) cocktail in growth medium over the period of three days. Gradual dilution of MASH-cocktail minimizes the shear and cellular stress on the spheroids.

### Collection of the liver spheroids

Timing: 1 h (Day 14 or 21)

**Table 5. Composition of 1× MetALD-cocktail**

| Reagent | Stock concentration | 1× MetALD-cocktail concentration |
|---|---|---|
| OA : PA | 3 mM : 3 mM | 160 μM : 160 μM |
| Fructose | 1 M | 10 mM |
| Glucose | 0.55 M | 5.5 mM |
| LPS | 5 mg/mL | 2 μg/mL |
| TGF-β1 | 25 μg/mL | 1 ng/mL |
| Ethanol | 10 M | 100 mM |

On day 14 (normal or MASH-induced spheroids) or day 21 (fibrosis regression-induced spheroids), human liver spheroids are collected for analysis.

*Note:* Human liver spheroids can be maintained in a 96-well ULA plate for a maximum of one month. It is not recommended to passage them to a new plate.

34. Harvest the spheroids by gently transferring them with a 200 μL pipette into 1.5 mL Eppendorf tubes.

*Note:* Collect 16 spheroids per condition for real-time qPCR analysis, and 48 spheroids per condition for Western blotting or immunofluorescent staining.

⚠ CRITICAL: Place the tube in a rack and wait for 1 min until all the spheroids settle to the bottom of the tube by gravity.

35. Remove the supernatant from the Eppendorf tube by pipetting and add 1 mL of PBS to remove residual medium.

⚠ CRITICAL: Place the tube in a rack and wait for 1 min until all the spheroids settle to the bottom of the tube by gravity. Make sure not to disturb the spheroids at the bottom.

36. Remove the PBS and lyse the spheroids (qRT-PCR and western blot analysis) with lysis buffer (Buffer RLT Plus and RIPA lysis buffer) or fix the spheroids (immunofluorescent and BODIPY staining) with 4% paraformaldehyde for further analysis.[10]
37. Collect 100 μL of the medium from each well by pipetting for the biochemical analysis of the supernatant.

*Note:* Do not touch the bottom of the 96-well ULA plate.

## EXPECTED OUTCOMES

### Characterization of normal human liver spheroid composition and function

Co-cultured hepatocytes, NPCs, and HSCs in growth medium spontaneously develop 3D normal human liver spheroids. Normal human liver spheroids are characterized at days 7, 9, 11 and 14. Bright-field microscopy demonstrates that human liver spheroids exhibit round shape and robust structural integrity (Figure 1B). To determine cellular composition, liver spheroids are formalin fixed and OCT embedded, and analyzed by immunocytochemistry. The number of hepatocytes (visualized by immunostaining for HNF4α), HSCs (Vimentin) myeloid cells (CD68), natural killer cells (NCR1), and endothelial cells (PECAM) are monitored at day 14 (Figures 2A and 2B). To assess the viability of normal human liver spheroids, growth medium is collected on days 7, 9, 11, and 14. Medium is then analyzed using ATP-based Cell-Titer Glo assays, which measures the amount of ATP. Increased amount of ATP correlates with the number of viable cells (Figure 2C). Human liver spheroids remain viable until day 14, as shown by the lack of ATP in the supernatant. Lysed spheroids served as a positive control for the ATP content from cells. Normal liver spheroids maintain normal function, as shown by sustained secretion of urea and albumin to the growth medium (Figures 2D and 2E). The protein expression of albumin and mRNA expression of hepatocyte markers *CK18*, *HNF4α*, *RBP4*, and *CYP2E1* (with the exception of *CYP3A4*) in human 3D liver spheroids (HLS) are similar to those observed in human primary plated hepatocytes (2D) cultured for one day. In particular, the mRNA expression of CYP3A4 increased in a time-dependent manner, as HSCs could promote liver sinusoidal structure development and enhance the activity of CYP3A4[11] (Figures 2F and 2G).

### Characterization of the human liver spheroids with MASH

Development of metabolic injury is assessed in human liver spheroids cultured in MASH-cocktail (between days 7–14 post spheroid formation vs. normal spheroids), and is associated with

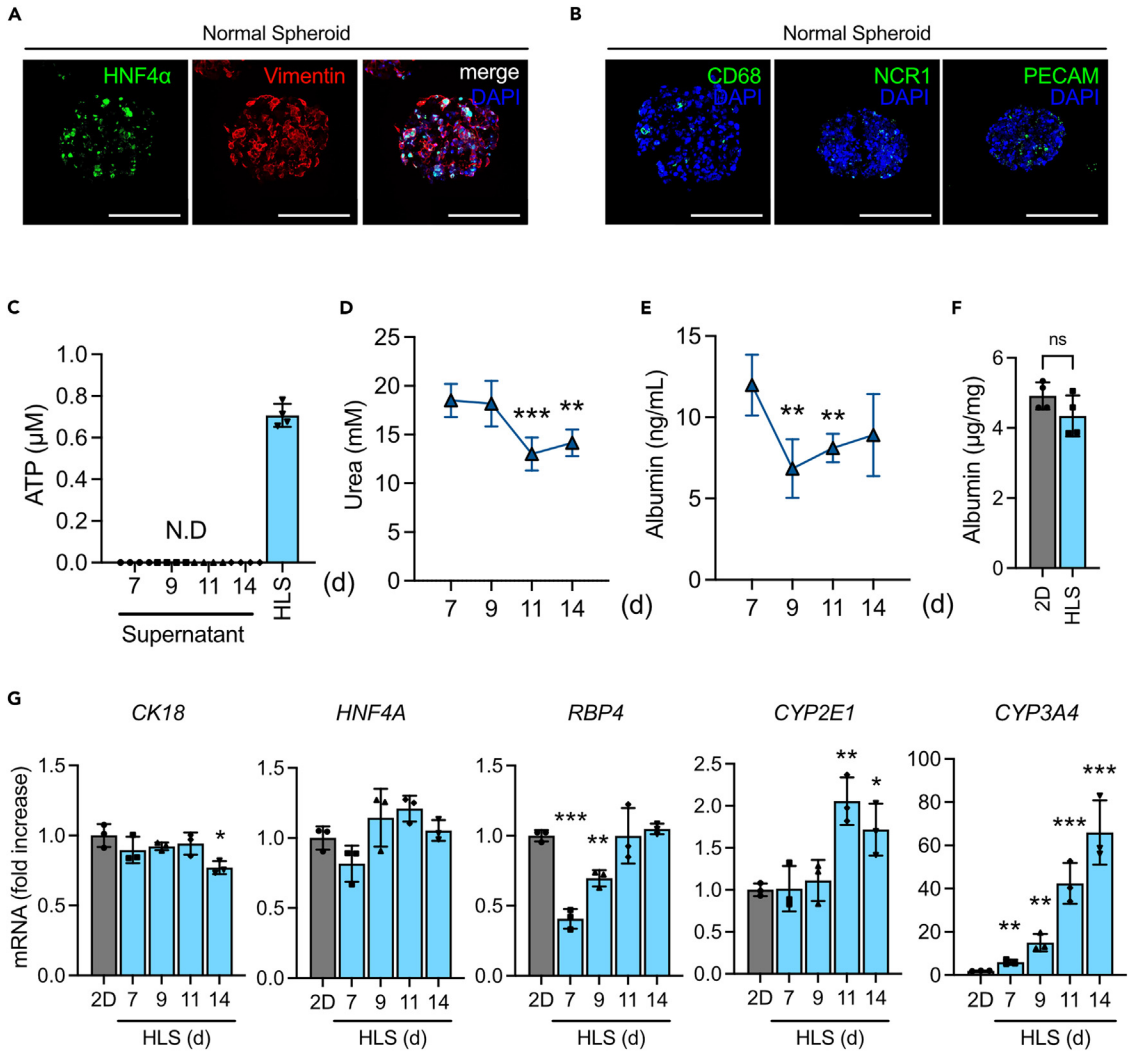


**Figure 2. Characterization of normal human liver spheroids**

Immunofluorescent images of spheroids stained for (A) Hepatocyte nuclear factor 4α (HNF4α), Vimentin and DAPI; or (B) CD68, NCR1, PECAM, and DAPI (scale bar = 250 μm).

(C) ATP and (D) urea levels in the supernatant of human liver spheroids. N.D., not detected, HLS, human liver spheroids. Quantification of (E) albumin secretion on days 7, 9, 11, 14. Data are the mean ± SD ($n$ = 4) and analyzed by Student's $t$-test compared to the supernatant from day 7. (F) Albumin production in human liver spheroids (spheroid lysis on day 14) is compared with primary human hepatocytes cultured for one day (normalized by total protein level). Data are the mean ± SD ($n$ = 4) and analyzed by Student's $t$-test compared to 2D-cultured primary human hepatocyte (2D); HLS, human liver spheroids.

(G) mRNA expression levels of the hepatocyte marker in primary human hepatocytes (2D) and human liver spheroids (HLS). Spheroids are assessed on days 7, 9, 11, 14. Data are the mean ± SD ($n$ = 3) and analyzed by Student's $t$-test compared to 2D, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

hepatic steatosis, inflammation, and fibrosis. MASH-induced human liver spheroids upregulate lipid accumulation (BODIPY-positive staining, Figures 3A and 3B), expression of free fatty acid-regulating enzymes *ACSL4* and *CPT1A* (Figure 3C), and increase production of inflammatory chemokines *CXCL1*, *IL6*, *IL8*, and *CCL5* mRNA (Figure 3D), causing activation of fibrogenic responses in HSCs. The number of DESMIN$^+$ COL1A1$^+$ HSCs is strongly increased in MASH human liver spheroids, and correlates with upregulation of fibrogenic genes *ACTA2*, *COL1A1*, *COL1A2*, *SERPINE1*, *TGFBR1* mRNA and protein expression (Figures 3E–3G). Moreover, increased secretion of Collagen Type I indicates fibrosis development in MASH human liver spheroids[12] (Figure 3H).

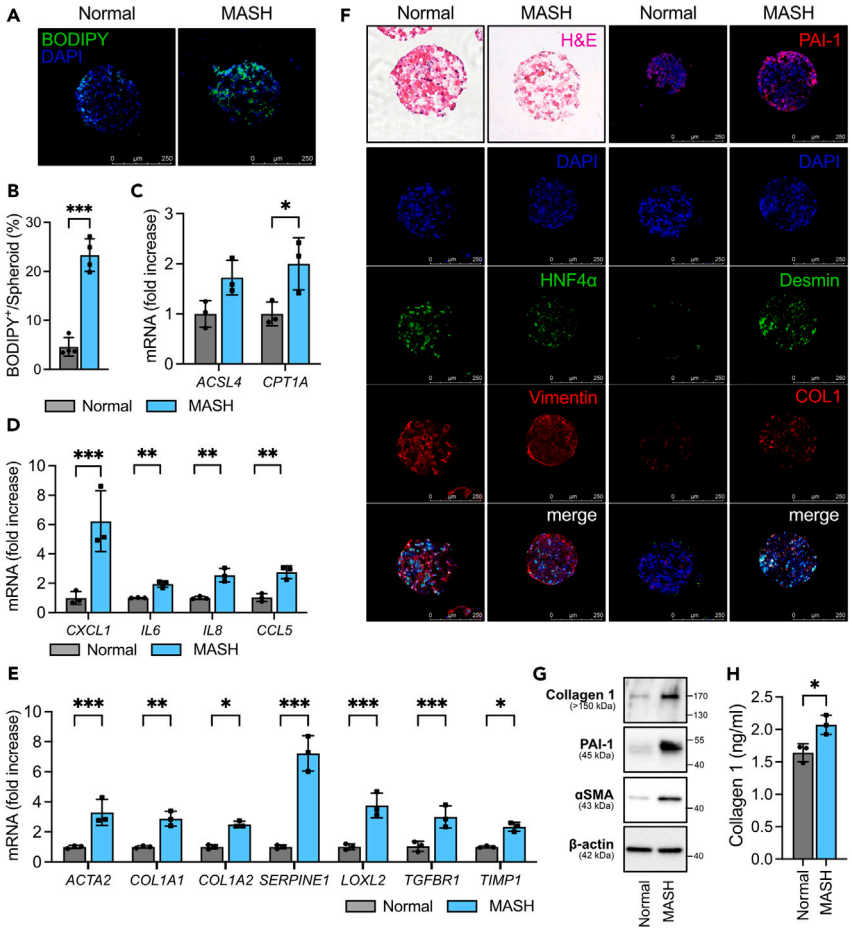


**Figure 3. Characterization of MASH-induced human liver spheroids**

(A) Micrographs represent human liver spheroids stained with BODIPY and DAPI (scale bar = 250 μm).

(B) BODIPY-positive area is normalized by spheroid area. Expression of (C) fatty acid-modifying enzymes, (D) inflammatory, or (E) fibrogenic genes in MASH-induced human liver spheroids (vs. normal human liver spheroids).

(F) H&E and immunofluorescent images of spheroids stained for hepatocyte nuclear factor 4α (HNF4α), Vimentin, PAI-1, Desmin, Collagen Type 1 or DAPI (scale bar = 250 μm).

(G) Protein expression of fibrogenic markers in MASH-induced human liver spheroids detected by Western blotting.

(H) Collagen Type I secretion from MASH-induced human liver spheroids. Data are the mean ± SD ($n$ = 3 or 4) and analyzed by Student's $t$-test compared to control, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

## Characterization of the human liver spheroids with MetALD

The development of MetALD is induced in human liver spheroids by culturing in MASH-cocktail supplemented with 100 mM of ethanol (between days 7–14 post spheroid formation). As expected, exposure to ethanol strongly accelerates expression of fibrogenic genes (*SERPINE1* and *TIMP1*) and proteins (Collagen Type I, αSMA), and hepatocyte injury (*CYP2E1*) in MetALD (vs. MASH) human liver spheroids (Figures 4A and 4B), suggesting that 3D human liver spheroids serve as a model to study MetALD.

## Regression of MASH-induced fibrosis in human liver spheroids

Cessation of metabolic liver injury often results in regression of liver fibrosis and inactivation of HSCs, which is associated with downregulation of fibrogenic gene expression and upregulation of some but not all quiescent-associated genes.[9] Here we test if MASH-induced aHSCs can inactivate. For this purpose, the MASH-cocktail is exchanged for growth medium on day 14, and the MASH-injured human liver spheroids continue to incubate in growth medium which does not support metabolic

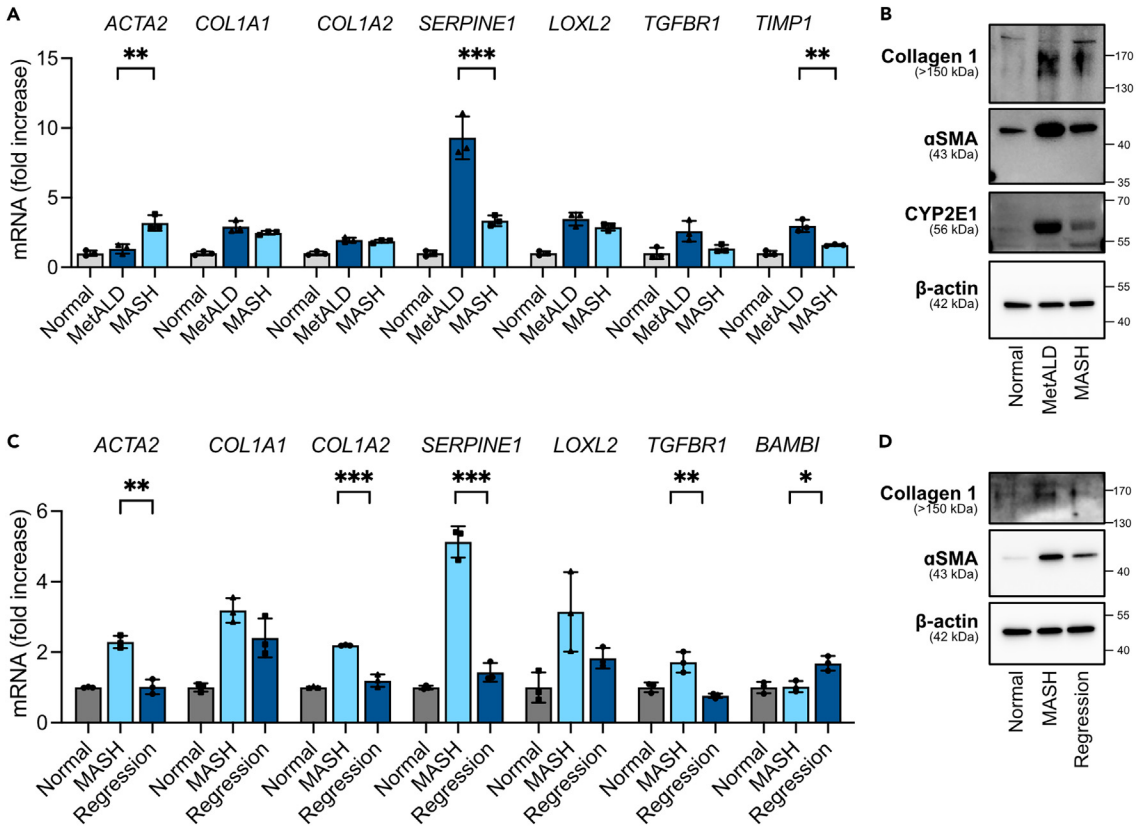


**Figure 4. MetALD induction and regression of MASH-induced fibrosis in human liver spheroids**

Expression of fibrogenic markers in the MASH- or MetALD-induced human liver spheroids is evaluated using (A) qRT-PCR analysis, or (B) Western blotting. Regression of MASH fibrosis is evaluated by expression of fibrogenic genes and proteins in the MASH and regression-induced human liver spheroids using (C) qRT-PCR analysis, or (D) Western blotting. Data are the mean ± SD ($n = 3$) and analyzed by Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

and fibrogenic stimulation (between days 14–21 post metabolic injury). The expression of fibrogenic genes (*ACTA2*, *COL1A1*, *COL1A2*, *SERPINE1*, *LOXL2*, and *TGFBR1*) rapidly decreases in human liver spheroids upon cessation of metabolic injury, while HSC-specific inactivation marker *BAMBI* is upregulated (Figure 4C). We also observed fibrosis resolution, as shown by suppressed expression of Collagen Type I and αSMA protein, indicating that activated human HSCs can inactivate in our spheroid system (Figure 4D). Human liver spheroids may become a model of choice to study inactivation of HSCs and regression of metabolic liver injury.

## Applications

The human liver spheroids serve as a useful tool to study the pathogenesis of metabolic liver diseases or responses to treatment in 3D "human livers in a dish", which are advantageous over 2D liver cell cultures or cultured liver slices.[13,14] The role of selected genes in specific cell populations can be studied in human liver spheroids using dsiRNA-knockdown approach. Gene knockdown can be achieved by transfection of human hepatocytes, HSCs, or myeloid cells with gene-targeting dsiRNA or control (dsi-negative control) siRNA prior to spheroid formation.[15] Transfected cells are then used for the formation human liver spheroids ± MASH-cocktail to study cell-specific functions of selected genes. For example, transfection of human HSCs with TGFβR1-targeting siRNA significantly (90%) downregulate expression of *TGFBR1* mRNA and suppresses the development of liver fibrosis in MASH human liver spheroids, as shown by downregulation of fibrogenic genes (*ACTA2*, *COL1A1*, *COL1A2*, *SEPRINE1*, *TIMP1*, *TGFB1*, and *LOXL2*) and protein expression (PAI-1 and αSMA) (Figures 5A and 5B).

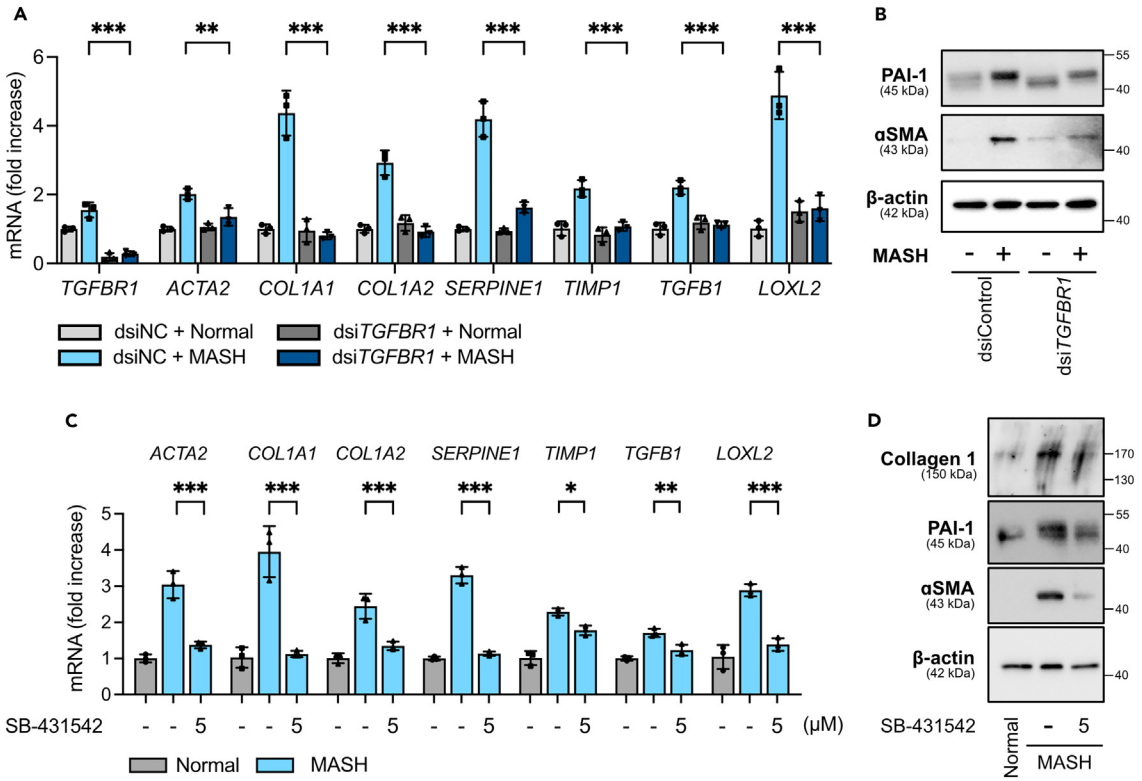


**Figure 5. Use of human liver spheroids for translational research**

Normal and MASH-induced liver spheroids containing dsiNC- or dsiTGFBR1-transfected HSCs are assessed using (A) qRT-PCR, and (B) Western blotting; dsiNC, dsi-negative control. The effect of ALK5 inhibitor SB431542 (5 μM) on expression of fibrogenic genes is tested in MASH (vs. normal) liver spheroids using (C) qRT-PCR, and (D) Western blotting. Data are the mean ± SD ($n$ = 3) and analyzed by one-way ANOVA followed by Tukey's test, *$p$ < 0.05, **$p$ < 0.01, and ***$p$ < 0.001.

Alternatively, human liver spheroids can be successfully used in various platforms for drug and therapeutic target screening for the liver diseases. MASH- or MetALD-induced human liver spheroids can be treated with potential inhibitors (days 7–14 post spheroid formation) to study the development and outcome of metabolic liver injury. This approach is suitable for high-throughput screening of therapeutic agents. For example, treatment with ALK5 inhibitor (SB-431542, a selective inhibitor of the TGF-β1 receptor) reduces expression of fibrogenic markers (*ACTA2*, *COL1A1*, *COL1A2*, *SEPRINE1*, *TIMP1*, *TGFB1* and *LOXL2*) and proteins (Collagen Type I, PAI-1, and αSMA) in MASH-induced human liver spheroids (Figures 5C and 5D).

## Advantage

Animal models do not recapitulate the development of metabolic injury in patients with MASH or MetALD. Recent guidelines from the Food and Drug Administration Modernization (Act 2.0) permitted to substitute animal studies with human organoids for drug development and screening.[16] Thus, generation of human multicellular liver spheroids, which are composed by all liver cell types, provides an alternative method for assessment of drug efficacy in human liver cells. Human liver spheroids can be designed based on the disease-specific experimental needs. Fully differentiated adult human primary hepatocytes and other liver cells are routinely used for generation of human liver spheroids. Some liver diseases can be better recapitulated in human liver spheroids composed of patient-derived pluripotent stem cells, or embryonic stem cells.[17–19] They have been used for modeling liver disease and testing drug efficacy and toxicity.[13,18,20] Moreover, hepatocyte-like cells can be differentiated from the precursor cells (blood cells, fetal liver cells, or embryonic stem cells) and used for human liver organoid systems.[17] While these hepatocyte-like cells

express mature hepatocyte markers, such as cytochrome P450 and albumin, they often maintain some hepatoblast-like characteristics, such as the expression of α-fetoprotein.[17]

## LIMITATIONS

Spheroids generated from distinct donors exhibit variability, including differences in the induction of fibrosis and the response to stimuli, such as production of free fatty acids and secretion of TGF-β1. These donor-specific variations may be associated with different donor SNPs (single nucleotide polymorphisms) and genotypes. For example, accelerated development of MASH in human liver spheroids can be associated with SNPs in genes known as "genetic risk factors" for MASH (PNPLA3, TM6SF2[21]). Therefore, assessment of donor-specific SNPs can be helpful in selection of donor liver cells to improve experimental outcome. Conversely, using liver cells from donors with known genetic risk factors may provide the opportunity to further develop precision medicine for liver diseases.[13] Although unlikely, the generation of spheroids using hepatic cells obtained from different donors may lead to unexpected immune responses. The formation of spheroids using liver cells from the same donor can prevent this potential pitfall. Limited availability of human livers minimizes the number of vendors that are able to provide freshly isolated and cryopreserved liver cells of high quality, particularly important for cryopreserved hepatocytes. Cell quality and viability of human liver cells should be tested prior to spheroid formation. In our experience only a limited number of frozen hepatocyte preparations can generate spheroids.

## TROUBLESHOOTING

### Problem 1

Differences in size of the spheroids upon formation (related to step 7).

### Potential solution

When seeding liver cells into the ULA plate, if the hepatic cells in the suspension are not distributed homogeneously, it will lead to variations in size. It is highly recommended to use a pipetting reservoir and a multichannel pipette for a consistent distribution of hepatic cells across the wells. Rock the pipetting reservoir for a homogeneous cell suspension, especially multiple plates are prepared simultaneously.

### Problem 2

Poor aggregation of the hepatic cells into spheroids (related to steps 10–16).

### Potential solution

The number and quality of the hepatic cells are crucial factors for generating compact human liver spheroids.

- Optimize the number of hepatic cells per well, which often involves increasing the cell number.
- Make sure to use primary hepatocytes with high viability. While freshly isolated hepatocytes are difficult to obtain, they serve as a best source for spheroid formation.
- It is required to shake the plate on day 1 and 2 to promote cell aggregation and facilitate spheroid formation.

### Problem 3

Unsuccessful induction of lipid accumulation and fibrogenic gene expression, as determined by BODIPY staining and qRT-PCR analysis (related to steps 19–24).

### Potential solution

- Components of the MASH-cocktail have a limited shelf life. It is strongly recommended to check the storage conditions and use freshly prepared solutions.

- Increase TGF-β1 concentration for fibrogenic gene expression or OA concentration for lipid accumulation.

### Problem 4

Contamination of the spheroids

### Potential solution

Spheroids are vulnerable to contaminations as they require frequent media change. Make sure to use sterile tips and reservoirs for the experiment.

### Problem 5

Loss of spheroids while washing the collected spheroids with PBS (related to step 35).

### Potential solution

A centrifugation step at low speed may help settle the spheroids and make complete PBS removal easier. However, centrifugation is not recommended for the spheroids collected for immunofluorescent imaging, as it can affect spheroid morphology.

### Problem 6

Poor yield of protein or mRNA from the spheroids (related to step 36).

### Potential solution

Spheroids need to be fully dissociated and lysed to extract protein or mRNA for further analysis (qRT-PCR and western blot analysis). Vigorously vortex the lysis buffer containing the spheroids until they are completely dissociated and no longer visible.

## RESOURCE AVAILABILITY

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### Materials availability

Not applicable.

### Data and code availability

Not applicable.

## ACKNOWLEDGMENTS

This research was supported by the National Institutes of Health R01DK111866, R56DK088837, DK099205, AA028550, DK101737, AA011999, DK120515, AA029019, DK091183, P42ES010337, R44DK115242 (T.K. and D.A.B.), and R01CA285997 (D.A.B.) and by Sanford Stem Cell Fitness and Space Medicine Center at Sanford Stem Cell Institute (UCSD) (T.K.). H.Y.K. was supported by basic science research program through the National Research Foundation of Korea RS-2023-00245179.

We thank Lifesharing OPO and UCSD microscopy core (NINDS P30NS047101) for their support. The authors used BioRender.com to create the illustrations.

## AUTHOR CONTRIBUTIONS

H.Y.K. and W.L. developed the protocols and wrote the manuscript; X.L., H.J., S.S., R.C.G.W., S.A.K., and C.M.M. worked on the protocol development and cell analysis; K.D. worked on

technique description and wrote the manuscript; and T.K. and D.A.B. provided supervision and funds and wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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