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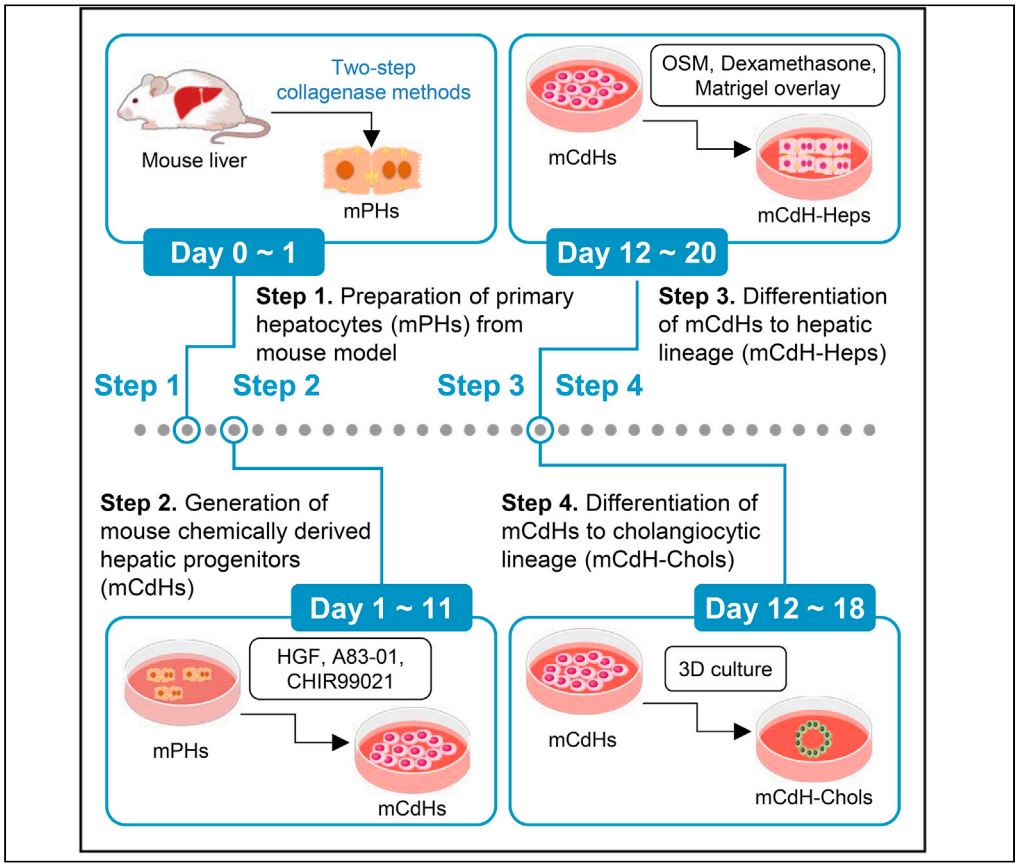
Generation and differentiation of chemically derived hepatic progenitors from mouse primary hepatocytes



This protocol describes the generation of bipotent chemically derived hepatic progenitors (mCdHs) from mouse primary hepatocytes and their subsequent differentiation into either hepatic or cholangiocytic lineages. The reprogrammed mCdHs have a high proliferation capacity and express progenitor markers in long-term passages. Differentiated mCdHs show the characteristics of either hepatic or cholangiocytic genes. This protocol has potential application for regenerative medicine, including *ex vivo* gene therapy, disease modeling, drug screening, and personalized medicine.

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Highlights

Generation of bipotent hepatic progenitors from mouse primary hepatocytes

Long-term maintenance of hepatic progenitor cells

Bi-potent differentiation capacity into hepatic or cholangiocytic lineage

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Generation and differentiation of chemically derived hepatic progenitors from mouse primary hepatocytes

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SUMMARY

This protocol describes the generation of bipotent chemically derived hepatic progenitors (mCdHs) from mouse primary hepatocytes and their subsequent differentiation into either hepatic or cholangiocytic lineages. The reprogrammed mCdHs have a high proliferation capacity and express progenitor markers in long-term passages. Differentiated mCdHs show the characteristics of either hepatic or cholangiocytic genes. This protocol has potential application for regenerative medicine, including *ex vivo* gene therapy, disease modeling, drug screening, and personalized medicine.

For complete details on the use and execution of this protocol, please refer to Kim et al. (2021).

BEFORE YOU BEGIN

Before following these methods, prepare the materials referred in the key resources table.

Preparation of collagen-coated plates, reprogramming medium, and differentiation media

Timing: 1 h to 1 day

Note: recipe tables are provided in the "materials and equipment" section.

1. Collagen-coated plates
   a. Dilute the collagen solution to 250 μg/mL in ice-cold 0.1% acetic acid.
   b. Add to the plate at a concentration of 10 μg/cm^2 and coat by spreading the collagen solution over the surface of the plate.
   c. Incubate for 1 h at 37°C or overnight (10–16 h) at 4°C.
2. Reprogramming medium (for primary hepatocytes in general)
   a. Add 1% fetal bovine serum (FBS), 1× insulin-transferrin-selenium, 0.1 μM dexamethasone, 10 mM nicotinamide, 50 μM β-mercaptoethanol, 20 ng/mL epidermal growth factor (EGF), 20 ng/mL hepatocyte growth factor (HGF), 4 μM A83-01, 3 μM CHIR99021, and 1% penicillin/streptomycin to DMEM/F-12 medium.
   b. Filter the medium through 0.45 μm cellulose acetate membranes. Store for up to 1 month at 4°C.
3. Hepatic differentiation medium (for re-differentiating general hepatocytes into hepatic lineage)
   a. Add 20 ng/mL oncostatin M and 10 μM dexamethasone to the reprogramming medium. Store for up to 1 month at 4°C.

4. Cholangiocytic differentiation medium
   a. Add 10% FBS, 20 ng/mL HGF, and 1% penicillin/streptomycin to DMEM/F-12 medium. Store for up to 1 month at 4°C.

Isolation of primary hepatocytes from mouse liver

Timing: 1–2 h

5. Prepare the perfusion solutions and medium
   a. Solution A: 0.19 g/L EDTA, 8 g/L NaCl, 0.4 g/L KCl, 0.078 g/L $NaH_2PO4 \cdot 2H_2O$, 0.151 g/L $Na_2HPO_4 \cdot 12H_2O$, and 0.19 g/L HEPES in 1 L sterilized tertiary distilled water. Adjust the pH to 7.4. Store for up to 1 month at 4°C.
   b. Solution B: 0.3 g/L collagenase, 0.56 g/L $CaCl_2$, 8 g/L NaCl, 0.4 g/L KCl, 0.078 g/L $NaH_2PO_4 \cdot 2H_2O$, 0.151 g/L $Na_2HPO4 \cdot 12H_2O$, and 0.19 g/L HEPES in 1 L sterilized tertiary distilled water. Adjust the pH to 7.4. Store for up to 1 month at 4°C.

Reagent	Solution A	Solution B
NaCl	8 g/L	8 g/L
KCl	0.4 g/L	0.4 g/L
$NaH_2PO4 \cdot 2H_2O$	0.078 g/L	0.078 g/L
$Na_2HPO4 \cdot 12H_2O$	0.151 g/L	0.151 g/L
HEPES	0.19 g/L	0.19 g/L
EDTA	0.19 g/L	N/A
Collagenase	N/A	0.3 g/L
$CaCl_2$	N/A	0.56 g/L
Sterilized tertiary distilled water	Up to 1L	Up to 1L

   c. Hepatocyte culture medium: Add the Primary Hepatocyte Maintenance Supplements and 1% penicillin/streptomycin to 500 mL of Williams' medium E.

6. The animal experiments are performed with permission of the IACUC (Institutional Animal Care and Use Committee). Anesthetize the 6–8 weeks-old mice (both male and female) with 2%–3% isoflurane and 4% O_2.
7. Make a U-shaped abdominal incision and find the portal vein.
8. Insert the 24G intravenous catheter into the portal vein and fasten the catheter in place with surgical thread.
9. Inject Solution A at a rate of 6 mL/min for 5 min at 37°C and cut the inferior vena cava (IVC).
10. Inject Solution B at a rate of 6 mL/min for 8 min at 37°C and extract the liver carefully.
11. Chop the liver with 50 mL of ice-cold Williams' medium E without Primary Hepatocyte Maintenance Supplements on a petri dish and filter with a 100 μm cell strainer.
12. Wash the filtrate by centrifugation at 50 g for 5 min at 4°C. Discard the supernatant and resuspend the pellet by adding 50 mL of ice-cold Williams' medium E. Repeat this step twice.
13. Discard the supernatant, add 50 mL of ice-cold 25% Percoll solution (diluted with DPBS), and centrifuge at 50 g for 5 min at 4°C without brake.
14. Discard the supernatant, add 5–10 mL of the hepatocyte culture medium with 1% penicillin/streptomycin to resuspend the pellet, and count the cell number. (The pellet consists of primary hepatocytes.)
15. Seed the cells in collagen-coated plates at 2,000 cells/$cm^2$for further studies and incubate in a humidified atmosphere containing 5% CO_2 at 37°C (Figure 1A).

Note: Collagenase can be used interchangeably with other collagenases (type 1+2, type 4, type H) etc.

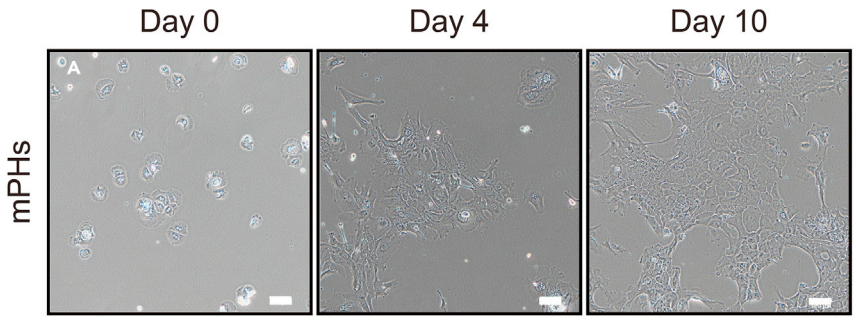

Figure 1. Generation of mouse chemically derived hepatic progenitors (mCdHs)

Morphological changes during the generation of mCdHs at day 0, 4, and 10 from adult mouse primary hepatocytes. Scale bars, 100 μm.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-Albumin (1:100 dilution)	Abcam	Cat#ab19194, RRID:AB_777886
Mouse anti-E-cadherin (1:100 dilution)	Abcam	Cat#ab76055, RRID:AB_1310159
Rabbit anti-Epcam (1:100 dilution)	Abcam	Cat#ab32392, RRID:AB_732181
Hoechst 33342 (1:10000 dilution)	Invitrogen	Cat#H3570
Mouse anti-Cytokeratin 19 (1:100 dilution)	Santa Cruz Biotechnology	Cat#sc-376126, RRID:AB_10988034
Rabbit anti-Sox9 (1:200 dilution)	Abcam	Cat#ab185966, RRID:AB_2728660
Donkey anti-Goat, Alexa Fluor 488 (1:250 dilution)	Thermo Fisher Scientific	Cat#A-11055, RRID:AB_2534102
Goat anti-Mouse, Alexa Fluor 488 (1:500 dilution)	Thermo Fisher Scientific	Cat#A-11001, RRID:AB_2534069
Donkey anti-Rabbit, Alexa Fluor 594 (1:500 dilution)	Thermo Fisher Scientific	Cat#A-21207, RRID:AB_141637
Chemicals, peptides, and recombinant proteins		
A83-01	Sigma-Aldrich	Cat#SML0788
Acetic acid	Fisher Scientific	Cat#984303
$CaCl_2$	Sigma-Aldrich	Cat#C1016
CHIR99021	Sigma-Aldrich	Cat#SML1046
Collagen Solution	STEMCELL Technologies	Cat#04902
Collagen type I	Gibco	Cat#A10483-01
Collagenase	Worthington Biochemical	Cat#LK002066
Dexamethasone	Sigma-Aldrich	Cat#1756
DMEM/F-12	Gibco	Cat#10565-018
DMSO	Sigma-Aldrich	Cat#D8418
DPBS	Welgene	Cat#LB001-02
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	Cat#E4884
Epidermal growth factor (EGF)	Peprotech	Cat#315-09
Fetal bovine serum	Gibco	Cat#16000-044
Gentamicin	Gibco	Cat#15710064
HEPES	Sigma-Aldrich	Cat#H3375
Hepatocyte growth factor (HGF)	PeproTech	Cat#100-39H
Insulin-transferrin-selenium	Gibco	Cat#51500056
Isoflurane	Piramal Critical Care	Cat#NDC66794-017
KCl	Duchefa Biochemie	Cat#P0515
Matrigel	Corning	Cat#356230
$Na_2HPO4 \cdot 12H_2O$	Sigma-Aldrich	Cat#71649
NaCl	Duchefa Biochemie	Cat#S0520
$NaH_2PO4 \cdot 2H_2O$	Sigma-Aldrich	Cat#71505
Nicotinamide	Sigma-Aldrich	Cat#N3376

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oncostatin M	Prospec	Cat#cyt-231
Penicillin/streptomycin	Gibco	Cat#15070-063
Percoll (pH 8.9)	Sigma-Aldrich	Cat#17-5445-02
Primary Hepatocyte Maintenance Supplements	Gibco	Cat#CM4000
TrypLE Express Enzyme	Gibco	Cat#12563029
TRIzol™ Reagent	Thermo Scientific	Cat# 15596018
Williams' medium E	Gibco	Cat#A12176-01
β-mercaptoethanol	Sigma-Aldrich	Cat#M3148
Critical commercial assays		
PAS stain kit	Abcam	Cat#ab150680
qPCR PreMix	Dyne Bio	Cat#DYRT1202
Experimental models: Organisms/strains		
Mouse: C57BL/6N (6–8 weeks)	Orient	N/A
Oligonucleotides		
Aat: Forward (AATGGAAGAAGCCATTCGAT); Reverse (AAGACTGTAACTGCTGCAGC)	Macrogen	N/A
Ae2: Forward (GACTCCTTTCCCTGTGTGGA); Reverse (GAAGCATCCGCTCTTTCTTG)	Macrogen	N/A
Alb: Forward (GGCTACAGCGGAGCAACTGA); Reverse (GCCTGAGAAGGTTGTGGTTGTG)	Macrogen	N/A
Asgr1: Forward (CAGCTCTGTGAGGCCTTGGA); Reverse (GGGCCCGTTCTGGTCAGTTA)	Macrogen	N/A
Aqpr1: Forward (CTGTGCGTTCTGGCTACCAC); Reverse (GCACAGCAGAGCCAAATGAC)	Macrogen	N/A
Aqpr9: Forward (CTCAGTCCCAGGCTCTTCAC); Reverse (TAAGACCTCCCAGGAAAGCA)	Macrogen	N/A
Cftr: Forward (GGTCATAGAGCAGGGCAATG); Reverse (TGCACTTCTTCCTCCGTCTC)	Macrogen	N/A
Cps1: Forward (TGAGACAGGCCAAAGAGATTGGGT); Reverse (TGCTCCTGGCCATTGTAGGTAACA)	Macrogen	N/A
Cyp1a2: Forward (AGGAGCTGGACACGGTGGTT); Reverse (AGGTGTCCCTCGTTGTGCTG)	Macrogen	N/A
Cyp2c9: Forward (TGACTTGTTTGGAGCTGGGACAGA); Reverse (GAAGCATCCGCTCTTTCTTG)	Macrogen	N/A
Fxr: Forward (TGTGAGGGCTGCAAAGGTT); Reverse (ACAGCATCTGTGTAGGGCATGT)	Macrogen	N/A
Grhl2: Forward (GTTCGATGCTCTGATGCTGA); Reverse (GCAGCCCGTACTTCTCAGAC)	Macrogen	N/A
Gapdh: Forward (CCAATGTGTCCGTCGTGGAT); Reverse (TTGCTGTTGAAGTCGCAGGAG)	Macrogen	N/A
Hnf4α: Forward (ATCGRCAAGCCTCCCTCTGC); Reverse (GACTGGTCCCTCGTGTCACATC)	Macrogen	N/A
Krt19: Forward (TTCCGGACCAAGTTTGAGAC); Reverse (CCTCGTGGTTCTTCTTCAGG)	Macrogen	N/A
Oct: Forward (TCCTGCTCAACAAGGCAGCTCTTA); Reverse (TCACGGCCTTTCAGCTGTACTTGA)	Macrogen	N/A
Ttr: Forward (AGTCCTGGATGCTGTCCGAG); Reverse (TTCCTGAGCTGCTAACACGG)	Macrogen	N/A
Software and algorithms		
GraphPad Prism 7	GraphPad Software	Version 7.04
Leica TCS SP5	Leica	https://www.leica-microsystems.com/products/confocal-microscopes/p/leica-tcs-sp5/
Other		
10 cm^2 plate	Corning	Cat#CLS430167
100 μm Cell strainer	Life Sciences	Cat#352360
6-well plate	Life Sciences	Cat#3516

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Freezing container	Thermo Scientific	Cat#5100-0001
I.V. Catheter	BD	Cat#382412
Surgical thread (Black Silk 6-0)	AILEE	Cat#SK617
Vacuum filter	Life Sciences	Cat#430770

MATERIALS AND EQUIPMENT

10 mL of collagen solution for collagen-coated plates

Reagent	Stock concentration	Final concentration	Amount
Collagen solution	3 mg/mL	250 μg/mL	830 μL
Acetic acid	0.1% acetic acid	N/A	9.15 mL
Total			**10 mL**

Note: Store up to 1 week at 4°C

500 mL of reprogramming medium

Reagent	Stock concentration	Final concentration	Amount
DMEM/F-12	N/A	N/A	480 mL
FBS	N/A	1%	5 mL
Insulin-transferrin-selenium	100×	1×	5 mL
Dexamethasone	25.5 mM	0.1 μM	1.96 μL
Nicotinamide	1 M	10 mM	5 mL
β-Mercaptoethanol	140 mM	50 μM	178.57 μL
EGF	100 μg/mL	20 ng/mL	100 μL
HGF	100 μg/mL	20 ng/mL	100 μL
A83-01	10 mM	4 μM	200 μL
CHIR99021	10 mM	3 μM	150 μL
Penicillin/streptomycin	N/A	1%	5 mL
Total			**500 mL**

Note: Store up to 1 month at 4°C

50 mL of hepatic differentiation medium

Reagent	Stock concentration	Final concentration	Amount
DMEM/F-12	N/A	N/A	48 mL
FBS	N/A	1%	500 μL
Insulin-transferrin-selenium	100×	1×	500 μL
Dexamethasone	25.5 mM	10 μM	19.6 μL
Nicotinamide	1 M	10 mM	500 μL
β-Mercaptoethanol	140 mM	50 μM	17.86 μL
EGF	100 μg/mL	20 ng/mL	10 μL
HGF	100 μg/mL	20 ng/mL	10 μL
A83-01	10 mM	4 μM	20 μL
CHIR99021	10 mM	3 μM	15 μL
Penicillin/streptomycin	N/A	1%	500 μL
Oncostatin M	100 μg/mL	20 ng/mL	10 μL
Total			**50 mL**

Note: Store up to 1 month at 4°C

50 mL of cholangiocytic differentiation medium			
Reagent	Stock concentration	Final concentration	Amount
DMEM/F-12	N/A	N/A	44.5 mL
FBS	N/A	10%	5 mL
HGF	100 μg/mL	20 ng/mL	10 μL
Penicillin/streptomycin	N/A	1%	500 μL
Total			50 mL

Note: Store up to 1 month at 4°C

STEP-BY-STEP METHOD DETAILS

Generation and maintenance of chemically derived hepatic progenitor cells

Timing: 7–10 days

This step describes the generation of CdHs from mouse primary hepatocytes (mPHs). The protocol was adapted from the generation method for human CdHs (Kim et al., 2019).

1. Resuspend the mPHs in hepatocyte culture medium. Seed the freshly isolated mPHs on a collagen-coated plate at 2,000 cells/cm^2 and incubate overnight (10–16 h) with 5% CO_2 at 37°C.
2. Change hepatocyte culture medium to the reprogramming medium. Change the reprogramming medium every two days.
3. Culture the cells until they reach 75%–80% confluence (Figure 1).

Note: Generation of CdHs is usually completed between 7 and 10 days. When cells reach 75%–80% confluence, move on to the passage stage.

⚠ CRITICAL: Confluence of CdHs greater than 75%–80% led to a decrease in cell proliferation capacity, passaging efficiency and differentiation capacity. Passage CdHs before the cells are over-confluent (Figure 1C).

4. Passage the cells at a ratio of 1:4
   a. Prewarm DPBS and TrypLE to room temperature (20°C–25°C). Prepare a collagen-coated plate, aspirate the collagen solution, wash the plate using DPBS, and add 7 mL of reprogramming medium (standard in 10 cm^2 plate).
   b. Aspirate the cultured medium and wash the cells with 2–3 mL DPBS.
   c. Add 2 mL TrypLE Express Enzyme and incubate for 5 min at 37°C.
   d. Detach the cells by gently pipetting and transfer to a 15 mL tube with 7 mL of reprogramming medium.
   e. Centrifuge at 200 *g* for 5 min at 4°C and aspirate the supernatant.
   f. Resuspend the pellet with 4 mL reprogramming medium and seed 1 mL of cells into a prepared 10 cm^2 plate.
   g. Incubate in a humidified atmosphere with 5% CO_2 at 37°C.
5. Cryo-preservation
   a. Count 1 × 10^5 cells and add the reprogramming medium until the total volume is 900 μL.
   b. Add 100 μL DMSO and transfer each sample to a cryo-tube.

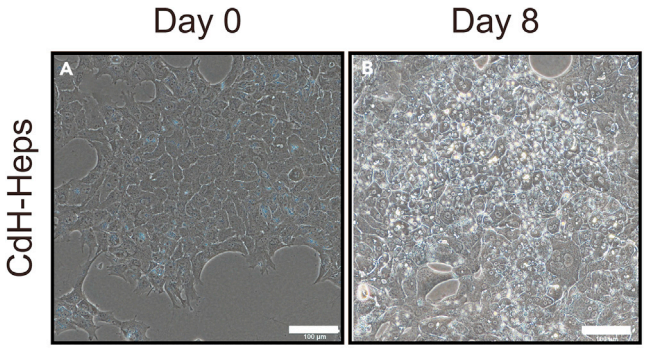

Figure 2. Hepatic differentiation of mouse chemically derived hepatic progenitors (mCdHs)
Morphological changes during the hepatic differentiation of mCdHs at day 0 (A) and 8 (B). Scale bars, 100 μm.

c. Place the cryo-tubes into a freezing container and store at −80°C deep freezer.

Note: For longer storage, transfer samples to liquid nitrogen after one day

6. Thawing frozen CdHs
   a. Prewarm the reprogramming medium and water bath. Prepare a collagen-coated plate, aspirate the collagen solution, wash the plate using DPBS, and add 7 mL of reprogramming medium (standard in 10 cm^2 plate).
   b. Thaw the frozen CdHs in a 37°C water bath and slowly transfer into a 15 mL tube with prewarmed 9 mL reprogramming medium.
   c. Centrifuge at 200 *g* for 5 min at 4°C and aspirate the supernatant.
   d. Resuspend the pellet in 1 mL reprogramming medium and seed the cells into a prepared 10 cm^2 collagen-coated plate.
   e. Incubate in a humidified atmosphere with 5% CO_2 at 37°C.

Note: Usually you can get the cells with 80%–90% viability after thawing. To increase the cell viability, change the medium after 1 day of thawing.

Hepatic differentiation

Timing: 9 days

This step describes the differentiation of CdHs into hepatocyte-like cells (CdH-Heps). The protocol was adapted from the hepatic differentiation method for human CdHs (Kim et al., 2019).

7. Prewarm the reprogramming medium and water bath set to 37°C. Prepare a collagen-coated plate, aspirate the collagen solution, wash the plate using DPBS, and add 1.5 mL of reprogramming medium (standard in 6-well plate).
8. Seed the CdHs on collagen-coated plates at 1,000 cells/cm^2 and incubate for 1 day in a humidified atmosphere with 5% CO_2 at 37°C.
9. After a 1-day incubation, change the reprogramming medium to the hepatic differentiation medium. The hepatic differentiation date begins at this point and is counted as day 0.
10. Change the medium every two days.
11. After 6 days, aspirate the culture medium and overlay the CdHs with the Matrigel mixture.
    a. Thaw the Matrigel in ice and prepare the ice-cold hepatic differentiation medium.
    b. Dilute the Matrigel with ice-cold hepatic differentiation medium at a 1:7 ratio on ice.
12. Incubate in a humidified atmosphere with 5% CO_2 at 37°C for 2 days (Figure 2).

Note: If differentiation is successfully completed, the differentiated cells will show the binuclear and bile canaliculi structures characteristic of hepatocytes.

Note: If you plan to conduct experiments such as measuring albumin secretion during the hepatic differentiation process, collect the conditioned medium—before changing the medium—on days 0, 2, 4, and 6. However, do not collect the medium on day 8 because it contains Matrigel.

13. Sampling for mRNA isolation.
    a. Gently add the ice-cold DMEM/F-12(1 mL / 1,000 cells) to the cells and incubate for 5 min at room temperature.
    b. Gently aspirate the Matrigel mixture using a pipette.

⚠ CRITICAL: Be careful not to aspirate the cells with Matrigel mixture.

    c. Gently add the ice-cold DPBS (1 mL / 1,000 cells) to the cells and aspirate it.
    d. Add 1 mL of Tryp/LE and incubate for 3 min at 37°C.
    e. Detach the cells from the collagen-coated plate and transfer to a 15 mL tube with 9 mL of ice-cold DMEM/F-12.
    f. Centrifuge at 200 *g* for 5 min at 4°C and aspirate the supernatant.

Note: After centrifugation, if there is still an excessive quantity of Matrigel, add ice-cold DPBS, resuspend the pellet, and centrifuge again.

    g. Isolate the mRNA using TRIzol (Rio et al., 2010).

14. Sampling for immunocytochemistry
    a. Gently add 1 mL of ice-cold DPBS to the cells and incubate for 5 min at room temperature.
    b. Gently aspirate the Matrigel mixture using a pipette.

⚠ CRITICAL: Be careful not to aspirate the cells with Matrigel mixture. If the Matrigel mixture remains, repeat steps 14a and 14b.

    c. Fixation with 4% paraformaldehyde, methanol, etc., according to the experimenter's immunocytochemistry protocol.
    d. Proceed to Immunocytochemistry.

Cholangiocytic differentiation

Timing: 7 days

This step describes the differentiation of CdHs into cholangiocyte-like cells (CdH-Chols). The protocol was adapted from the cholangiocytic differentiation method for human CdHs (Kim et al., 2019).

15. Harvest the CdHs using Tryp/LE in the same manner as the passaging method.
16. Resuspend the pellet with cholangiocytic differentiation medium at a density of 1×10^5 cells/well in a 6-well plate.

⚠ CRITICAL: Seeding with too high confluence makes it difficult for cells to form tubular-like structures.

17. Mix the cells with an equal volume of collagen type I (pH 7.0) on ice.

⚠ CRITICAL: If the mixture does not solidify, even after incubation for 30 min, the pH is not 7.0. Adjust collagen type I to pH 7.0, using NaOH or HCl, and mix with the cells.

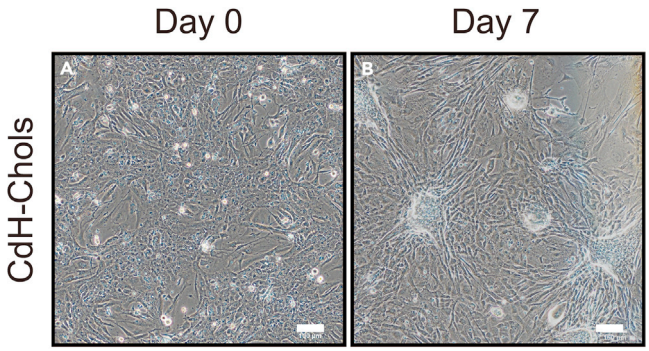

Figure 3. Cholangiocytic differentiation of mouse chemically derived hepatic progenitors (mCdHs)
Morphological changes during the cholangiocytic differentiation of mCdHs at day 0 (A) and 7 (B). Scale bars, 100 μm.

18. Incubate for 30 min at 37°C.
19. Gently add 1 mL of the prewarmed cholangiocytic differentiation medium.
20. Incubate for 7 days in a humidified atmosphere with 5% CO_2 at 37°C (Figure 3). Change the medium every two days using fresh cholangiocytic differentiation medium.

Note: If the differentiation process is done well, the cells form the tubular-like structure.

⚠ CRITICAL: Be careful not to aspirate the cells with the collagen mixture when changing the medium.

21. Sampling for mRNA isolation.
    a. Gently aspirate the cultured medium and wash with 1 mL DPBS.
    b. Aspirate the DPBS and add TRIzol or lysis buffer depending on the mRNA isolation methods.
22. Sampling for immunocytochemistry
    a. Gently aspirate the cultured medium and wash with 1 mL DPBS.
    b. Aspirate the DBPS gently.
    c. Fixation with 4% paraformaldehyde, methanol, etc., according to the experimenter's immunocytochemistry protocol.
    d. Proceed to Immunocytochemistry.

EXPECTED OUTCOMES

Primary hepatocytes cannot proliferate and be passaged *in vitro*. If the reprogramming of primary hepatocytes to chemically derived hepatic progenitors proceeded well following this protocol, the cells will have acquired proliferative capacity (Figures 4A and 4B) and the ability to express progenitor/stem cell-specific markers (Figure 4C). Mouse CdHs (mCdHs) stably proliferate, can be cloned, and maintain the expression of progenitor/stem cell-specific markers even at passage 20 or more (Kim et al., 2021). These characteristics of mCdHs suggest that they can be used for *ex vivo* gene therapy and for bioartificial liver research that requires many cells (Strain and Neuberger, 2002).

Progenitors can differentiate into their organs of fate; specifically, hepatic progenitors can differentiate into hepatocytes and biliary epithelial cells (Dolle et al., 2010; Kim et al., 2019; Seaberg and van der Kooy, 2003). Therefore, if mCdHs reprogrammed through this protocol produced hepatic progenitors, they would differentiate into hepatocyte- and cholangiocyte-like cells according to the method described in this paper (Figures 2 and 3). mCdHs that have undergone hepatocyte differentiation show increased expression of hepatocyte-specific markers including *Alb*, *Asgpr1*, *Hnf4α*, *Ttr*, and Cytochrome P450 (Figure 5A). Also, they show the functional characteristics of mature

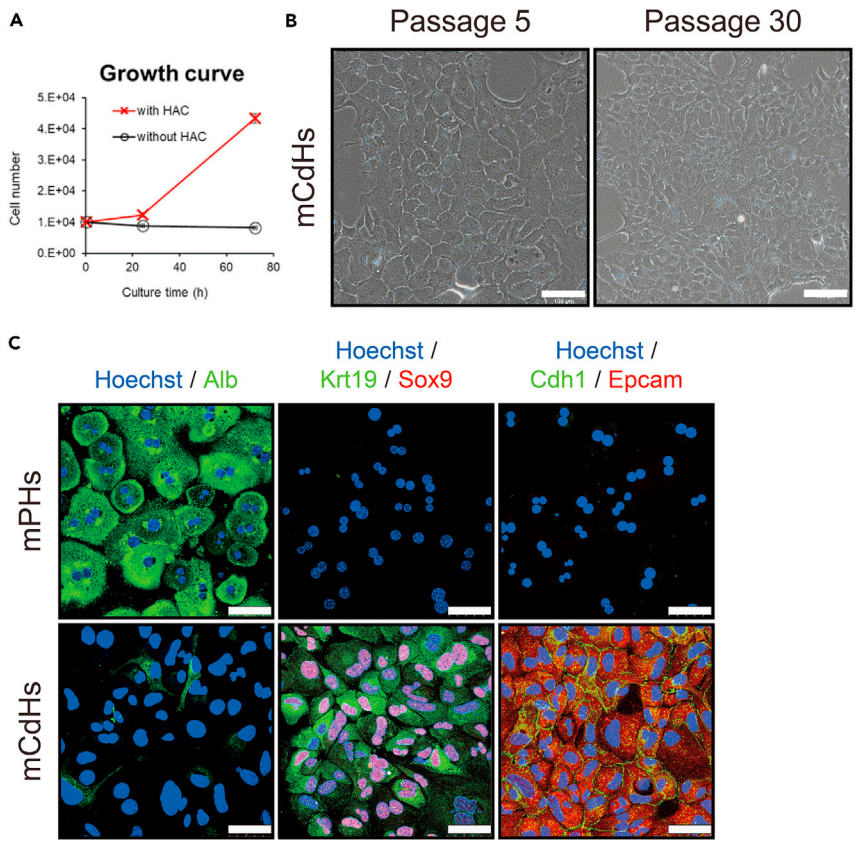

Figure 4. Characterization of mouse chemically derived hepatic progenitors (mCdHs)

(A) Generation curve of mCdHs derived from mPHs in the presence or absence of HAC (HGF, A83-01 and CHIR99021) for 72 h. Data are mean ± SD (n=3).

(B) Bright-field images of mCdHs at early (passage 5) and late (passage 30) passages. Scale bars, 100 μm.

(C) Immunofluorescence staining of mature hepatocyte marker (Alb) and hepatic progenitor markers (Krt19, Sox9, Cdh1, and Epcam). Nuclei were counterstained with Hoechst 33342. Scale bars, 50 μm.

hepatocytes, including albumin secretion, cytochrome P450 activity, PAS staining, and ICG uptake (Kim et al., 2021). In addition, mCdHs can stably differentiate into hepatocytes even in long-term passages, and their gene expression profiles are similar to those of primary hepatocytes after differentiation (Kim et al., 2021). mCdH-Chols, which are differentiated into cholangiocyte lineages, show the expression of cholangiocytic-specific markers (Figure 5B). Bipotent differentiation capacity confirmed that mCdHs can differentiate into both hepatocytes and biliary epithelial cells *in vivo* after transplantation into a liver injury mouse model (Kim et al., 2019).

LIMITATIONS

In this paper we have described the most efficient reprogramming and differentiation methods that we have developed. However, there are many different methods for differentiating hepatocyte- and cholangiocyte-like cells from stem cells (Aurich et al., 2009; Ogawa et al., 2015; Sampaziotis et al., 2017; Sancho-Bru et al., 2011). In the case of cholangiocyte differentiation, 2D and 3D differentiation methods exist, and various small molecules and growth factors are used. Cholangiocytes display a tubular formation *in vivo*. Our protocol also uses the 3D culture method, but studies to improve differentiation efficiency by treatment of cells with additional growth factors and/or small molecules as proposed in other research papers are also needed to be explored.

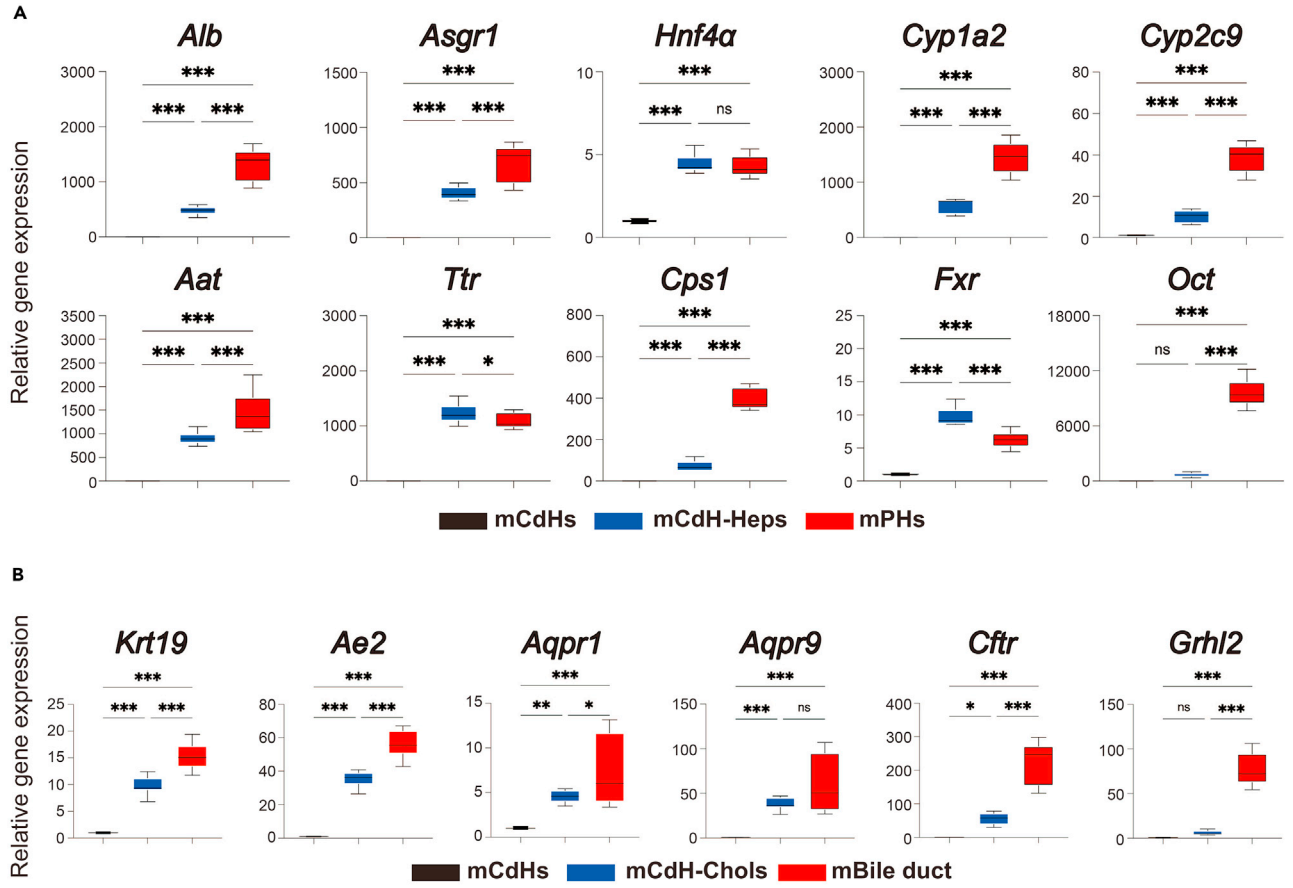

Figure 5. Bipotent differentiation capacity of mouse chemically derived hepatic progenitors (mCdHs)

Gene expression levels of (A) mature hepatocyte-specific markers and (B) cholangiocyte-specific markers in mCdHs, differentiated mCdHs and positive control determined by RT-qPCR. mPHs and mouse bile duct were used as positive control, respectively. Gapdh was used as an internal control. Data are mean ± SD (n=9). Data were analyzed by one-way ANOVA with post-hoc (Tukey), *p < 0.05, **p < 0.01, and ***p <0.001.

TROUBLESHOOTING

Problem 1

At step 3, if the cells are continuously cultured beyond 75%–80% confluence, the cells will continue to divide, the cell size will tend to decrease, and finally the cells will detach from the plate (Figure 6).

Potential solution

Passaging should be performed before the confluence reaches 80%–90%. If it will be a long period of time before the cells are needed, it is recommended to freeze the cells and thaw again when needed.

Problem 2

At steps 13 and 14, if the Matrigel mixture is aspirated without completely dissolving, the cells may be aspirated along with the Matrigel mixture.

Potential solution

When aspirating the Matrigel mixture or adding ice-cold DMEM/F-12 or DPBS, carefully aspirate and add along the wall of the plate. In addition, if the Matrigel mixture is not completely dissolved, dissolve the Matrigel mixture as much as possible by repeating the aspiration and addition of ice-cold DMEM/F-12 or DPBS steps.

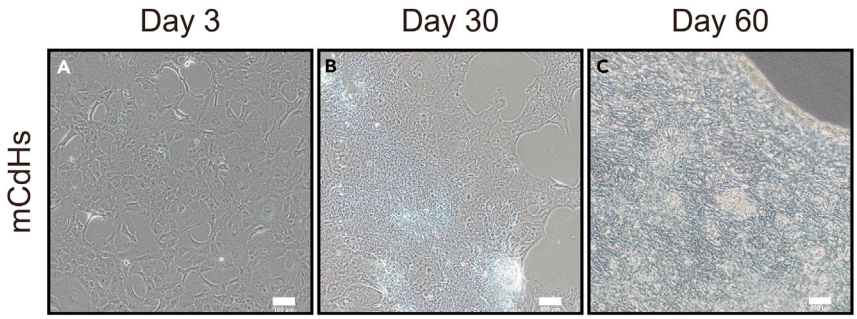

Figure 6. Results of over-confluence of mCdHs

Morphological changes in cells during culture at over 75%–80% confluence for 60 days.

(A) Mouse chemically derived hepatic progenitor (mCdHs) after reaching the appropriate confluence for passaging.

(B) Dense growth of mCdHs in the middle of a colony, accompanied by decrease in cell size.

(C) Detachment of cells from the collagen-coated plate. Scale bars, 100 μm.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dongho Choi (crane87@hanyang.ac.kr).

Materials availability

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability

This study did not generate any unique data sets or code.

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AUTHOR CONTRIBUTIONS

Y.K. conceived the study protocol and wrote the manuscript. J.J. and D.C. designed and supervised the study.

DECLARATION OF INTERESTS

Y.K., J.J., and D.C. filed a patent application based on this work.

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