Selective proliferation of rat hepatocyte progenitor cells in serum-free culture

Qijie Chen, Junko Kon, Hidekazu Ooe, Kazunori Sasaki & Toshihiro Mitaka

Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-Ku, Sapporo 060-8556, Japan. Correspondence should be addressed to T.M. (tmitaka@sapmed.ac.jp).

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This protocol details a method of obtaining selectively proliferated hepatocyte progenitor cells using hyaluronic acid (HA)–coated dishes and serum-free medium. A small hepatocyte (SH) is a hepatocyte progenitor cell of adult livers and has many hepatic functions. When the rat SH begins to proliferate, CD44 is specifically expressed. To define the purification of SH, CD44 and cytokeratin 8 are used as marker proteins. The growth of SHs is faster on HA-coated dishes than on other extracellular matrix–coated ones. The use of both DMEM/F12 medium and HA-coated dishes allows the selective proliferation of SHs in culture. The purification of SHs is approximately 85% at day 10.

INTRODUCTION

Small hepatocytes (SHs) are a subpopulation of hepatocytes that have high growth potential in culture[1]. Although the cells are less than half the size of mature hepatocytes (MHs), they possess hepatic characteristics[1]. SHs can clonally proliferate to form a colony and can differentiate into MHs by interacting with hepatic non-parenchymal cells (NPCs)[2,3] or as a result of treatment with gel derived from Engelbreth-Holm-Swarm sarcoma[4]. Thus, we consider that SHs may be 'committed progenitor cells' that can further differentiate into MHs. The standard method to obtain an SH-rich fraction, which is a mixture of SHs and NPCs from a liver cell suspension after collagenase perfusion, has used multistep centrifugation. However, with this method, although SHs account for more than 60% of the cells at day 10, it is very difficult to inhibit the growth of NPCs.

Recently, gene expression analysis revealed that CD44 is specifically expressed in SHs but not in MHs[5]. The CD44 gene encodes for a family of alternatively spliced multifunctional molecules, and CD44 plays a role in adhesion of cells to an extracellular matrix such as hyaluronic acid (HA), collagen (Col) or fibronectin (FN)[6]. CD44 standard form (CD44s) is composed of a short cytoplasmic tail, a transmembrane region and two extracellular domains, and ten variant forms (v1–v10) exist. SHs have been shown to express CD44s and v6 (ref. 5). CD44s in SHs appears from 3 d after plating; expression increases with the expansion of the SH colony, but decreases with the maturation of SHs. The appearance of CD44v6 is delayed compared with that of CD44s. The expression also disappears with maturation of SHs. Although CD44 is expressed in cultured SHs, no $CD44^+$ hepatocytes are found in the normal rat liver. When the rat liver is severely injured by hepatotoxins such as galactosamine and 2-acetylaminofluorene, $CD44^+$ hepatocytes transiently appear in the periportal regions of the liver lobules. Using an anti-CD44 Ab, we have isolated $CD44^+$ cells from the galactosamine-treated rat liver[5]. These $CD44^+$ cells possess the characteristics of SHs. However, as we were not able to isolate $CD44^+$ SHs from either a normal adult liver or from a liver with two-thirds removed, we tested HA as a ligand for separating a population of SHs.

HA, a linear polymer of (1-β-4)-D-glucuronic acid (1-β-3)-*N*-acetyl-D-glucosamine, is a large glycosaminoglycan that can reach a molecular size of 10^7 Da. It is found in the tissue matrix and body fluids of all vertebrates and has diverse biological roles. These include acting as a vital structural component of connective tissues and playing roles in the formation of loose hydrated matrices that allow cells to divide and migrate, immune cell adhesion and activation, and intracellular signaling[7]. Such diversity results from the large number of hyaluronan-binding proteins (termed hyaladherins), which exhibit significant differences in their tissue expression, cellular localization, specificity, affinity and regulation. Three HA synthase (HAS) genes, coding for HAS-1, 2 and 3, are recognized to synthesize HA[8]. HAS is located at the inner cell membrane, where the newly synthesized HA is extruded into the extracellular space[9]. Synthesized HA is degraded locally in the tissues where it is produced or by the lymph nodes, and the reminder enters the bloodstream[10]. More than 90% of circulating HA is degraded by hepatic sinusoidal endothelial cells (SECs) through a receptor recycling pathway. Hyaladherins, LYVE-1 and stabilin-1 and 2, but not CD44, are expressed in SEC[11,12]. Furthermore, a relationship between serum HA levels and liver diseases has been reported[13]. HA may be related to the induction of SHs in the liver.

We found that SHs cultured on HA-coated dishes could selectively proliferate to form colonies and that the contamination by NPCs with this method was much less than with our previous method. Although we do not know in detail why a population of SHs can be isolated from a normal liver and selectively proliferate on HA-coated dishes, the HA–CD44 interaction may enhance the growth of SHs. In addition, the combination of DMEM/F12 medium and HA-coated dishes allows us to exclude FBS from the culture. Using this protocol, we have isolated human SHs from a normal adult liver. Taking into consideration the application of hepatic stem/progenitor cells to regenerative medicine, the use of proteins derived from an animal, particularly FBS, should be avoided in the culture of the cells. This protocol for isolating and culturing SHs may help researchers in this field to progress in their own investigations.

MATERIALS

REAGENTS

- Male F344 rats (Sankyo Lab Service, Tokyo, Japan) weighing 150–200 g
  ! CAUTION All animal experiments must comply with national and institutional regulations.
- Ascorbic acid-2 phosphate (Asc2P; Wako Pure Chemical Industries, Osaka, Japan, cat. no. 013-12061) (see REAGENT SETUP)
- BSA (30% solution;Serological Proteins, IL, cat. no. 82-046-3)
- Collagenase (Wako Pure Chemical Industries, cat. no. 034-10533; Yakult Pharmaceutical Industry, cat. no. YK-101; Sigma, St. Louis, MO, cat. no. C5138)
- Dexamethasone (Wako Pure Chemical Industries, cat. no. 041-18861) (see REAGENT SETUP)
- DMEM/nutrient mixture Ham F-12 (DMEM/F12) (Sigma, cat. no. D8900)
- Nicotinamide-supplemented medium[1] (to grow MHs)
- L15 medium[14] supplemented with a growth factor (to grow MHs)
- Epidermal growth factor (EGF; BD Biosciences, Bedford, MA, cat. no. 354001) (see REAGENT SETUP)
- EGTA (Sigma, cat. no. E-0396)
- Gentamicin solution (50 mg ml^{-1}; Sigma, cat. no. G1397)
- Culture medium stock (see REAGENT SETUP)
- HA derived from human umbilical cords (Biozyme Laboratories, cat. no. HA1NaL), bovine vitreous humor (Sigma, cat no. H7630), pig skin (Seikagaku Kogyo, cat. no. 400720), rooster comb (Sigma, cat. no. H5388) and *Streptococcus* (Sigma-Aldrich, cat. no. 53747) (see REAGENT SETUP)
- HANKS' balanced salt solution (HANKS; Sigma, cat. no. H9269)
- 10× Ca^{2+}, Mg^{2+}-free HANKS (Sigma, cat. no. H4641)
- Wash solution (see REAGENT SETUP)
- MH wash solution (see REAGENT SETUP)
- Phenol red–free HANKS
- HEPES (Dojindo, Kumamoto, Japan, cat. no. 342-01375)
- Insulin (Sigma, cat. no. I-5500) (see REAGENT SETUP)
- Insulin–transferrin–selenium (ITS; GIBCO, cat. no. 0459)
- $NaHCO_3$ (Kanto Chemical, cat. no. 37116-00)
- Nembutal, 50 mg ml^{-1} (Dainippon Pharmaceutical, Tokyo, Japan, cat. no. 132141)
- Nicotinamide (Sigma, cat. no. N3376) (see REAGENT SETUP)
- Penicillin–streptomycin solution (Sigma, cat. no. P-4333)
- Percoll PLUS (GE Healthcare Bio-Sciences, Piscataway, NJ, cat. no. 17-5445-01) (see REAGENT SETUP)
- L-Proline (Sigma, cat no. P5607)
- Trypan blue (Chroma Technology, VT, cat. no. 1B187) (see REAGENT SETUP)
- Pre-perfusion solution (see REAGENT SETUP)

EQUIPMENT

- Dishes, 100, 60 and 35 mm (Corning Glass Works, Corning, NY)
- Non-charged (NC) 60-mm dish (Kord-Valmark, Ontario, Canada, cat. no. 2901)
- Autoclaved 250-μm nylon filter net (Nippon Rikagaku Kikai, Tokyo, Japan)
- Cell strainer, 70- μm filter (BD Falcon, cat. no. REF352350)
- 0.2- μm filter (Mediakap-2; Spectrum Laboratories, CA, cat. no. MEM2M-02B-12S)
- Paper filter no. 3 (Advantec, Tokyo, Japan)
- Sterilized 10-cm Petri dish (glass or plastic)
- Water bath (Teitec Co., Tokyo, Japan, cat. no. EX-B2015250)
- Vascular clamp, bulldog type (Fine Science Tools, Foster City, CA, cat. no. 18050-35)
- Peristatic pump (Tokyo Rika Instruments, Tokyo, Japan, RP-1000)
- Silicon tube (ϕ4.76 × 7.94 mm^2)
- Butterfly needle, 18 gauge (Terumo, Osaka, Japan, cat. no. SV-18CLK)
- O_2 gas (95% O_2/5% CO_2)
- Neubauer improved hemocytometer (Sigma, cat. no. Z359629)

REAGENT SETUP

HA solution Measure flakes or powder of HA; UV irradiate HA on a plastic tray for 1 h. After irradiation, place HA into a 50-ml tube and adjust the concentration of HA stock solution to 10 mg ml^{-1} by adding sterilized PBS. For UV irradiation, use a standard clean bench or safety cabinet equipped with a UV lamp. ! CAUTION UV is harmful to skin and eyes.

1,000× insulin stock solution (500 μg ml^{-1}) Add 100 mg insulin to 100 ml ddH_2O and then add 1.2 ml 1 N HCl. Adjust to 200 ml with ddH_2O and filter with a 0.2-μm filter. ▲ CRITICAL Insulin dissolves in acidic solution.

Pre-perfusion solution Add approximately 850 ml ddH_2O into a 1,000-ml graduated cylinder; add 100 ml 10× Ca^{2+}, Mg^{2+}-free HANKS, 190 mg EGTA, 1 ml 1,000× insulin stock solution and stir and adjust pH to 7.5 with 7 ml 1 M $NaHCO_3$. Adjust to 1,000 ml and filter with a 0.2-μm filter. Distribute into each bottle (150 ml). Store at 4 °C until use. ▲ CRITICAL EGTA solution should be warmed to 37 °C before use.

Perfusion solution To 200 ml HANKS, add 1 ml 1,000× insulin stock solution and collagenase (100 U ml^{-1}). Shake gently. ▲ CRITICAL Prepare HANKS with insulin before the experiment. Add collagenase to pre-warmed perfusion solution just before use and immediately dissolve by gentle shaking.

Wash solution To 500 ml HANKS, add 0.5 ml 1,000× insulin stock solution, 2 ml penicillin–streptomycin solution and 0.5 ml gentamicin solution.

MH wash solution To 500 ml HANKS, add 0.5 ml 1,000× insulin stock solution, 3.3 ml BSA, 2 ml penicillin–streptomycin solution and 0.5 ml gentamicin solution.

Percoll solution[15] Add 2.4 ml 10× HANKS and 21.6 ml Percoll into a 50-ml conical tube. Mix gently upside down several times. Store at 4 °C until use.

Trypan blue stock solution (0.1%) Add 100 mg trypan blue to 100 ml phenol red–free HANKS. Filter with a paper filter.

Dexamethasone stock solution Add 39.2 mg dexamethasone to an autoclaved brown bottle. Add 10 ml ethanol (10^{-2} M stock solution); dilute to 100× (10^{-4} M) with autoclaved ddH_2O. Store at 4 °C until use. ▲ CRITICAL 10^{-4} M stock solution must be used within 3 months.

EGF stock solution (10 μg ml^{-1}) Add 10 ml autoclaved ddH_2O to an EGF vial. Store 1-ml aliquots in microcentrifuge tubes at −20 °C until use.

Nicotinamide stock solution (1M) Add 12.21 g nicotinamide to 100 ml PBS. Filter with a 0.2-μm filter and store at 4 °C until use.

Asc2P stock solution (100 mM) Add 2.90 g Asc2P to 100 ml PBS, filter with a 0.22-μm filter and store at 4 °C in a 100-ml brown bottle until use.

Culture medium stock Add the following reagents to a 1,000-ml beaker: DMEM/F12 (15.56 g), HEPES (1.20 g), L-Proline (30 mg), penicillin–streptomycin (8 ml), ddH_2O up to 1,000 ml. Mix using a magnetic stir bar, add 2.20 g $NaHCO_3$, adjust pH to 7.6 with 1 N NaOH and filter with a 0.2-μm filter. Store at 4 °C until use.

Preparation of culture medium Mix DMEM/F12 stock medium (500 ml), BSA (1.67 ml), nicotinamide stock solution (5.50 ml), Asc2P stock solution (5 ml), ITS (5 ml), EGF stock solution (0.5 ml), dexamethasone stock solution (0.5 ml) and gentamicin (0.5 ml).

EQUIPMENT SETUP

Perfusion system See **Figure 1a**. The system is composed of a water bath, vascular clamp, peristatic pump, silicon tube (ϕ4.76 × 7.94 mm^2) and 18-gauge butterfly needle.

PROCEDURE

Preparation of HA-coated dishes ● TIMING At least 1 d before the experiment

1| Dilute the stock HA solution to 100 μg cm^{-2} with PBS, fill Petri dishes and incubate at 37 °C overnight. The next day, discard the solution and wash once with PBS. Aspirate PBS and leave to dry on a clean bench with UV irradiation for 30 min.
! CAUTION UV is harmful to skin and eyes.
? TROUBLESHOOTING

Isolation of liver cells ● TIMING 1–1.5 h

2| Settle the perfusion apparatus in the warmed water bath (**Fig. 1a**). Pour the pre-perfusion solution into the apparatus before the experiment and bubble it with 95% O_2/5% CO_2 gas at a flow rate of 0.5 l min^{-1}.

▲ CRITICAL STEP The equipment should not be left for more than 30 min before proceeding to the next step.
? TROUBLESHOOTING

3| After light anesthesia by ether, anesthetize a rat with an i.p. injection of nembutal (5 mg per 0.1 ml per 100 g body weight).
? TROUBLESHOOTING

4| Cut the abdominal wall using surgical scissors and open the abdominal cavity to look at the portal vein.

5| Ligate a common bile duct and splenic vein together using a surgical thread at the portion nearest the portal vein (**Fig. 1b**).
▲ CRITICAL STEP This is to avoid loss of the solution.
? TROUBLESHOOTING

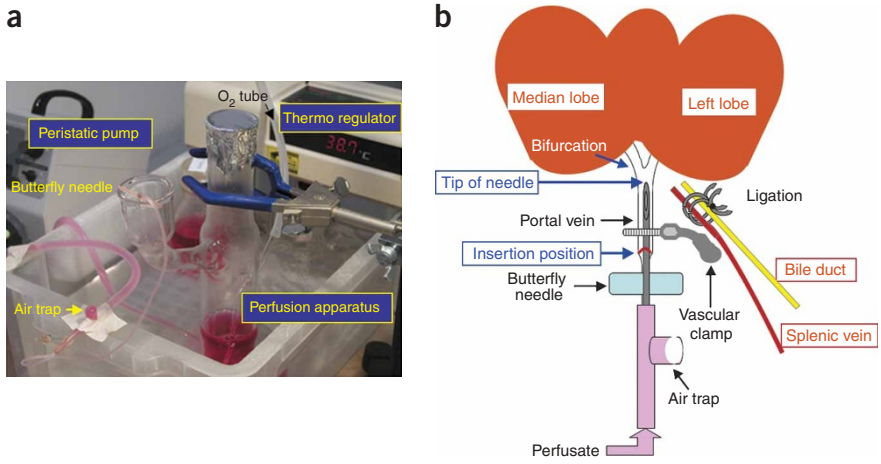

Figure 1 | Experimental setup. (**a**) Setting up the perfusion apparatus. Pre-perfusion solution is warmed to approximately 39 °C and bubbled with O_2. (**b**) Position of the inserted needle in the portal vein of a rat.

6| Insert a butterfly needle filled with pre-perfusion solution into the portal vein 1.5–2.0 cm from the bifurcation of the portal vein, stop the tip of the needle at a position close to the bifurcation of the portal vein and clamp the needle with a surgical clip (**Fig. 1b**).
? TROUBLESHOOTING

7| Start the perfusion at a flow rate of 30 ml min^{-1}.

8| Cut the inferior vena cava and heart as soon as the flow starts.
▲ CRITICAL STEP Cut the inferior vena cava at the portion beneath the right kidney and the thoracic cavity, and then cut the heart to flow the perfusate out of the cadaver. Washing out the blood completely from the liver and preventing an increase of intra-hepatic pressure is important to the success of the preparation.
? TROUBLESHOOTING

9| When the amount of the pre-perfusion solution becomes small, add collagenase to the perfusion solution and then pour it into the perfusion apparatus.
▲ CRITICAL STEP To avoid a decrease in collagenase activity, dissolve it after the pre-perfusion solution flows. Do not mix vigorously.
? TROUBLESHOOTING

10| Flow the solution at a flow rate 15–20 ml min^{-1}.
▲ CRITICAL STEP The flow rate should be decided by rat body weight.
? TROUBLESHOOTING

11| Stop the flow before air bubbles move into the liver when the solution flows out from the reservoir.
? TROUBLESHOOTING

12| Cut the liver from the abdominal cavity and transfer it to a sterilized Petri dish.

13| Prepare a 100-ml beaker with 70–80 ml wash solution and add a small amount of the wash solution to the Petri dish.
▲ CRITICAL STEP From this step onward, all procedures should be done in sterilized conditions.

14| Peel the hepatic capsule as carefully as possible and, to drop the digested cells, shake the liver into the beaker.
? TROUBLESHOOTING

15| Filter the cell suspension through a 250-μm nylon filter net into a new 100-ml beaker.

16| Filter the cell suspension through a 70-μm filter into four 50-ml conical tubes using a 25-ml pipette and then adjust each tube to an equal volume with the wash solution (approximately 40 ml).

17| Centrifuge the tubes at 50*g* for 1 min at 4 °C.
? TROUBLESHOOTING

18| Collect supernatants and transfer to new conical tubes. Repeat Steps 15–17 a total of 3 times.
▲ CRITICAL STEP Many SHs are included in the supernatant after low-speed centrifugation. This procedure is carried out to remove the majority of MHs from the cell suspension.
■ PAUSE POINT Cells in tubes may be kept on ice for 1–3 h. Avoid long-term preservation.

19| If you want to isolate MHs, proceed directly to Step 19B. To isolate SHs, proceed with Step 19A.

(A) Isolation of SHs ● TIMING **1–1.5 h**

(i) Centrifuge the supernatant at 50*g* for 5 min at 4 °C.
▲ CRITICAL STEP This procedure is used to remove hematopoietic cells.
? TROUBLESHOOTING

(ii) Discard the supernatant and add 40 ml wash solution to the tubes.
? TROUBLESHOOTING

(iii) Centrifuge at 50*g* for 5 min at 4 °C after gentle pipetting to dissociate the cell pellet.

(iv) Add 40 ml wash solution and then centrifuge at 150*g* for 5 min at 4 °C.
▲ CRITICAL STEP This procedure is carried out to damage some MHs contained in the suspension.
? TROUBLESHOOTING

(v) Discard the pellet, add 40 ml wash solution and then centrifuge at 150*g* for 5 min at 4 °C.

(vi) Discard the supernatant, pour 20 ml culture medium into each tube and gather the suspension into two 50-ml conical tubes.

(vii) Centrifuge the suspension at 50*g* for 5 min at 4 °C.
? TROUBLESHOOTING

(viii) Add 10 ml culture medium to each tube and gather the suspension into one tube.

(ix) Add 0.5 ml cell suspension to 1.5 ml trypan blue solution and pipette gently.
▲ CRITICAL STEP Tubes should be on ice.

(x) Count the viable cells as soon as trypan blue is added. For counting, use a Neubauer improved hemocytometer. Count the number of cells with trypan blue–negative nuclei which exist in nine masses. Use this formula to obtain the cell number: the number of viable cells (cells ml^{-1}) = [(number of cells inside nine masses)/9] × 4 × 10^4.
▲ CRITICAL STEP Cells with trypan blue–positive nuclei are dead. Count the number of cells that are smaller than typical MHs and larger than NPCs (15–20 μm in diameter). The overall viability of cells will not be good, but most small cells, including SHs, are viable. As SH colony formation depends on the cell density, the number of viable cells, which have the potential to attach to the dishes, is important in this step.
■ PAUSE POINT Cells in tubes may be kept on ice for 1–3 h. Avoid long-term preservation.

(xi) Adjust the concentration of the cells to 1 × 10^5 cells ml^{-1} and then seed them on HA-coated dishes.
? TROUBLESHOOTING

(xii) Place dishes in a 5% CO_2/95% air incubator at 37 °C.

(xiii) Replace the medium with fresh medium after 3 h.
▲ CRITICAL STEP This procedure excludes unattached cells.

(xiv) Renew medium every other day.
▲ CRITICAL STEP Two times a week is enough when the number of attached cells is small.

(B) Isolation of MHs ● TIMING **1–1.5 h**

(i) Add 20 ml MH wash solution to a pellet in each tube (from Step 17) and gather the suspension into two 50-ml conical tubes.

(ii) Centrifuge the tubes at 50*g* for 1 min at 4 °C after gentle pipetting to dissociate the cell pellet.
▲ CRITICAL STEP As MHs are more labile to a shock-like pipetting or centrifugation than SHs, gentle pipetting is important.
? TROUBLESHOOTING

(iii) Discard the supernatant and add 40 ml MH wash solution to each tube.

(iv) Centrifuge at 50*g* for 1 min at 4 °C.
? TROUBLESHOOTING

(v) Discard the supernatant and add 25 ml MH wash solution to each tube.

(vi) Pipette gently to dissociate the cell pellet.

(vii) Pour 25 ml cell suspension into Percoll solution and gently mix (upside down several times).

(viii) Centrifuge at 50*g* for 15 min at 4 °C.
▲ CRITICAL STEP This step is performed to remove dead MHs. After centrifugation, dead MHs and NPCs float on the solution.
? TROUBLESHOOTING

(ix) Discard the supernatant and add 40 ml MH wash solution to each tube.

(x) Centrifuge the tubes at 50*g* for 1 min at 4 °C after gentle pipetting to dissociate the cell pellet.
? TROUBLESHOOTING

(xi) Discard the supernatant and add 40 ml culture medium to each tube.
(xii) Centrifuge the tubes at 50*g* for 1 min at 4 °C after gentle pipetting to dissociate the cell pellet.
? TROUBLESHOOTING
(xiii) Add 20 ml culture medium to each pellet and gather the suspension into one tube.
(xiv) Add 0.5 ml cell suspension to 1.5 ml trypan blue solution and suspend.
(xv) Count the number of viable and dead MHs as described in Step 19A(x).
▲ CRITICAL STEP Keep cells in tubes on ice.
(xvi) Adjust cell density to 2–6 × 10^5 viable cells ml^{-1} and then seed the cells on dishes.
? TROUBLESHOOTING
(xvii) Place the dishes in a 5% CO_2/95% air-incubator at 37 °C.
(xviii) 1–2 h later, replace the medium with fresh medium.
▲ CRITICAL STEP If researchers need to grow MHs, we suggest using nicotinamide-supplemented medium[1] or L15 medium[14] supplemented with a growth factor.
? TROUBLESHOOTING

● TIMING
Steps 2–18, isolation of liver cells: 1–1.5 h
Steps 19A(i–xiv), isolation of SHs: 1–1.5 h
Steps 19B(i–xviii), isolation of MHs: 1–1.5 h

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Reagent setup	Reagent bacterial or viral contamination or lost activity	Inadequate sterilization	To sterilize hyaluronic acid (HA) solution, avoid using an autoclave or filter. Instead, use UV-irradiation and HA will then dissolve in sterilized PBS
		Storage conditions	Stock solution is kept at 4 °C. To avoid bacterial contamination, a small amount of the stock solution should be prepared for several experiments. Avoid long-term storage (more than 1 mo)
Reagent setup	HA insolubility	Short time	As HA solution has high viscosity, HA is dissolved overnight at 37 °C
Reagent setup	Crystallization of nicotinamide	Concentrated	Crystals are sometimes formed in 1 M nicotinamide stock solution. As they may damage cells, do not use the solution. Make new stock solution
	Quantity of collagenase	Difference between lots and between suppliers	Adjust the quantity of collagenase by units, not percentage
Step 1	Dish coating	Unequally distributed	Shred the solution using a non-charged dish. If there is not enough HA solution to cover the dish surface, add more PBS. HA-coated dishes can be stored at 4 °C, but use within 1 mo
Step 2	O_2 gas bubbling	Changes of solution color	Too much O_2 gas flow causes the buffer to become yellowish. If the color changes, stop the O_2 gas. Viability of small hepatocytes (SHs) decreases in acidic conditions
Step 3	Animal moving	Light anesthesia	Inject a small additional amount of nembutal
Steps 5, 6, 8–11	Poor yield or viability, and cell aggregation	Collagenase	(i) Collagenase activity is best at 37 °C. To keep the perfusion-solution at 37 °C, carefully adjust the temperature of the water bath and solution at the tips of tubes (ii) Long-term storage (more than 1 y) causes loss of activity (iii) Storage should be at 4 °C
		Length of perfusion time	(i) Retention of collagenase solution in the abdominal cavity causes the early rupture of hepatic capsules and inadequate digestion

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
			(ii) Although collagenase works efficiently at a slow flow rate, too slow a flow causes partial perfusion of the liver, and the yield and viability of the cells have a tendency to be bad
		Flow rate	The flow rate depends on the weight of the rat. When the weight is more than 250 g, use the maximum flow rate of 20 ml min^{-1}. Liver weight is not correlated to body weight in bigger rats. A relatively high flow rate may cause the early rupture of hepatic capsules
		Embolisms	(i) Inside of the needle is not filled with perfusate (ii) Do not shake vigorously after collagenase is added to perfusion solution (iii) Tube sometimes generates bubbles and causes air embolisms in the liver. An air-trap apparatus is necessary. A rapid change of liver color from red-brown to light brown is a sign of good circulation of the perfusate. Red spots in the liver may result from embolisms
Step 14	Shortage of cells	Insufficient extraction of cells	Comb the liver remnant carefully using tweezers at Step 12
Steps 17, 19A(i), (ii), (iv) and (vii), 19B(ii), (iv), (viii), (x) and (xii)	Recovery of cells	Time of centrifugation	Centrifugation should be adjusted to g_{max}. The times shown are from pushing the 'Start' button to pushing the 'Stop' button
Step 19A(xi)	Poor growth of SHs	Few SH colonies	(i) Avoid plating too small a number of cells. We found that fewer than 2.5×10^3 cells cm^{-2} resulted in a smaller number of SH colonies than we expected (ii) Total number of SH colonies is dependent on rats. Cells from younger rats make many colonies per dish[19]
Step 19B(viii)	No pellet	Poor mixture	Mix the cell suspension again and repeat Step 8. Good mixing of cells and Percoll solution is important to obtain high viability and purity of mature hepatocytes (MHs)
Step 19B(xvi)	Poor attachment	Too many cells	9×10^5 cells and 2×10^6 cells are confluent in 35-mm and 60-mm dishes, respectively
		Few cells	Avoid using fewer than 1×10^4 cells cm^{-2}. MHs need cell–cell contact to survive. Confluence is important to maintain hepatic differentiated functions and cell longevity
		Dish	In serum-free culture, use extracellular matrix (collagen type I or IV, fibronectin, laminin and vitronectin)–coated dishes. Type I collagen (rat tail collagen) is sufficient for good attachment of MHs. You can also use pre-incubation of serum-supplemented medium for 1 h
Step 19B(xviii)	No MH growth	Medium	(i) Avoid the combination of L15 medium and nicotinamide because many cells may die (ii) Please refer to review article[1]
		Growth factors	Epidermal growth factor, hepatocyte growth factor and transforming growth factor-α have similar growth effects on MHs. MHs require high-quality water. Water in some places may not be appropriate for the culture even if other cells can grow
		Water	Deionization may not solve this problem. We recommend using purchased water; otherwise you can obtain it from a successful laboratory

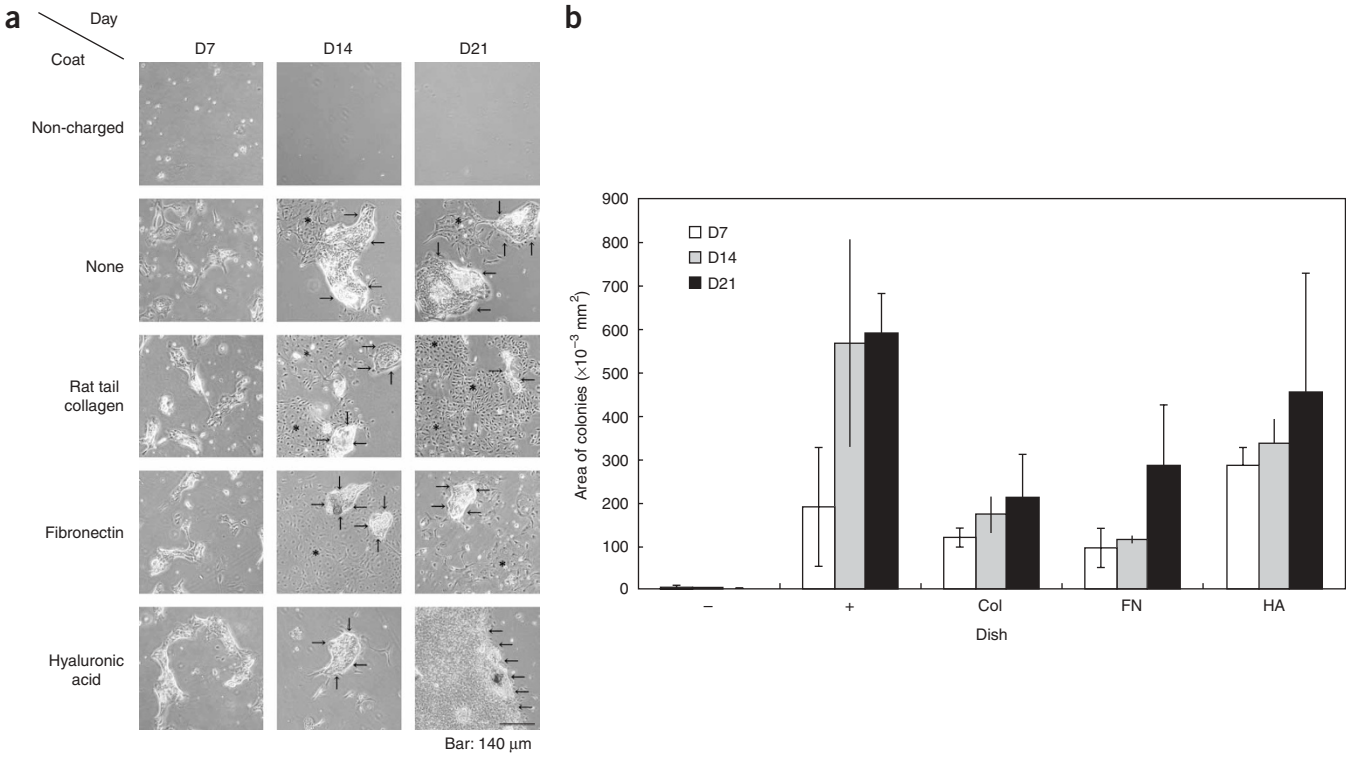

Figure 2 | Growth of liver cells on dishes coated with extracellular matrices. (**a**) Phase-contrast micrograph of small hepatocytes (SHs) and non-parenchymal cells at days 7, 14 and 21 after plating. Cells are cultured in DMEM supplemented with 10% FBS. Arrows show SH colonies and asterisks show NPCs. The expansion of SH colonies is clear and there is little contamination by NPCs on the hyaluronic acid (HA)–coated dish compared with other dishes. At day 21 on HA-coated dishes, proliferated cells, which are marked by arrows, are all SHs. The magnification of the photos is the same; scale bar = 140 μm. (**b**) The growth of SH colonies on various dishes. Columns and bars show the average and s.d. of three dishes, respectively.

TABLE 2 | Purity of small hepatocytes cultured in serum-free medium and on hyaluronic acid–coated dishes.

Medium	$CD44^+$ cells (%)	$CD44^-$ cells (%)
DMEM/F12-FBS (HA-coated dish)	84.5 ± 3.2	15.5 ± 3.2
DMEM+FBS (Normal dish)	64.1 ± 8.0	35.9 ± 8.0

The numbers show the average ± s.d. of three dishes. Cells were cultured for 10 d and immunostained for CD44. After counterstaining with hematoxylin, both $CD44^+$ and $CD44^-$ cells were counted. $CD44^-$ cells include sinusoidal endothelial, stellate, liver epithelial and Kupffer cells.

ANTICIPATED RESULTS

A critical component of this method is to use HA-coated dishes and serum-free medium for SH culture. As CD44 is not expressed in MHs but is expressed in SHs, we first examined whether SHs could selectively proliferate on HA-coated dishes under our standard culture conditions (DMEM + 10% FBS). As shown in **Figure 2a**, compared with the NC-, non-, rat tail Col- and FN-coated dishes, SHs could selectively proliferate on HA-coated dishes. However, there was much less growth of NPCs in HA-coated dishes than in other dishes. The average size of SH colonies on HA-coated dishes was larger than that of SH colonies on NC-, Col- and FN-coated dishes (**Fig. 2b**). Although the SH growth on non-coated dishes was as good as that on HA-coated dishes, NPCs also grew (**Table 2**).

Next, we examined whether SHs could proliferate in serum-free medium.

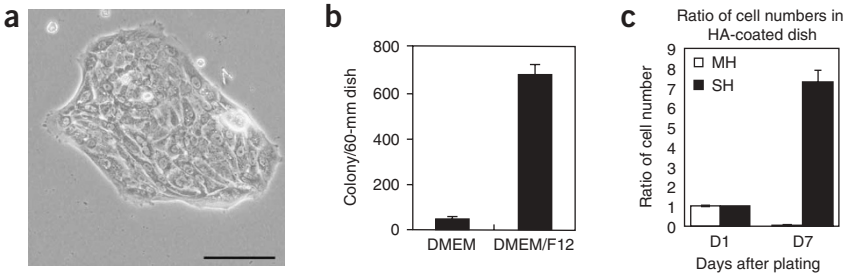

Figure 3 | The growth of small hepatocyte (SH) colonies on hyaluronic acid (HA)–coated dishes cultured in serum-free medium. (**a**) Phase-contrast micrograph of a typical SH colony at day 7. Scale bar = 100 μm. (**b**) The number of SH colonies on HA-coated dishes cultured in serum-free DMEM or DMEM/F12 medium at day 10. Many colonies grew in the serum-free DMEM/F12 medium, whereas there were few in the serum-free DMEM. Columns and bars show the average of the total number of colonies per 60-mm dish and s.d. of three dishes, respectively. (**c**) Few mature hepatocytes (MHs) can survive in the serum-free medium on HA-coated dishes. The growth of MHs and SHs is shown as the ratio day 7/day 1.

Although we reported that SH colonies could expand in serum-free medium after re-plating SH colonies[4,16], it was very difficult for SHs in serum-free medium to grow to form colonies on conventional dishes. When we used serum-free DMEM/F12 and HA-coated dishes, many colonies developed (**Fig. 3a**), whereas few colonies grew in serum-free DMEM (**Fig. 3b**). We usually use HA derived from human umbilical cords. However, the effect of HA is not different among commercially available forms of HA derived from pig skin, bovine vitreous humor, rooster comb and *Streptococcus* (data not shown). To observe the appearance of SHs in serum-free culture, both nicotinamide and growth factors (either a single factor or a combination of EGF, hepatocyte growth factor and transforming growth factor-α) must be included in the medium[1]. These results suggest that ingredients contained in Ham F12 medium are important for SHs to grow in the serum-free culture, because the use of DMEM alone without FBS cannot support the growth of SHs. However, when the specific agents included in Ham F12 medium were added to DMEM and SHs were cultured in the serum-free medium, the growth of SHs was neither enhanced nor inhibited (data not shown). Therefore, we now hypothesize that the balance of ingredients in Ham F12 medium may be important for SHs to grow in serum-free culture. On the other hand, adding transferrin and selenium is different from the original recipe for culturing rat SHs[2]. Although SHs appear and grow without those agents, both are necessary to maintain their growth in serum-free culture. Without transferrin and selenium, the growth of SHs becomes worse after 1–2 weeks of culture (current data not shown; refer to ref. 1). **Figure 3a** shows a typical SH colony in serum-free culture. Although it was difficult to avoid MH survival in DMEM with FBS, the use of HA-coated dishes and serum-free medium suppressed MH survival. Most attached MHs died within 1 week (**Fig. 3c**). In this protocol, the purity of SHs was 84.5% ± 3.2% at day 10 (**Table 2**). Some SECs ($SE1^+$), stellate cells ($desmin^+$), liver epithelial cells ($vimentin^+$) and Kupffer cells ($ED1/2^+$) were observed.

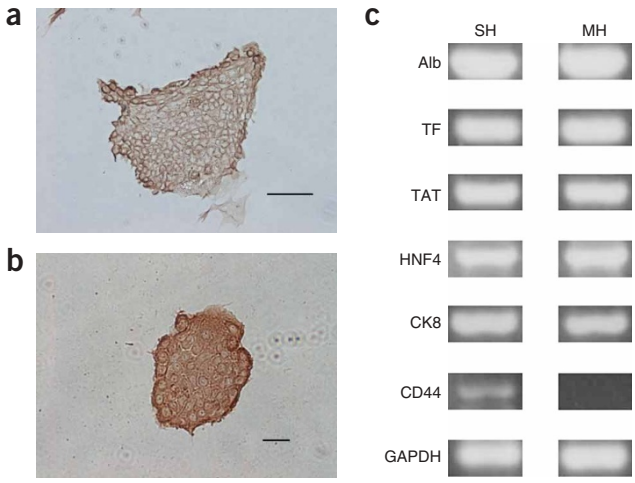

Figure 4 | Characteristics of small hepatocyte (SH) colonies on hyaluronic acid (HA)–coated dishes. Immunocytochemistry for (**a**) CD44 and (**b**) CK8 at day 10. Scale bars = 100 μm. (**c**) Expression of mRNAs of hepatic marker genes. SHs were cultured in serum-free medium on HA-coated dishes at day 7. RNA of mature hepatocytes (MHs) is derived from isolated MHs. SHs express many hepatic marker genes. CD44 is expressed in SHs but not MHs. These are representative data.

Characterization of SHs on HA was carried out using immunocytochemistry and RT-PCR. All SHs were immunocytochemically stained with anti-CD44 (**Fig. 4a**) and anti-CK8 Abs (**Fig. 4b**). As shown in **Figure 4c**, RT-PCR shows that SHs express mRNAs of hepatic marker genes such as albumin, transferrin, tyrosine aminotransferase, hepatocyte nuclear factor-4α and CK8 as plentifully as MHs at day 10. In addition, CD44 is not expressed in MHs but is expressed in SHs. These results reveal that cell colonies grown in serum-free DMEM/F12 on HA have quite similar characteristics to SHs observed using our standard culture method (DMEM/FBS). Until now, we have not observed any difference between the cells.

The SH is a hepatocyte progenitor cell that is committed to differentiate into an MH. Manipulation of the growth and maturation of SHs is easy, and they can be cryopreserved[17]. Cryopreserved SHs can maintain the abilities of growth and maturation[18]. Maturated SHs in serum-free culture can be used for studies of hepatic drug metabolism, liver diseases and regenerative medicine.

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