Chapter 4

Isolation and Purification Method of Mouse Fetal Hepatoblasts

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Abstract

During development, liver precursors constitute a valuable source of pluripotent stem cells that present the ability to differentiate into both a hepatic and biliary lineage. In the present chapter, we report an experimental procedure developed by our group to isolate mouse fetal hepatoblasts (MFHs) with high purity. The method is based on a selective harvesting of the hepatic parenchymal cells from fetuses (E 14.5), followed by the sorting of E-cadherin$^+$ progenitors through the use of magnetic beads and specific antibodies. This protocol allows the isolation of bipotent liver stem cells expressing both hepatic and biliary markers. Primary cultures of purified MFHs can be maintained under proliferation until confluence, leading to promotion of the differentiation process in the presence of hepatotrophic factors. By using a quantitative real-time polymerase chain reaction approach, we show the hepatospecific phenotype and the progressive maturation of MFHs, delineating early (α-fetoprotein), mid (albumin), and late (glucose-6-phosphatase) hepatic markers. Consequently, the model appears to be a valuable cell system for the study of molecular and cellular aspects occurring in hepatic differentiation.

Key words: Mouse fetal hepatoblasts, MFHs, E-cadherin, Cell sorting, Bipotent stem cells, Hepatic differentiation

1. Introduction

Effective accessibility to cell resources appears as a major challenge in modern hepatology regarding fundamental research and therapeutic management of liver diseases. An increasing number of reports have focused on the remarkable potential of liver stem cells and their ability to give rise to the hepatic lineage (1). Liver progenitors are known as "oval cells," in reference to their oval-shaped nucleus and scant cytoplasm. These liver-specific stem cells, also called hepatoblasts, exhibit bipotent capacities and are characterized

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by an intermediary phenotype between biliary epithelial cells and hepatocytes (2, 3). During embryonic development, hepatoblasts are able to proliferate in order to permit liver morphogenesis and finally differentiate into mature hepatocytes. At that time, the establishment of cell–cell interactions is crucial for organogenesis and implicates a category of glycoprotein named cadherins. In particular, the epithelial cadherin (E-cadherin) represents a key calcium-dependent cell adhesion molecule ensuring the integrity of epithelial tissues (4). In conjunction with hepatotrophic factors, the E-cadherin signaling pathway promotes the onset of a hepatic phenotype and the maturation of embryonic stem cells (5, 6). In contrast to cell differentiation, the alteration of E-cadherin is frequently correlated with cancer progression as reported in numerous human tumors (7). Thus, a reduced expression of the E-cadherin/catenin complex leads generally to the disruption of cell–cell contacts which can promote epithelial to mesenchymal transition, invasiveness and the metastatic potential of a variety of cancers, including hepatocellular carcinoma (8–10).

The molecular and cellular mechanisms leading to the proliferation/differentiation of hepatoblasts are still poorly understood, mainly because of the lack of hepatoblast-like cell lines, as well as the difficulty in isolating liver bipotent progenitors and differentiating primary cultures into mature hepatocytes and/or cholangiocytes. In addition, no technical reports consistently describe the experimental procedures required for the isolation of hepatoblasts and the characterization of their bipotency. Nevertheless, recent studies have focused on a number of markers expressed in hepatic precursors that can be suitable for the specific selection of liver stem cells (11–16). In the present chapter, we report in detail an efficient method for an accurate purification of mouse fetal hepatoblasts (MFHs), based on the sorting of E-cadherin$^+$ cells through the use of specific antibodies and magnetic beads. Through controlled stimulation with hepatotrophic growth factors, we characterized isolated E-cadherin$^+$ MFHs and demonstrated the suitability of this marker for the specific isolation of hepatoblasts from the fetal liver.

2. Materials

2.1. Animals

C57BL/6J-Jcl pregnant mice (CLEA Japan, Tokyo, Japan). Embryonic livers are harvested at 14.5 days of gestation (see Note 1).

2.2. Specific Materials

1. EasySep magnet. StemCell Technologies (Cat. # 18000).
2. Autoclaved nylon filter with a 60-μm pore size. MILLIPORE (Cat. # NY6000010).
3. Collagen type I-coated dishes (35 mm diameter). IWAKI (Cat. # 4000–010).
4. Other materials required: 8-cm diameter funnel, 10-cm dishes (noncoated), sterilized surgical scissors and forceps, 50-mL round-bottom polypropylene tubes for centrifugation, and 200-mL Erlenmeyer for cell decantation.

2.3. Reagents

A complete list of the reagents employed for the isolation of MFHs is provided in Table 1.

Table 1
List of reagents

Description	Source	Cat. number
Cell sorting		
EasySep biotin selection kit (Mouse)	StemCell Technologies	# 18556
Biotin anti-CD324 (E-cadherin)	eBioscience	# 13-3249
Primary culture of hepatoblasts		
William's medium E	Gibco	# 12551-032
Fetal bovine serum (Fetalclone)	HyClone Thermo Scientific	# SH30088
L-Glutamine (200 mM)	Gibco	# 25030-081
Penicillin (5,000 U/mL)/streptomycin (5,000 μg/mL)	Gibco	# 15070-063
Insulin from bovine pancreas (50 mg)	Sigma	# I5500
Mouse recombinant oncostatin M (25 μg)	Sigma	# O1637
Human recombinant hepatocyte growth factor (10 μg)	PreproTech	# 100-39
Human recombinant epidermal growth factor (0.2 mg)	Sigma	# E9644
Hydrocortisone 21-hemisuccinate (100 mg)	Sigma	# H2270
Dexamethasone (100 mg)	Sigma	# D2915
Other reagents		
Liberase TM Research Grade	Roche	# 05401119001
HEPES buffer solution (1 M)	Gibco	# 15630-080
Sodium chloride (NaCl)	Sigma	# S9888
Potassium chloride (KCl)	Sigma	# P5405
Sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$)	Wako	# 196-02835
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	Sigma	# C7902
D-PBS	Sigma	# D8537

2.4. Reagents Preparation

2.4.1. Culture Medium

To prepare the hepatoblast basal medium, add the following to a 500-mL final volume of William's E medium: 50 mL of fetal bovine serum (FBS) corresponding to a final concentration of 10%, 5 mL of L-glutamine 200 mM (final concentration: 2 mM), and 5 mL of the antibiotic solution.

To prepare the complete culture medium optimized for primary culture of MFHs, prepare and add the following factors to the basal medium as mentioned below (see Fig. 1):

1. HGF (25 ng/mL): Add 1 mL of sterile water in two vials (2×10 µg) after having centrifuged the tubes to obtain a solution with a concentration of 20 µg/mL. Make aliquots and store at −20°C (see Note 2 regarding growth factor stability). 625 µL of the stock solution are required to obtain a final concentration of 25 ng/mL for a 500-mL final volume.
2. EGF (25 ng/mL): After having centrifuged the tube, add 1 mL of sterile water in one vial (0.2 mg) to obtain a 200 µg/mL concentration solution. Use 62.5 µL of the stock solution to obtain a 25 ng/mL concentrated culture medium.
3. OSM (12.5 ng/mL): Centrifuge the vial prior to opening. Reconstitute the lyophilized oncostatin M (25 µg) in 1-mL sterile water to a concentration of 25 µg/mL. Make aliquots and store at −20°C (see Note 2). Use 250 µL to get appropriate working concentration in 500 mL of medium.

Prepare stock solutions of the following factors mentioned below:

1. Insulin stock solution (5 mg/mL): Reconstitute the 50 mg of lyophilized powder in a 50-mL tube by adding 10 mL of sterile

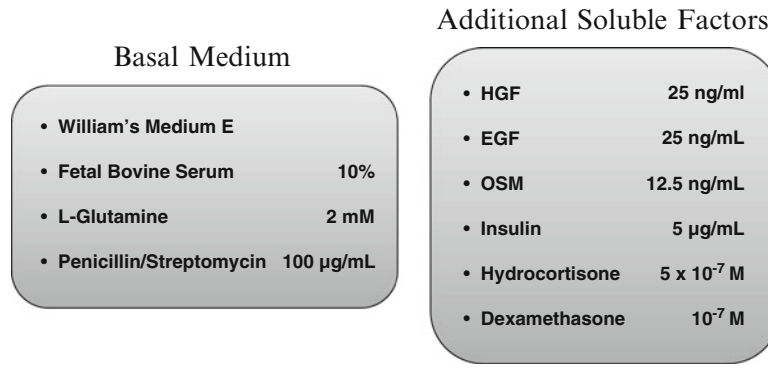

Fig. 1. Complete medium used for the primary culture of mouse fetal hepatoblasts.

water. After dissolution of the insulin, the solution becomes whitish. To obtain a perfectly limpid solution, add one or two drops of 1 N HCl (see Note 3). Perform filtration of the solution with a 0.22-µm filter. Make aliquots and store at –20°C (see Note 4).

2. Hydrocortisone-21-hemissuccinate and dexamethasone stock solutions (10^{-2} M): Add appropriate volumes of sterile water to reconstitute both corticoids at 10^{-2} M. Make aliquots and store at –20°C after filtration of the solutions (see Note 4).

Finally, to obtain the complete medium used for hepatoblast differentiation into mature hepatocytes add, respectively: 500 µL of insulin (final concentration: 5 µg/mL), 25 µL of hydrocortisone (5×10^{-7} M), and 5 µL of dexamethasone (10^{-7} M). Keep the complete medium at 4°C (see Note 5).

2.4.2. HEPES Buffer Solution

Prepare the HEPES buffer by adding the following compounds to 750 mL of autoclaved distilled water: 8 g NaCl, 0.2 g KCl, 0.1 g $Na_2HPO_4 \cdot 12H_2O$, and 10 mL of HEPES commercial solution (equivalent to 2.38 g). Then, mix well using a magnetic rotating system; adjust volume at 1 L and pH at 7.65. Finally, store HEPES buffer at 4°C after filtration of the solution with a 0.22-µm filter.

2.4.3. Liver Dissociation Solution

1. Reconstitute the lyophilized liberase (Roche, Cat. # 05401119001, collagenase amount: 5 mg) in 2 mL of sterile water to get a solution with an enzyme activity equal to 13 U/mL. Place the vial on ice for 30 min to rehydrate the enzyme and gently shake the solution every few minutes until complete dissolution. Immediately store the unused stock solution in single-use aliquots at –20°C (see Note 6 regarding important remarks about liberase storage and stability).
2. We use a working concentration of liberase equivalent to 0.13 U/mL. To prepare 10 mL of dissociation buffer (sufficient for the digestion of approximately 15–20 fetal livers), 100 µL of the stock solution are required. Ten minutes before performing liver dissociation, dilute the concentrated liberase into the HEPES solution containing calcium (see Note 7). To prepare 100 mL of calcium–HEPES buffer, dissolve 56.25 mg of $CaCl_2 \cdot H_2O$. Store at 4°C after 0.22-µm filtration.

2.4.4. E-Cadherin Cell Sorting Medium

The recommended medium for the isolation of E-cadherin positive cells is a calcium–HEPES buffer solution supplemented with 2% FBS (see Note 8).

3. Method

The following experimental protocol describes the usual method used to isolate MFHs from fetuses (E 14.5) of pregnant mice. The purification of hepatic progenitors relies on two consecutive steps (1) isolation of parenchymal cells after an enzymatic dissociation of the fetal livers; (2) sorting of isolated cells by using the E-cadherin surface marker (see Fig. 2).

3.1. Collection of Mouse Fetal Livers

1. Before starting the harvesting procedure, place 10 mL of calcium-supplemented HEPES buffer into a warming water-bath (37°C). Throughout dissection, use cold buffers (4°C)

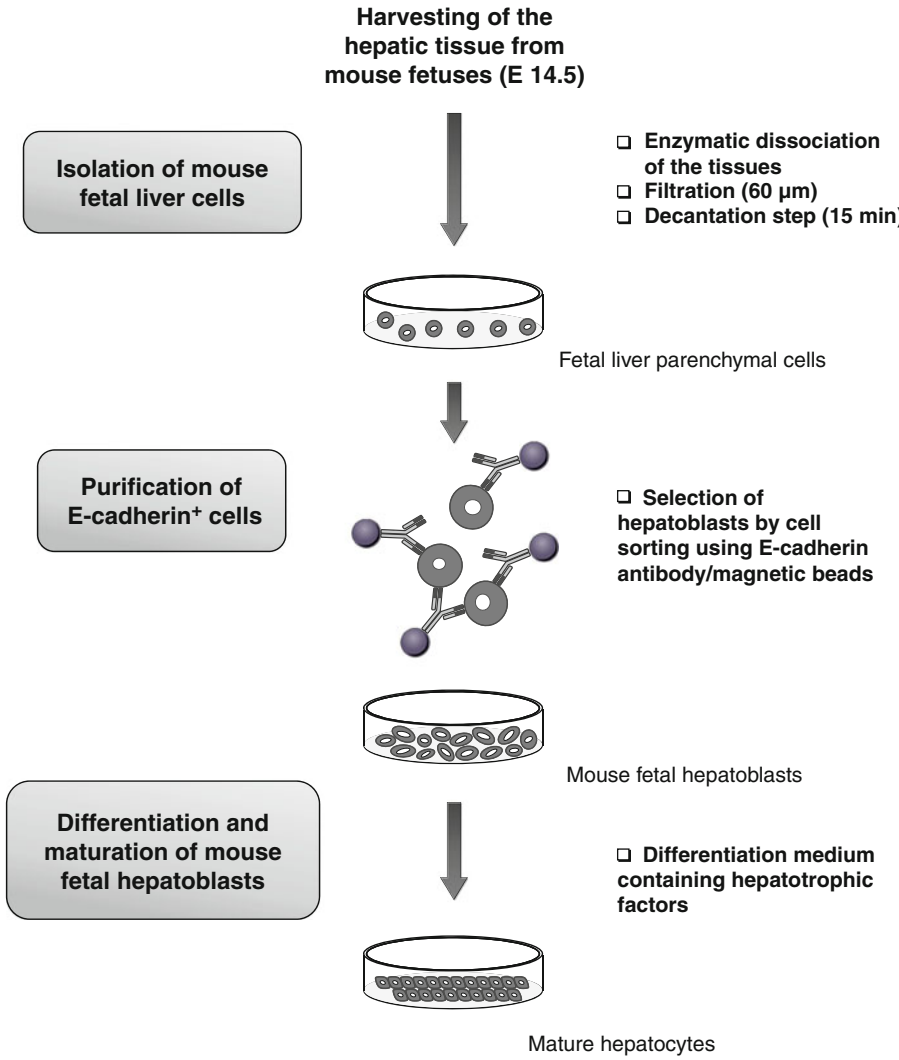

Fig. 2. Schematic representation of the MFHs isolation/purification method.

and keep the fetal livers in HEPES solution on ice. Prepare in advance all the required material as mentioned above.

2. After deep anesthesia, two pregnant mice (E 14.5) are sacrificed by cervical dislocation. Following opening of the abdominal cavity with surgical scissors, localize uterus containing the fetuses. Remove uterus carefully to avoid injury to the fetuses and store it in PBS on ice.
3. Extract each fetus with sterile thin forceps and transfer them to a 10-cm Petri dish containing cold HEPES buffer. Typically, 15–20 fetuses can be collected from two pregnant mice.
4. Using a stereo microscope system, dissect each fetus to isolate the liver, taking care to systematically remove all the surrounding/adjacent tissues that could contaminate the culture (see Note 9). Recognizing the fetal liver is simple due to its strong dark/red coloration (important presence of blood) compared to the rest of the fetus (white).
5. Next, transfer the livers into another dish containing clear cold HEPES and meticulously peel the hepatic capsule.
6. Finally, keep the harvested tissues in HEPES on ice until the end of the collection procedure.

3.2. Isolation of Parenchymal Hepatic Cells from Fetal Liver

1. Before starting the isolation protocol, add 100 μL of liberase enzyme (working aliquot) to the 10 mL of sterile warmed calcium–HEPES solution in order to make the dissociation buffer. Keep at 37°C. From this point, all experimental procedures have to be carried out under sterile conditions.
2. After transferring the collected livers into a 1.5-mL tube without medium, hash the samples thoroughly with thin and sterile surgical scissors.
3. Perform enzymatic dissociation as follows: Suspend tissue fragments in 10 mL of the liberase/calcium/HEPES digestion buffer (use a 50-mL tube) and incubate at 37°C for 5 min. Shake the tube gently every minute. Next, a physical dissociation step is required by softly pipetting the lysate several times using a 2-mL serological pipette. The final step to achieve the dissociation of liver fragments is an additional digestion for 5 min at 37°C (see Note 10). From this point onward, absolutely avoid any bubbles that could damage the isolated cells.
4. Add 25 mL of warmed basal medium (without growth factors) to dilute the enzyme and stop the digestion procedure.
5. Perform one filtration of the cell suspension through a 60-μm nylon filter. Wash the filter thoroughly with the basal medium to reach a final approximate volume of 100 mL.
6. Carry out one decantation by simply reserving the cell suspension in a 200-mL Erlenmeyer for 15 min at room temperature (see Note 11 for further information about this essential point).

7. Carefully discard the upper phase (approximately 3/4 of the total volume) with a 25-mL serological pipette.
8. Resuspend cells contained in the 20-mL remaining medium after adding up to 50 mL of the basal medium.
9. Distribute the suspension into two 50-mL round-bottom polypropylene tubes using a 25-mL pipette, afterward adjusting each tube to an equal volume of 40 mL.
10. Centrifuge the tubes at 1,000 rpm ($22 \times g$) for 2 min at 4°C.
11. Remove the supernatants and add 2× 250 µL of HEPES buffer plus calcium and 2% FBS on cells. A moderate pipetting using a 2-mL pipette is necessary to completely dissociate the cell pellet (see Note 12). Pool the suspensions contained in both tubes and keep in a single 50-mL round-bottom polypropylene tube at room temperature.
12. Count cells using a numeration system, typically an improved Neubauer hemocytometer, and determine the cell density (see Note 13). Use trypan blue to assess the viability of isolated cells (see Note 14).

3.3. Purification of E-Cadherin Positive MFHs

The number of gathered cells after the dissociation/decantation phase can greatly vary regarding the number of fetuses collected and/or the experimental conditions applied during the liberase treatment. To achieve an effective cell sorting, cell density needs to be precisely adjusted. Here, we describe the method used for processing a cell suspension at the concentration of 10^7 cells for 100 µL for a total volume between 0.5 and 2.5 mL ($5–25 \times 10^7$ cells) (see Fig. 3).

1. First, prepare and place the cells in a 5-mL round-bottom polystyrene tube (12 × 75 mm) after performing the appropriate dilution in the cell sorting medium (HEPES–calcium–2% FBS).
2. Add 1 µL/10^7 cells of the mouse blocking antibody provided with the kit to the suspension and mix well by tapping the tube (see Note 15).
3. Add the biotinylated primary antibody (anti-E-cadherin) at a final concentration of 10–20 µg/mL (2–4 µL/10^7 cells/100 µL, Biotin anti-CD324, eBioscience) and mix carefully (see Note 16). Incubate at room temperature for 15 min. Tap the tube every few minutes to put sedimented cells into suspension.
4. Put into the cell suspension the biotin selection cocktail at 10 µL/10^7 cells. Keep 15 min at room temperature and sometimes homogenize.
5. Add 5 µL/10^7 cells of magnetic nanoparticles, mix well, and incubate at room temperature for 10 min taking care to resuspend the cell pellet when necessary. Ensure the homogeny of the

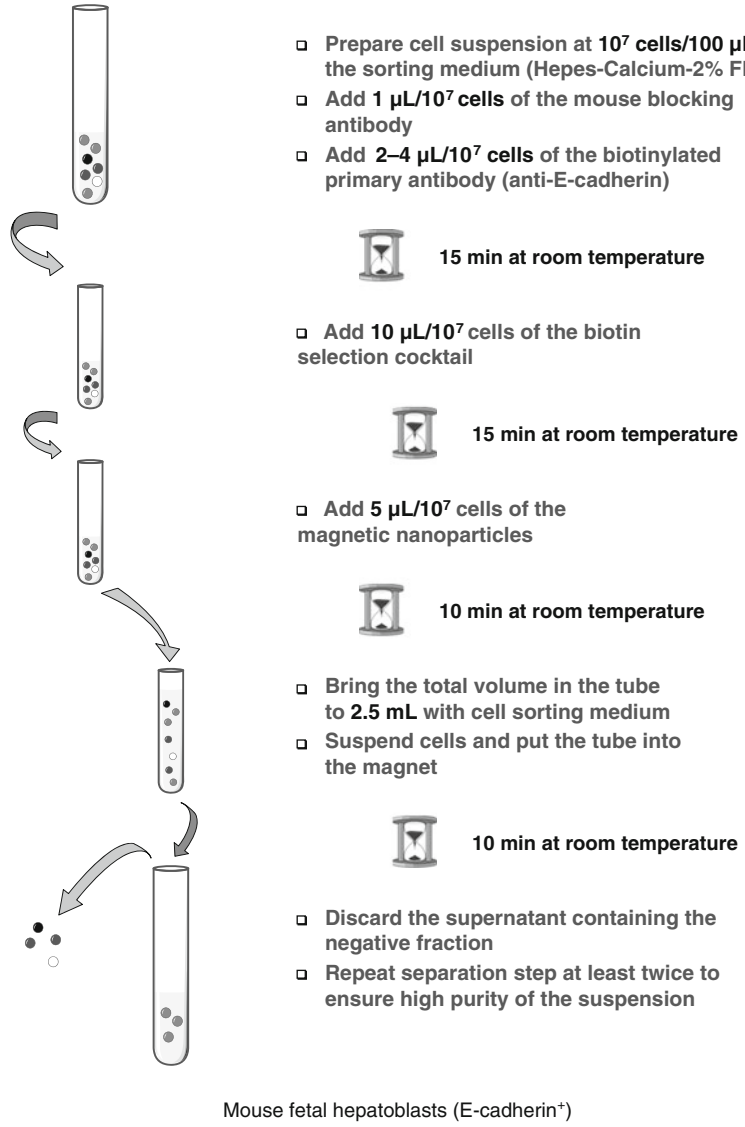

Fig. 3. Experimental protocol for MFHs sorting.

beads by pipetting the solution vigorously and several times before use.

6. Bring the total volume of the cell suspension to 2.5 mL by adding an adequate volume of cell sorting medium.
7. Resuspend cells by pipetting up and down three times with a 2.5-mL serological pipette. Immediately, put the uncapped tube into the magnet and keep at room temperature for 10 min.
8. Discard the negative fraction by inverting the magnet and the tube for 3 s (see Note 17). Keep the negative fraction if needed.

9. Remove the tube from the magnet and immediately add 2.5 mL of HEPES/calcium/2% FBS medium. Suspend cells by gently pipetting as mentioned above and place the tube back into the magnet for 10 min.
10. Repeat steps 7–9 at least once to ensure effective cell purification (see Note 18 for further information relating to purity).
11. Resuspend the positive selected cells in 500 µL of complete medium containing the hepatic factors, transfer the enriched sample into a new tube and determine the cellular density and viability.

3.4. Differentiation and Maturation of MFHs

The E-cadherin$^+$ cells are seeded on collagen type I-coated dishes with a 35-mm diameter at a density of 2×10^5 cells/cm^2. 24 h after plating, the attached cells are washed twice with the basal medium (devoid of growth factors). Then, the basal medium is replaced by 2 mL of the complete medium in each dish. As described in the previous section, the medium employed for primary culture is based on a mixture of William's E medium, L-glutamine, and 10% of FBS, supplemented with appropriate factors promoting hepatic differentiation that include HGF, EGF, and OSM. During the culture period, the cells are incubated at 37°C in a humid atmosphere containing 5% CO_2. The medium is changed entirely every each days. After cell attachment, MFHs do not require FBS and EGF anymore, and these growth factors can be removed from the medium after 48 h of culture. Following plating on collagen type I, MFHs proliferate to rapidly reach confluence after 3 or 4 days of culture and adopt a compact morphology exhibiting a significant cuboidal appearance (Fig. 4a). In the presence of hepatic factors, undifferentiated cells undergo relative changes of phenotype which include the reduction of growth and the appearance of an oval cell-like shape characterized by a large elliptical nucleus and a slight cytoplasm (Fig. 4b). Finally, oval cells form pronounced cell aggregates and adopt the typical morphology of mature hepatocytes with a small round nucleus and a dark cytoplasm (Fig. 4c, d).

3.5. Characterization of MFHs

Hepatotrophic factors and cellular density both appear to condition the terminal differentiation of MFHs *in vitro*. By using a defined hepatospecific medium, we demonstrated that isolated E-cadherin$^+$ liver fetal cells express increased levels of albumin, whereas α-fetoprotein rates decline (Fig. 5). The profiles displayed by both markers are specific to the transition scheme observed in the case of liver regeneration or when hepatic progenitors differentiate into mature hepatocytes during development. Furthermore, the expression of glucose-6-phosphatase which characterizes typically adult mature hepatocytes is strongly enhanced during the induced-differentiation process of purified MFHs (Fig. 6). Notably, MFHs are also characterized by a concomitant expression of α-fetoprotein and cytokeratin 19 (CK19) at the early stage of culture. CK markers are commonly

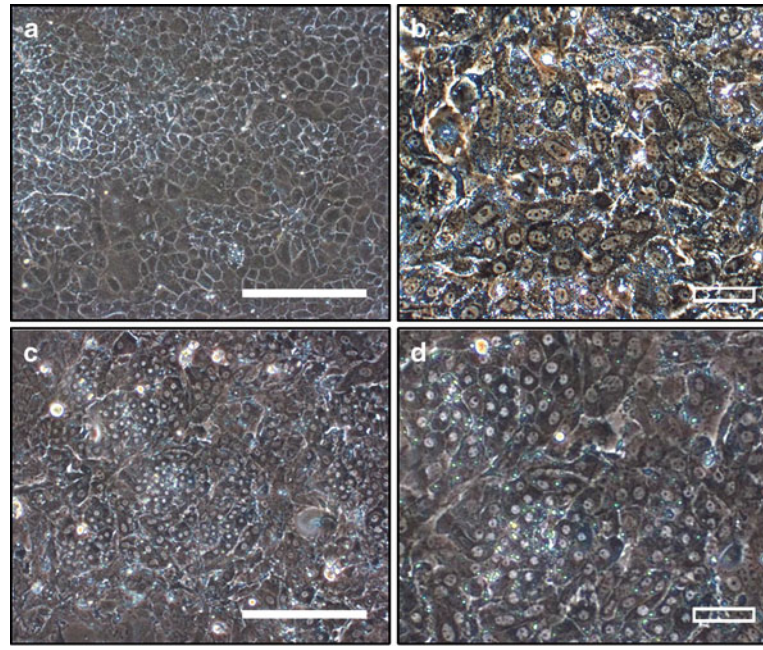

Fig. 4. Primary cultures of mouse fetal hepatoblasts after E-cadherin cell sorting. (**a**) Confluent monolayer formed by the hepatic progenitors 3 days after seeding. (**b**) In the presence of hepatotrophic factors, MFHs undergo radical changes of phenotype from day 5 and adopt an oval cell-like morphology. (**c**, **d**) Mature hepatocytes derived from E-cadherin$^+$ MFHs after 12 days of culture. *Scale bars* are respectively 200 μm (**a**, **c**) and 50 μm (**b**, **d**).

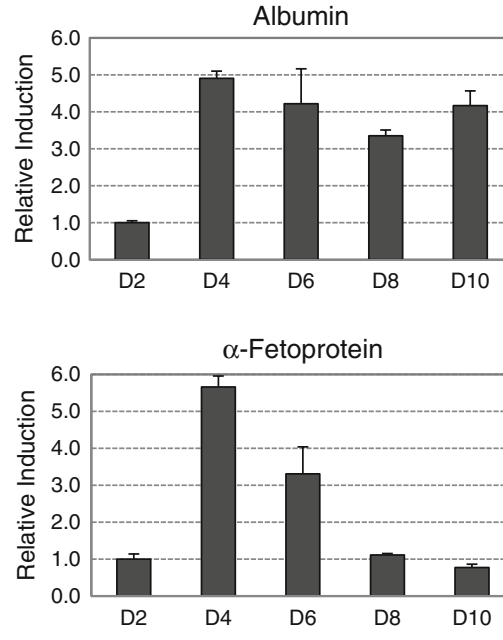

Fig. 5. Expression levels of albumin and α-fetoprotein during MFHs maturation. E-cadherin$^+$ MFHs were isolated from E 14.5 fetal livers after performing a purification procedure. Total RNAs were harvested after cell seeding at the indicated times. cDNA were synthesized from 1 μg of total RNA of each fraction. After an initial denaturation at 95°C for 2 min, the thermal cycles of quantitative real-time PCR were repeated 40 times as follows: 95°C for 15 s, 60°C for 30 s. The housekeeping gene GAPDH was used as an internal control to normalize the amount of cDNA.

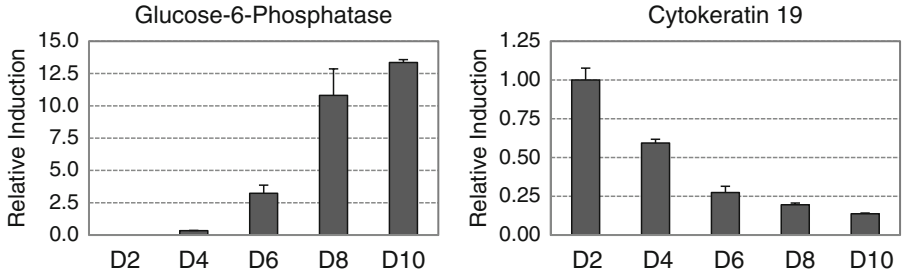

Fig. 6. Expression levels of the hepatospecific enzyme glucose-6-phopshatase and the biliary epithelial cell marker cytokeratin 19 (CK19) during the maturation process of MFHs.

associated with epithelial cells of the biliary tract, such as cholangiocytes. This point argues for the bimodal ability of MFHs to differentiate into the hepatic and the biliary lineage. To summarize, the present data based on the expression profiles of the major hepatic markers show that E-cadherin cell sorting from the embryonic liver is a valuable method for making the isolation of bipotent hepatoblasts possible. MFHs are able to proliferate and are also responsive to growth factor stimulation leading to the induction of hepatic maturation.

4. Notes

1. Animals were maintained in an isolator unit under a constant temperature of 20°C and a 12-h light–dark cycle. Mice received standard sterilized food and water ad libitum. All experiments were carried out in accordance with national laws and institutional regulations.
2. Reconstituted HGF, EGF, and OSM are stable at least 3 months when stored at –20°C and up to 15 days at 4°C. Avoid repeating freezing/thawing cycles by making appropriate aliquots.
3. The bovine insulin requires acidic conditions in order to be dissolved.
4. After reconstitution, working aliquots of insulin, dexamethasone, and hydrocortisone can be stored at 4°C up to 3 weeks as well as several months at –20°C. Repeated freezing and thawing is not recommended.
5. The complete medium for hepatoblast primary culture must be used within 2 weeks of its preparation. To avoid degradation of growth factors, warm at 37°C only the volume required for each experiment (cell seeding or medium change).
6. The reconstituted liberase is stable at –20°C for up to 3 months. To avoid repeated freezing and thawing, highly damageable for the enzyme, make stock solution in single-use aliquots.

Furthermore, prepare the liberase–HEPES solution just before performing liver dissociation to avoid enzyme degradation.

7. Calcium is necessary to insure the enzymatic activity of liberase. This cation acts as a cofactor and stabilizes the enzyme. In addition, protease inhibitors, serum, and bovine albumin inhibit the performance of liberase and must be excluded from the dissociation medium.
8. E-cadherin is a transmembrane glycoprotein exhibiting a basic structure in its extracellular portion that is composed of repeating domains, each with two consensus Ca^{2+}-binding motifs. Consequently, the addition of calcium in the cell sorting medium ensures E-cadherin integrity.
9. Tissues surrounding the fetal liver represent a major cause of contamination by fibroblasts in primary cultures, making it crucial to perform the most complete dissection of each liver under a microscope system to remove contaminants (portal vein, intestines, etc.).
10. The duration of the enzymatic digestion can vary slightly depending on the aspect of the solution containing tissue fragments. As a rule, the dissociation medium must become cloudy and brown at the end of the procedure traducing the effective dissociation of the hepatic parenchyma. If not, increase the time of enzymatic treatment or use a fresh liberase solution.
11. The decantation procedure is essential to remove from the cell suspension all hematopoietic and nonparenchymal cells. Indeed, the fetal liver is a transient niche of hematopoiesis during the embryonic development and produces massive amounts of blood cells. The decantation step enables a soft sedimentation of the hepatoblasts which typically aggregate to form heavy clusters descending to the bottom. On the contrary, single blood cells and nonparenchymal cells stay in the upper phase of the suspension and can easily be discarded.
12. As mentioned above, hepatic progenitors tend to form aggregates in the presence of divalent cations such as Ca^{2+} that is contained in the medium or HEPES buffer. In consequence, a soft physical dissociation phase is necessary. However, avoid the use of 1-mL tips that could damage the cells, and opt for an automatic system with a 2-mL serological pipette.
13. To prevent the formation of clusters that occurs after tissue dissociation. To facilitate cell counting, use trypsin solution (0.05%+EDTA) when diluting the cell suspension in order to prevent the formation of clusters that occurs after tissue dissociation.
14. Several factors can condition the viability of hepatic progenitors after the isolation and the purification stages. However, the number of viable cells observed remains suitable when following the standard procedure described previously. See Subheading 5 for further details regarding cell viability.

15. It is essential during the procedure to limit cell clumping and sedimentation that could decrease the purification/recovery rate. To ensure cell suspension, tapping the tube is highly preferable to several pipetting steps.
16. In order to achieve an optimal purity and recovery, it is necessary to perform antibody titration prior to carrying out cell sorting. The number of selected cells may be higher with an increased amount of antibody. However, an antibody excess may reduce the purity.
17. During removal of the negative fraction and during washing steps, it is recommended not to shake or blot off any drops that might form at the top of the tube.
18. At least three separation stages into the magnet are essential to isolate E-cadherin-positive cells with high purity. Additional separation rounds may improve the purity. However, it will decrease recovery.

5. Important Remarks

1. Purified collagenase that compounds liberase enzyme requires calcium. The exposure of liberase to divalent cation chelators like EDTA removes calcium leading to the inactivation of the enzyme.
2. Numerous critical points can contribute to reducing the viability of isolated cells. In order to obtain a higher viable cell rate (1) limit ischemia by performing the isolation/purification protocol in the briefest time; (2) absolutely avoid the formation of bubbles during pipetting; (3) employ the use of 2-mL serological pipettes rather than 1-mL tips; and (4) adhere to the concentration of liberase and the incubation conditions during the enzymatic dissociation of the fetal livers.
3. Cellular density is a crucial point conditioning hepatic differentiation of liver progenitors. During seeding, take care to ensure optimal homogeny of plated cells in order to avoid high density areas that correlate to a quick over-confluence, as well as low density zones leading to a significant delay in the differentiation and the maturation process of hepatoblasts.

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