

A ROBUST, FAST, AND REPRODUCIBLE METHOD FOR ISOLATING HEPATIC STELLATE CELLS FROM C57/BL6 MICE

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Manuscript received: November 2017

Abstract

Fibrosis is defined by the scarring of various tissues and is attributed to excess deposition of extracellular matrix (ECM) components. One of the main contributors to ECM deposition during liver fibrosis, the hepatic stellate cells (HSCs), are localized in the space of Disse, a space situated between the basolateral side of the hepatocytes and the endothelial cells. During liver fibrosis, in response to aggressive factors that induce liver injury, HSCs activate, contributing to an exaggerated deposition of scar tissue in the liver. The aim of this study was to develop an improved HSC isolation technique in order to obtain a constant number of pure and viable HSCs from C57/BL6 mice. Using our protocol we managed to isolate, in average, $(1.02 \pm 0.34 \times 10^6)$ HSCs *per* mouse ($n = 11$). Subsequently, the isolated HSCs were characterized, their purity was assessed, and they were used in different types of *in vitro* experiments. The improved HSC isolation protocol we developed was reproducible, less time-consuming, and relatively easy to perform.

Rezumat

Fibroza este definită prin cicatrizarea diferitelor țesuturi și este atribuită excesului de depozitare a componentelor matricei extracelulare (MEC). Printre principalii contribuabili la depunerea MEC în timpul fibrozei hepatice se numără celulele hepatice stelate (CHS), celule localizate în spațiul lui Disse, un spațiu situat între partea bazolaterală a hepatocitelor și al celulelor endoteliale. În timpul fibrozei hepatice, ca răspuns la factori agresivi, CHS se activează, contribuind la o depunere exagerată de țesut cicatricial în ficat. Scopul acestui studiu a fost de a dezvolta o tehnică îmbunătățită de izolare a CHS, folosită pentru a obține un număr constant de CHS viabile și cu o puritate acceptabilă de la șoareci de tip C57/BL6. Folosind protocolul nostru, am reușit să izolăm, în medie, $(1,02 \pm 0,34 \times 10^6)$ CHS *per* animal ($n = 11$). Ulterior, celulele izolate au fost caracterizate, puritatea lor a fost evaluată și acestea au fost utilizate în diferite tipuri de experimente *in vitro*. Protocolul nostru de izolare a CHS este reproductibil, rapid și relativ ușor de realizat în orice laborator care are echipamentele necesare.

Keywords: liver fibrosis, hepatic stellate cell, isolation protocol, auto-activation, myofibroblast, C57/BL6 mice

Introduction

Liver fibrosis is defined as the replacement of liver parenchyma with extracellular matrix (ECM). It has been proposed that 45% of deaths in the western world are related to fibro-proliferative diseases, including liver fibrosis [1]. Fibrosis is the wound-healing response to different aggressive factors such as infections, drugs or metabolic disorders. In healthy conditions, an acute hepatic injury is resolved in a short time after the removal of the aggressive factor. By contrast, persistent liver injury leads to a massive deposition of ECM, also known as scar tissue. It has been shown that the hepatic stellate cells (HSCs) are the main source of myofibroblasts contributing to the excessive ECM deposition during liver fibrogenesis [2]. HSCs are localized in the space of Disse, a space situated between the

basolateral side of the hepatocytes and the endothelial cells of the blood vessels [3]. They present a star-shaped morphology which allows their easy recognition [4, 5]. In response to liver injury, HSCs change their phenotype from a quiescent, lipid-droplet containing cell, to a myofibroblast-like, metabolically active and proliferative cell. This process, commonly called HSC activation, is responsible for the exaggerated deposition of scar tissue in the liver [6]. Interestingly, when freshly isolated HSCs are cultured, they auto-activate, mimicking the *in vivo* activation observed during hepatic fibrosis. This activation leads to the upregulation of fibrogenic genes of collagens such as type 1, $\alpha 1$ (Col1a1) and type 3, $\alpha 1$ (Col3a1), as well as for α smooth muscle actin (α SMA), considered to be the main markers of HSC activation [7]. It is important to point out that, although the *in vitro*

activation observed when culturing HSCs shows similarities to the *in vivo* process, the gene expression patterns are not exactly the same [8]. Nevertheless, isolating and culturing HSCs remains a valuable tool to understand the mechanisms involved in their activation and the identification of key factors which take part in this process. The isolation of HSCs was first achieved in rats but has recently focused mainly on mice due to a shift in the momentum of research towards a wide range of genetically modified animal models. Even though the BALB/c mice breed has been extensively used for isolation of HSCs due to higher yields, C57/BL6 are more often used to create transgenic mice. Therefore, developing a robust, simple, and reproducible method to isolate HSCs from these mice is needed.

Despite the fact that HSCs represent 10 to 15% of the total resident cell populations in a healthy liver, due to their peri-sinusoidal localization, their isolation is a process that requires multiple steps and therefore presents an important inter-individual variation in terms of cell yield and purity [9, 10]. In general, most protocols described in the literature are based on 3 consecutive steps: (1) *in situ* Pronase/Collagenase perfusion of the liver; (2) followed by *in vitro* digestion; and (3) density-based gradient separation of HSCs from the rest of the cell populations from the liver [11-13].

The aim of this study was to develop an improved protocol to isolate HSCs from C57/BL6 mice, which could be easily reproduced in other laboratories. One of our main objectives was to increase the total number of isolated HSCs by modifying centrifugation settings and digestion conditions, as well as using simple and easily obtainable consumables, reducing the cost of the protocol. The second objective was to develop a protocol which allowed the isolation of viable HSCs to be used for subsequent *in vitro* experiments such as cell behaviour studies (activation kinetics, treatment with different compounds, etc.) and gene manipulation (transduction with adenoviruses).

Materials and Methods

Isolation of HSCs from 4 healthy C57/BL6 mice.

This perfusion protocol is adapted for the isolation of HSCs from 4 healthy C57/BL6 mice. Rinse the tubing with the HEPES buffer and fill the bubble trap (pump flow at 5 mL/minute measured in a falcon tube using the timer), set the water bath in which you will keep all the solutions used for the perfusion at 40°C. Anesthetize the animal by intra-peritoneal injection with the ketamine-xylazine-PBS mix (4:1:5 ratio), around 150 - 200 µL for 1 mouse. Check the anaesthesia of the animal by paw pinching. Attach the animal on a dissection board and wet the abdominal part with pure medicinal

alcohol. Perform a laparotomy along a vertical line and the ventral sides. Incise the abdominal wall without touching the organs. Check that the liver is well detached from the abdominal wall (help it go down by lightly tapping on the abdominal wall). Open the sides of the abdominal cavity in a very wide angle (well clear at the chest). Using a cotton swab move the intestines to the right side to see the portal vein and inferior vena cava. Leave the swab on the intestines. Place a plier under the left paws of the animal to maintain the clamp in height. Moisten the opened abdominal cavity with PBS. The following 4 operations must be done as quickly as possible but without haste. Perforate the inferior vena cava with the winged needle, remaining parallel to the vein and with a steady hand motion. Attach the needle with the clamp, lock the infusion and put the clamp on the needle gently and slowly. Start the perfusion pump. Cut the portal vein (you should see the blood and buffer flow and an instant liver discoloration to clear brown (removal of the blood). Cut the diaphragm and trap the inferior vena cava with curved pliers. Check the flow, check the clamp is well attached, be careful not to move the tubes. Ligate with a thread. Wash the liver with 20 - 40 mL of S1. Quickly move the hose in the Pronase solution and perfuse the liver with 15 mL (for a fibrotic mouse use up to 25 mL of pronase). Quickly move the hose in the Collagenase solution and pursue the digestion of the liver with 15 mL (for a fibrotic mouse use up to 25 mL of pronase).

Recuperate the liver in a sterile crystallizer in 5 to 10 mL of the Pronase + Collagenase solution (P + C solution). Use a pair of scissors to notch the liver lobes and a pair of forceps to gently shake the liver in the solution in order to empty the lobes. Recuperate the solution in a 50 mL Falcon. Repeat this step until all the P + C solution (30 mL) is used. Recuperate as many cells from the liver as possible. Close the lid and add paraffin to waterproof the tube (this step should take from 4 to maximum 7 min). Put the cell solution in a water bath for 15 min at 37°C under low agitation speed. Prepare two 50 mL Falcons, one filter of 100 µm, and a funnel. Filter the cell solution first through the 100 µm filter. Distribute the filtered cell solution and split it in two Falcon 50 mL tubes. Add S2 up to 50 mL and centrifuge at 15°C for 8 min at 630 G. Remove slowly 35 mL of the supernatant. Add 100 µL of DNase I solution, resuspend the pellet gently and add solution S2 up to 50 mL. Centrifuge again at 15°C, 8 min, 630 G. In 2 gradient centrifugation tubes (SW41) add 5.5 mL of the gradient solution and keep in the fridge until the ultracentrifugation step. After the second centrifugation, slowly aspirate the supernatant until 5 mL of cell suspension is left in the tube. Gently resuspend all the cells from the white layer using a plastic pipette. Be certain that

the cell pellet is well resuspended, pool the two Falcon tubes and complete the volume to 12 mL with solution S2. Slowly add 6 mL of this cell solution to each gradient ultracentrifugation tube without mixing the 2 phases. Balance the tube with the solutions with the help of a Pan Balance or scales and place the tubes carefully in the ultracentrifugation rotor to not spill the cell solution. The parameters for the ultracentrifugation are speed = 20.000 G, time = 30 min, temp = 4°C, with slow acceleration and deceleration.

After the ultracentrifugation, take the tubes slowly out of the tube holder. You will notice a small

white band in the middle of the tube, as seen in Figure 1C. The white band represents the HSC fraction. Slowly aspirate the supernatant until the white band. Using a plastic pipette gently recuperate the entire white band from each of the 2 tubes and put the cell fraction in a 15 mL Falcon tube. Count the cells and plate them in culture in Dulbecco's modified essential medium (Life Technologies, USA) containing 10% foetal bovine serum, 1% HEPES buffer and 1% penicillin/streptomycin at a density of 2×10^4 cells/cm².

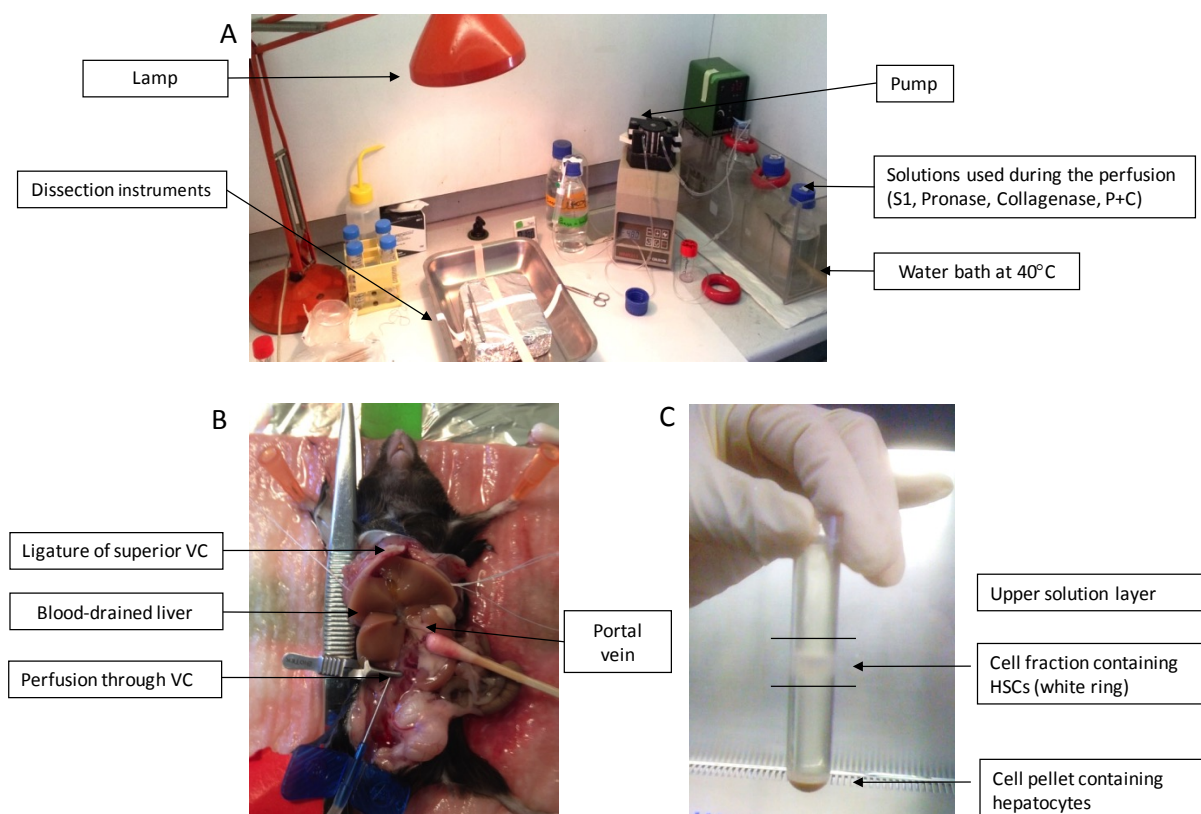


Figure 1.

Perfusion set-up and cell fractions after centrifugation

A. Perfusion set-up. Materials used for perfusion include dissection board, dissection instruments, perfusion needle and line, sterile Falcon tubes, sanitary alcohol for sterilization of surgical field, crystallizer (for *ex situ* liver digestion), duct tape, perfusion pump, lamp, water bath, and the solutions used for *in situ* washing and digestion of the liver (Solution S1, Pronase, Collagenase, and P + C solutions). **B.** *In situ* liver digestion. Liver perfusion was performed through the VC. The perfusion needle was clamped in order to maintain solution flow pressure. Ligation of the VC was performed through the rib cage to prevent loss of solution and to maintain pressure throughout the perfusion line. **C.** HSC fraction. Different cell fractions were obtained following gradient ultracentrifugation. In the middle part of the gradient solution, a characteristic HSC white ring can be observed. The upper part contains KCs and LSECs, while the cell pellet contains mainly dead hepatocytes. HSC, hepatic stellate cell; P + C, pronase plus collagenase solution; VC, vena cava; KC, Kupffer cells; LSEC, Liver sinusoidal endothelial cells.

Buffers preparation

Histodenz solution preparation. Dissolve 7.175 g of Histodenz (5-(N-2,3-Dihydroxypropylacetamido)-2,4,6-triiodo-N,N'-bis(2,3-dihydroxypropyl) isophthalamide, MW: 821.14, Sigma-Aldrich, USA) in 25 mL of

GBSS₁ 1X solution (Grey's balanced salt solution; calcium chloride 10% v/v; magnesium chloride 10% v/v, GBSS₁ 10X 10% v/v, HEPES (4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid) 1M, 1% v/v, as presented in Table I).

Table IGrey's balanced salt solution 1 10X (GBSS₁ 10X)

Reagent	Final concentration (mM)	Quantity (for 250 mL of ddH ₂ O)
KCl	49.6	925 mg
Na ₂ HPO ₄	4.1	300 mg
KH ₂ PO ₄	2.2	45 mg
D-glucose	55	2.5g
NaHCO ₃	27	567.6 mg

The solution is prepared by dissolving the components of the recipe in 250 mL of ddH₂O, the pH is adjusted to 7.3 - 7.4 and the solution is filtered through a 0.2 µm bottle top filter. KCl, potassium chloride; Na₂HPO₄, sodium diphosphate anhydrous; KH₂PO₄, potassium phosphate monobasic; NaHCO₃, sodium bicarbonate.

Mix 11.1 mL from this solution with 28.9 mL of GBSS₂ 1X solution (calcium chloride (CaCl₂) 10% v/v; magnesium chloride (MgCl₂), 10% v/v, GBSS₂ 10X, 10% v/v, HEPES 1M, 1% v/v, as presented in Table II) to prepare a volume of 40 mL Histodenz gradient at 8.2% final concentration (density = 1.043 ± 0.0023). Store at 4°C.

Table IIGrey's balanced salt solution 2 10X (GBSS₂ 10X)

Reagent	Final concentration (mM)	Quantity (for 250 mL of ddH ₂ O)
NaCl	1.37 M	20 g
KCl	49.6	925 mg
D-glucose	5.5	2.5 g
NaHCO ₃	2.7	557.6 mg
NaH ₂ PO ₄	2.5	75 mg
Na ₂ HPO ₄	4.1	300 mg

The solution is prepared by dissolving the components of the recipe in 250 mL of ddH₂O, the pH is adjusted to 7.3 - 7.4 and the solution is filtered through a 0.2 µm bottle top filter. NaCl, sodium chloride; KCl, potassium chloride; MgCl₂•6H₂O, magnesium chloride hexahydrate; MgSO₄•7H₂O, magnesium sulphate heptahydrate; Na₂HPO₄, disodium phosphate anhydrous; NaHCO₃, sodium bicarbonate; CaCl₂•H₂O, calcium chloride dihydrate; NaH₂PO₄, monosodium phosphate anhydrous.

Enzyme solution preparation (for 4 mice with solution reserve for 1 mouse)

DNase I solution. Prepare DNase I (10mM Tris-HCl, 2.5 mM MgCl₂, 0.5 mM CaCl₂, pH 7.6 at 25°C, Roche, Germany) solution at 2 mg/mL in enzyme buffer solution. Store at 4°C.

Pronase solution. Dissolve 30 mg of Pronase (7 U/mg; ref: 70456921, lot: 70456921, Roche, Germany) in 100 mL of Solution S₂. The solution needs to be prepared and used *ex tempore*.

Collagenase solution. Dissolve 20 mg of Collagenase P (1.6 U/mg, ref: 11213865001, lot: 10089625, Roche, Germany) and 54 mg of BSA (bovine serum albumin, Sigma-Aldrich, USA) in 100 mL solution S₂. The solution needs to be prepared and used *ex tempore*.

Pronase + Collagenase solution. Dissolve 75 mg of Pronase, 65 mg of Collagenase and 180 mg of BSA in 120 mL solution S₂ and add 1 mL of DNase solution. The solution needs to be prepared and used *ex tempore*.

Anesthesia solution. Prepare a solution of Ketamine (ketamine hydrochloride 50 mg/mL Bayer, Germany): Xylazine (xylazine hydrochloride, 12 mg/mL, Virbac, France): PBS (Phosphate buffer solution, 10 mM PO₄³⁻, 137 mM NaCl, and 2.7 mM KCl, Sigma-Aldrich, USA) with a mix ratio of 4:1:5.

Final pH must be between 7.3 and 7.4 at 37°C for the Pronase, Collagenase, Pronase + Collagenase, S₁ and S₂ solutions. All solutions need to be filtered through a 22 µm sterile filter.

The recipes for solutions S₁ and S₂ can be found in Tables III and IV respectively.

Animals. C57/BL6 mice were used at 32 weeks of age (Old Breeders from Charles River Laboratories). Mice were fed a standard chow diet and weighed around 28 - 30 g at the time of the experiments. All the animals were kept at CEF (*Centre d'Explorations Fonctionnelles* of the CRC, Agreement no. A75-06-12). They were maintained in a constant temperature and humidity in light controlled rooms with a 12 hours light cycle. They had free access to food (SAFE Laboratory) and tap water.

Table IIIRecipe for the preparation of Solution S₁

Reagent	Final concentration (mM)	Quantity (for 250 mL of ddH ₂ O)
NaCl	136.86	2 g
KCl	5.36	100 mg
NaH ₂ PO ₄ •2H ₂ O	0.55	22.04 mg
Na ₂ HPO ₄	0.84	30.11 mg
NaHCO ₃	4.16	87.5 mg
HEPES	9.98	595 mg
EGTA	0.49	47.5 mg
Glucose	4.99	225 mg

The solution is prepared by dissolving the components of the recipe in 250 mL of ddH₂O. The pH is adjusted to 7.3 - 7.4 and the solution is filtered through a 0.2 µm bottle top filter. NaCl, sodium chloride; KCl, potassium chloride; NaH₂PO₄•2H₂O, sodium phosphate monobasic dihydrate; Na₂HPO₄, sodium biphosphate anhydrous; EGTA, ethylene glycol bis(β-amino-ethyl ether)-N,N',N',N'-tetraacetic acid.

Table IVRecipe for the preparation of Solution S₂

Reagent	Final concentration (mM)	Quantity (for 1 L of ddH ₂ O)
NaCl	136.86	8 g
KCl	5.36	100 mg
NaH ₂ PO ₄ •2H ₂ O	0.55	88 mg
Na ₂ HPO ₄	0.84	120 mg
HEPES	9.98	595 mg
NaHCO ₃	4.16	350 mg
CaCl ₂ •H ₂ O	4.34	560 mg

The solution is prepared by dissolving the components of the recipe given in the table in 1 liter of ddH₂O. The calcium chloride dihydrate (CaCl₂•H₂O) is slowly added at the end, after all the other components were solubilized. The pH is adjusted to 7.3-7.4 and the solution is filtered through a 0.2 µm bottle top filter. NaCl, sodium chloride; KCl, potassium chloride; NaH₂PO₄•2H₂O, sodium phosphate monobasic dihydrate; Na₂HPO₄, sodium biphosphate anhydrous; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; NaHCO₃, Sodium bicarbonate; CaCl₂•H₂O, calcium chloride dihydrate.

Equipment. Perfusion pump (Glison, Minipulse 3, Model M312; Glison, USA); perfusion line, ref.: PER3F, lot: 0723-A (Didactic, France); blood vessel clamp (GP Supplies, UK); perfusion needles (BD Valu-Set, 23G) (GP Supplies, UK); ater bath (up to 40°C); syringes (1 mL) (GP Supplies, UK); sterile filters (100 µM) (ThermoFisher, France); hot plate with magnetic stirrer (Fisher Scientific, France); pH meter (Mettler Toledo, France); falcon tubes (Fisher Scientific, France); Ultra-Clear™ ultracentrifugation tubes $9/16 \times 3\frac{1}{2}$ IN (Beckman Coulter, USA); crystallizer (Fisher Scientific, France); EMD Millipore Steritop Sterile Vacuum Bottle-Top Filters (Merck, France); centrifuge for normal Falcon tubes; ultracentrifuge with an SW41 rotor (Beckman Coulter, USA); Pan Balance or scales.

Procedure. In the following paragraphs, we described the steps taken in setting-up the equipment, performing the perfusion and isolation procedures, as well as washing and culturing the obtained HSCs.

Equipment setup. Adjust the perfusion pump to a flow of 5 mL/min. Wash once with sterile water and fill the system with solution S₁. The temperature of the water bath needs to be maintained at 40°C during the whole procedure. An example of the equipment setup can be visualized in Figure 1A.

In situ digestion. Before starting the perfusion, pre-heat solution S₁, Pronase solution and Collagenase solution in the water bath at 40°C. Rinse the perfusion tubing with solution S₁, fill the bubble trap of the system and eliminate all the bubbles from the tubing. Anesthetize the mouse by intraperitoneal injection using anaesthesia solution (approximately 150 - 200 µL *per* mouse). Fix the mouse using tape to attach extremities on the dissection board and perform a laparotomy to expose the abdominal cavity with the liver. Move the visceral organs using a wet cotton swab to expose the inferior vena cava (IVC) and the portal vein. Cannulate the IVC with a 25G catheter, clamp it, and without delay cut the portal vein. Next, cut the diaphragm and ligate the IVC above the diaphragm to optimize the liver perfusion. At this point, the liver is discoloured and pale. Wash the liver with approximately 15 - 20 mL of solution S₁. Quickly switch to Pronase solution and perfuse the liver with 15 mL of Pronase solution. Change quickly to the Collagenase solution and continue to perfuse with 15 to 20 mL solution. Carefully remove the liver from the abdominal cavity (do not cut any part of the digestive tract to avoid contamination of the cell fraction). Place the liver in a sterile crystallizer and add 10 mL of the P + C solution. An example of the steps described above can be visualized in Figure 1B.

In vitro digestion of mouse liver. Empty the liver in the crystallizer, in 10 mL of P + C solution using a pair of scissors to notch the liver lobes and a pair of

forceps to gently shake the liver in the solution in order to transfer the liver cells into the solution. Recuperate the cell solution in a sterile 50 mL Falcon tube. Repeat this step 3 times with the remainder of P + C solution until the liver has no more cell mass (the emptying of the liver should last 4 to 7 min.). Incubate the 30 mL cell solution in a 50 mL Falcon tube in a preheated shaking water bath at 37°C for 15 min. Filter the solution through a 100 µm sterile filter, split the 30 mL of cell solution in two (in new 50mL falcon tubes), add 100 µL of DNase I solution *per* tube and complete with solution S₂ to 50 mL. Then centrifuge the cell solution for 8 min at 630 G (15°C). Aspirate 40mL of the supernatant, add 100 µL of DNase solution, resuspend the cell pellet gently with a sterile plastic Pasteur pipette, complete to 40 mL with solution S₂, and centrifuge the cell solution for 8 min at 630 G (15°C).

HSC cell fraction separation and culture or pellet

Cell fraction separation. Next steps are focused on separating the HSCs from the other liver cell types. Aspirate the supernatant until 7.5 mL, resuspend and pool the cell solution from the two tubes. Fill to 20 mL with solution S₂. Add 5.5 mL of cold Histodenz gradient solution to each of 4 ultracentrifugation tubes. On top of the Histodenz solution, slowly add approximately 5 mL of cell solution, without mixing the two phases. Next, balance the tubes two by two and ultracentrifuge the cell solution at 20,000 G for 30 min (4°C) with no break. Recuperate the white ring containing the HSC fraction situated in the middle of the tube.

Cell culture. After ultracentrifugation, use a sterile graded transfer pipette to collect the cells (2 to 4 mL *per* tube) and transfer them into a new 15 mL Falcon tube. HSCs were counted using a *Mallassez* counting cell and viability was assessed by vital staining method using trypan blue as a viability dye at a concentration of 0.1% (v/v). HSCs were subsequently cultured in Dulbecco's modified essential medium (DMEM, Life Technologies, USA) containing 10% foetal bovine serum (FBS, ThermoFisher), 1% HEPES buffer and 1% penicillin/streptomycin solution (referred as normal HSC medium). To avoid any loss of the cells number and viability by centrifugations for cleaning fraction from the gradient, plate the cells with gradient and change the medium 4 to 5 h after plating. Plate the cells into 12 well plastic dishes at a density of 2×10^4 cells/cm². Change the medium 4 to 5 hours after plating (in the case of non-adherent cells, wait up to 12 h to change the medium). Cell purity was assessed by analysing the expression of HSC specific cell markers, such as α -SMA and Desmin by RT-qPCR.

Cell pelleting. To directly extract the RNA after HSC isolation or stock the cells for later use (RNA or protein analyses), dilute the gradient solution containing HSC almost 5 times in DMEM and centrifuge for 8 min at 700 G at 4°C (preferably in a

15 mL Falcon tube). After the centrifugation, discard the supernatant slowly and freeze instantly in liquid nitrogen and store at -80°C . In case the supernatant contains a fraction of HSCs, do a second dilution in S2 buffer solution and centrifuge again, repeating the abovementioned steps.

Cell fraction purity. Freshly isolated HSCs pellets were lysed in Qiazol reagent (Qiagen, Germany) and were prepared for RNA extraction as *per* the Materials and Methods section. Subsequently, gene expression was quantified for specific cell markers, including Desmin for HSC, EGF-like module-containing mucin-like hormone receptor-like (F4/80) for Kupffer cells (KCs), and albumin for hepatocytes.

Activation kinetic. For activation experiments, mouse HSCs were isolated and cultured at a density of 2×10^4 cells/cm² on 12 well plastic dishes for up to 6 days in normal HSC medium (7.5×10^4 cells/well). They were harvested in Qiazol at days 2, 4, and 6 after plating for RNA extraction. The medium was changed 12 hours after plating, as well as every two days.

Adenoviral transduction. Cells were cultured in 500 μL of DMEM at a density of 2×10^4 cells/cm² and subsequently infected with an Adenovirus (AdEasy[®] recombinant virus system) expressing the green fluorescent protein (GFP) under the control of a CMV promoter at a concentration of 150 plaque-forming unit (pfu)/cell (mother solution concentration of 4×10^{10} Pfu/mL after amplification in HEK 293 cells and purification on a cesium chloride gradient). 500 μL of DMEM containing 2% HEPES buffer and 20% FBS and 2% penicillin/streptomycin solution was added to each well 2 hours after infection. At day 6, cells were harvested and RNA extraction was performed.

TGF β treatment. Cells were cultured at a density of 2×10^4 cells/cm² in normal HSC medium. After 24 hours of culture, cells were cultured in starvation medium (DMEM with 2% FBS, 1% HEPES buffer and 1% penicillin/streptomycin solution) and were treated at 24 and 48 h after medium change with mouse monoclonal TGF- β 1 recombinant protein (eBioscience ref: 14-8342-80 lot: 4281872) at 10 ng/mL (control were in the same starvation medium without TGF- β 1). Cells were harvested 72 hours after plating in Qiazol[®] for RNA extraction.

RNA extraction, RT and qPCR. Cells were lysed and total RNA was extracted from mouse livers, isolated primary cells (hepatocytes, KCs and HSC fractions) using Qiazol reagent. The first strand cDNA was synthesized from total RNA using M-MLV reverse transcriptase (ThermoFisher Scientific, USA) according to the manufacturer's protocol. Gene expression was measured by real-time PCR using cDNA, SYBR-Green real-time PCR Master Mix (Eurogentec, France). Relative target gene messenger RNA (mRNA) levels were calculated

and reported to the endogenous housekeeping genes cyclophilin A (Cyclo). Sequences of primers used can be found in Table V.

Table V

Primer sequences of main primers used to quantify gene expression

Gene	Primer sequence (F for forward and R for reverse)
18s	F: GGG AGC CTG AGA AAC GGC R: GGG TCG GGA GTG GGT AAT TT
Cyclophilin A	F: TGG AGA GCA CCA AGA CAG ACA R: TGC CGG AGT CGA CAA TGA T
F4/80	F: GCA GGC GAG GAA AAG ATA GTG T R: TGA CAA CCA GAC GGC TTG TG
Col1a1	F: CCC CGG GAC TCC TGG ACT T R: GCTCCGACACGCCCTCTCTC
α SMA	F: TTG GAA AAG ATC TGG CAC CAC R: GCA GTA GTC ACG AAG GAA TAG
Col3a1	F: CTC ACC CTT CTT CAT CCC ACT R: ACA TGG TTC TGG CTT CCA GAC

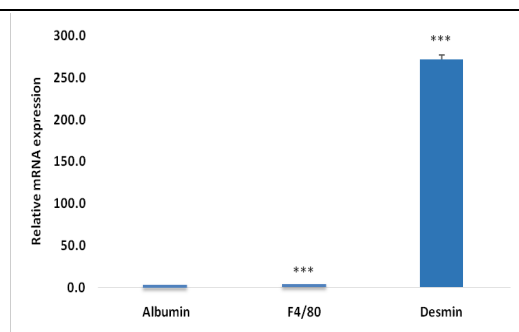
KC and Hepatocyte isolation protocol. The suspension of liver cells was acquired by *in situ* perfusion of the liver from C57/BL16 mice using a modified digestion protocol with Collagenase A (Roche, Germany). Non-parenchymal cells were separated from hepatocytes by centrifugation (50 G, 3 min). KCs were isolated by sedimentation in a two-step Percoll gradient as already described previously [14].

Ethics. All experiments were conducted in accordance with the institutional guidelines and the recommendations for the care and use of laboratory animals put forward by the Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes.

Results and Discussion

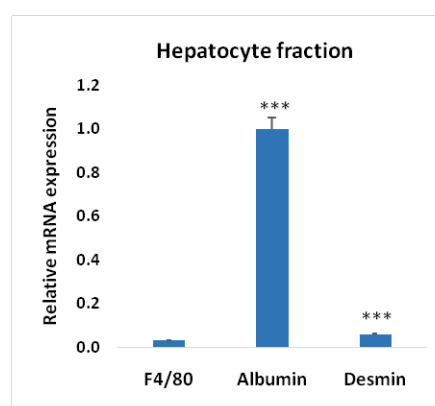
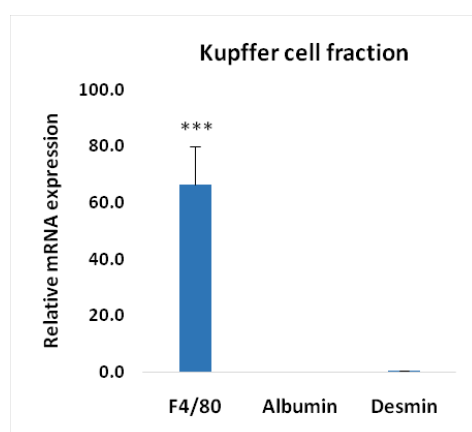
HSC number and purity. Using our modified protocol, we managed to isolate, in average, $(1.02 \pm 0.34 \times 10^6)$ HSCs *per* mouse ($n = 11$). HSC fraction purity was confirmed by comparing gene expression of cell markers used for the most abundant cell types obtained following an HSC isolation protocol. F4/80 was used as a cell marker for KCs, the most abundant type of immune cell contaminant, albumin for hepatocytes, and Desmin, a specific marker of HSCs as a positive control. Indeed, a low relative expression of both albumin (1 ± 0.24) and F4/80 (1.395 ± 0.32) can be observed in the isolated HSC fraction as compared to expression of Desmin (271.48 ± 7.8), as presented in Figure 2.

Since hepatocytes and KCs are the most abundant contaminants of HSC obtained using the perfusion/isolation protocol, this highlights the purity of the HSC fraction obtained using our modified protocol.

**Figure 2.****Purity of the HSC fraction**

Results were normalized to cyclophilin A and were expressed as fold induction (mean $\pm$ SEM) (n = 4) *** p < 0.001 using mean comparison Student test. mRNA, messenger ribonucleic acid; F4/80, EGF-like module-containing mucin-like hormone receptor-like 1; HSC, Hepatic stellate cell; SEM, standard error of mean.

To confirm that F4/80 and Albumin low expression reflect the absence of KCs and hepatocyte respectively, we isolated cell fraction enriched in hepatocytes and KCs and we estimated the relative mRNA expression of the two specific cell markers in these fractions by qRT-PCR. Results show that, in the KC enriched fraction, F4/80 mRNA expression is 66.25 ± 13.61 higher compared to Desmin expression (0.32 ± 0.1) and albumin expression (1.00 ± 0.27). In the hepatocyte enriched fraction, albumin expression (1 ± 0.06) is 33 times higher than F4/80 expression (0.03) and 16 times higher than Desmin expression (0.06), as presented in Figure 3.

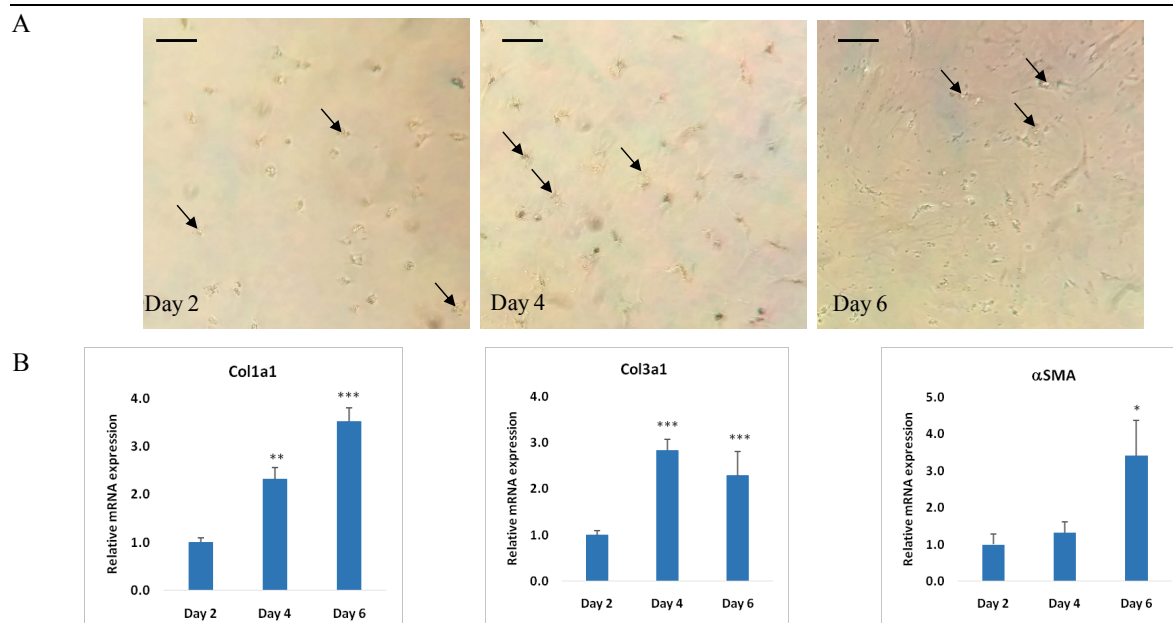
**Figure 3.****Expression of albumin, F4/80, and Desmin in the hepatocytes and Kupffer cell enriched fractions**

Results were normalized to cyclophilin A and were expressed as fold induction (mean $\pm$ SEM) (n = 4). mRNA, messenger ribonucleic acid; F4/80, EGF-like module-containing mucin-like hormone receptor-like 1; HSC, Hepatic stellate cell; KC, Kupffer cells; SEM, Standard error mean.

Auto-activation (kinetic study)

During the first days of HSC culture, the major visual characteristics of this cell type can be observed. As such, its characteristic star-like shape, as well as perinuclear lipid droplets can be clearly spotted, as presented in Figure 4A. Since HSC activation is the key event during liver fibrogenesis, we decided to perform an experiment that highlights this process *in vitro* by plating the cells on plastic dishes, an event which triggers HSC activation, and then

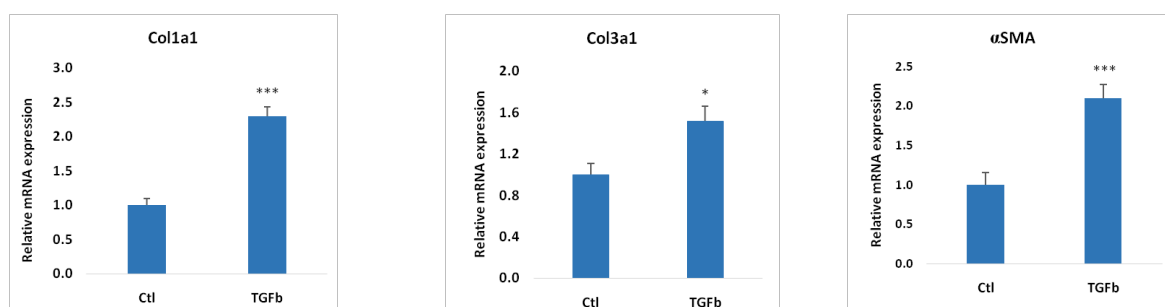
measuring the expression of specific markers of activated HSCs. Our results show that the expressions of α smooth-muscle actin (α SMA), type 1 collagen (Col1a1), and type 3 collagen (Col3a1) increased significantly over the 6 days culture period. As such, at day 6 after culture, mRNA expression was increased up to (3.41 ± 0.97) for α SMA, (3.52 ± 0.29) for Col1a1, and (2.29 ± 0.52) for Col3a1 compared to the expression of the same markers at Day 2 (Figure 4B).

**Figure 4.****HSC *in vitro* auto-activation**

A. Morphology of isolated primary mouse HSCs. The images depict cells 48 h after isolation and culture (Day 2) as well as after 96 and 144 h (Days 4 and 6 respectively). Freshly isolated HSCs present intracellular perinuclear lipid vesicles characteristic of quiescent cells (see corresponding black arrows). Following plating, HSCs auto-activate, losing their intracellular lipid droplets, transforming into a myofibroblast-like cell, via the process of HSC activation. (Magnification 100x; scale bars, 50 μ m); **B.** Markers of HSCs activation (mRNA expression levels of the HSC activation markers α -SMA, Col1a1, and Col3a1, at days 2, 4, and 6). Results were normalized to Cyclophilin A and were expressed as fold induction (mean $\pm$ SEM) (n = 5) * p < 0.05, ** p < 0.01, *** p < 0.001 respectively, using mean comparison student test. D 2-6, Day 2-6; HSC; hepatic stellate cells; mRNA, messenger ribonucleic acid; α -SMA, α smooth-muscle actin; Col1a1, type 1 collagen; Col3a1, type 3 collagen.

These results reflect the trans-differentiation process of HSCs from their quiescent-like, lipid-storing phenotype observed in a normal liver (observed at Day 2) towards the activated, myofibroblast-like, ECM producing cell type that can be found throughout the liver fibrogenesis process (observed at Day 6). *Cytokine induced activation (TGF β 1 treatment).* The second experiment which we performed in order to

induce HSC activation was to treat the cells with TGF β 1, the most potent cytokine involved in HSC activation. This treatment triggered an activation of the HSCs, measured by a significant increase in the mRNA expressions of α SMA (2.10 ± 0.18), Col1a1 (2.29 ± 0.14), and Col3a1 (1.52 ± 0.14), as illustrated in Figure 5.

**Figure 5.****TGF β treatment**

Markers of isolated primary HSC activation following TGF β 1 treatment. mRNA expression levels were measure for α -SMA, Col1a1 and Col3a1 at 48 h treatment with TGF β 1. Results were normalized to cyclophilin A and were expressed as fold induction (mean $\pm$ SEM) (n = 5) *p < 0.05, **p < 0.01, *** p < 0.001 respectively, using mean comparison student test. HSC; hepatic stellate cells; mRNA, messenger ribonucleic acid; α -SMA, α smooth-muscle actin; Col1a1, type 1 collagen; Col3a1, type 3 collagen; SEM, standard error of the mean.

Adenoviral transduction. In a final experiment we tested the HSCs adenoviral transduction susceptibility, one method commonly used for gene expression modulation experiments. As illustrated in Figure 6,

HSC isolated using our protocol can sustain an adenovirus transduction. A transduction yield of approximately 40 to 50% of was achieved, illustrated by the green fluorescence of the transduced cells.

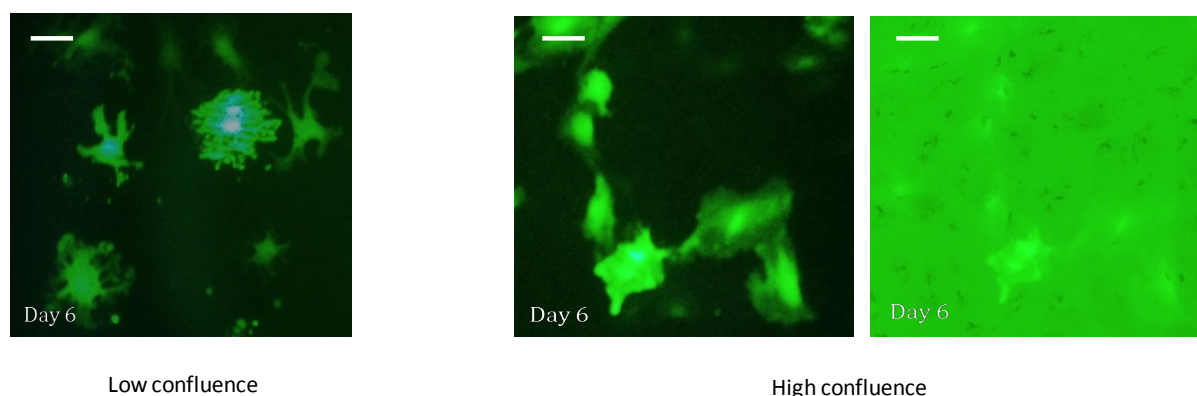


Figure 6.

HSC susceptibility to adenoviral transduction

Images illustrate the number of transfected cells in a low and a high confluence area (image of the left and of the right respectively). Medium transduction yield is around 40 to 50% of the total live cells. (Magnification 100 x; scale bars, 50 μ m).

HSCs are the main actors in hepatic fibrogenesis. Although HSCs are well represented in the liver (between 10 - 15% of all liver cell types), their localization in the perisinusoidal space of Disse contributes to the increased difficulty of their isolation. HSC isolation protocols have been published but they lack consistency in the protocol description and the number of isolated HSCs varies greatly from 2×10^5 to more than 2×10^6 HSC *per mouse* [11-13]. Taking this into consideration, we decided to develop a modified protocol for mouse HSC isolation which is consistent, reproducible, detailed, less time-consuming, and easier to implement. We performed several modifications in our protocol compared to previously published protocols. First, we adapted this protocol for C57/BL6 mice, the most common mouse breed used to generate murine genetic models. Secondly, we modified the characteristics of the *in situ* digestion of the liver by increasing the digestion temperature and decreasing the flow of the perfusion. These modifications were performed in order to obtain a faster and more complete digestion of the liver and without decreasing the yield of viable cells. We also increased the centrifugation speed and used an ultracentrifugation step in order to better separate the HSCs from the other cell types (hepatocytes and KCs), reducing the risk of contamination. Finally, we plated the cells directly after the ultracentrifugation with the gradient solution in cell culture medium without washing centrifugations, an important change which contributes substantially to the reasonably high HSC number.

Using this modified procedure, we obtained $1.02 \pm 0.34 \times 10^6$ HSCs *per mouse* ($n = 11$). The whole

procedure takes between 2 and 2.5 h *per mouse*, which is important to mention when working with bigger groups of animals. Alternatively, a parallel perfusion and isolation protocol allows the perfusion of two mice at the same time, using the same perfusion pump and 2 different sets of perfusion lines, reducing the isolation time when working with larger groups of animals. This procedure can either be performed in sequence by one person or by two people but should be carefully planned. Using the parallel method of perfusion, the protocol time can be reduced to 1 hour *per mouse*, a considerably reduced time as compared to other published protocols [11, 12].

The entity of isolated HSCs can be controlled by considering the major morphological characteristics of the cells: the star-like shape, perinuclear lipid vesicles and expression of HSC marker Desmin. Potential contaminants for HSCs obtained using this protocol include hepatocytes, KCs, and LSECs. F4/80 mRNA expression was used to exclude KC contamination, while albumin mRNA expression was used to exclude hepatocyte contamination. The low level of mRNA expression of F4/80 and albumin compared to the high level of Desmin mRNA expression of the cell fraction isolated using this protocol proves the high purity of the HSC fraction obtained. Exclusion of KC contamination also rules out LSEC contamination risk due to similar cell density [14]. Thus, no further enrichment or sorting methods are required. To prove that the HSCs isolated using this protocol are suitable to be used for gene modification experiments, we transduced mouse HSCs with an adenovirus expressing GFP with a transduction yield of 40 - 50%.

HSCs differentiate into myofibroblast-like cells due to their auto-activation capacity. It is worthwhile to mention that activated HSCs are very different from quiescent HSCs. During this dynamic process, several markers of HSC activation, such as α SMA, as well as different types of collagens (Col1a1 & Col3a1) are upregulated. We decided to reproduce this activation *ex vivo*, by plating freshly isolated HSC on plastic culture dishes. This type of auto-activation has common characteristics with the *in vivo* HSC activation process which takes place during liver fibrosis, although they are not identical when it comes to the pattern of gene expression [15, 16]. Nevertheless, HSCs obtained using our protocol can be used as a model of both quiescent and differentiated HSCs, depending on their activation stage.

In the literature, there are articles dealing with mice HSC isolation, but some lack crucial information, making them hard to reproduce. One protocol describes the isolation of more than 1×10^6 HSC *per* mouse from C57/BL6 mice, but the authors do not provide a detailed protocol [13]. For instance, in the article description, the authors state that average density at which HSCs can be found following centrifugation in gradient solution is 1.053. Since KCs can also be found at an average density of 1.060, this makes cell separation very difficult. Looking closely in the literature, it seems that the authors misinterpreted the results of Friedman *et al.* [14] when analysing their own data. In this last article, Friedman *et al.* realized that HSCs are less dense than the lowest gradient density fraction (1.053), which is found between the cell culture medium and the first gradient density fraction after the centrifugation. In contrast, KCs and LSECs are mainly retained in the gradient at a density between 1.058 and 1.080, with a very small cell fraction found between 1.053 and 1.058. Moreover, the authors analysed the cell fractions and showed that the lowest density fraction (density < 1.053) contained 2% hepatocytes and 98% HSCs, but no KCs nor LSECs. In our article, we used a gradient solution in which the HSCs are found at a density of 1.042, thus the risk of contamination due to KCs or LSECs is considerably reduced.

Another important point to highlight is the number of HSC isolated from one C57/BL6 mouse. In an article from 2011, Maschmeyer *et al.* provided a protocol for HSC isolation with an isolation yield of around 2×10^5 cells *per* mouse. Analysing the steps in the two protocols, it seems that this protocol has high cell loss due to the last washes following density gradient centrifugation, as well due to the characteristics of the *in situ* digestion procedure. Indeed, according to the Materials & Methods section of their protocol, the length of the perfusion tube, as well as the debit used for mice perfusion, are inadequate for achieving a corresponding

enzyme temperature needed for an optimal digestion temperature, thus decreasing the HSC isolation yield [11]. In the same paper, it has been shown that HSCs isolated following *in situ* perfusion and density gradient can be purified by sorting the cells using flow cytometry cell sorting by fluorescent activity (FACS). In the article from Mederacke and colleagues, a detailed protocols mentions the factors which can influence HSC isolation, including age, gender, and genetic background, as well as all the required perfusion equipment. Indeed, they obtain a high yield of isolated HSC, more than 2×10^6 HSCs *per* mouse. However, they used BALB/c mice, which can lead to higher HSC isolation yields, but which are not as easy to genetically engineer as C57/BL6 mice, and thus less prone to be used in genetic models of hepatic disorders or for studying pathways specific to HSC pathogenesis [12]. Moreover, they did not specify the perfusion flow used during the *in situ* liver perfusion. Given the fact that enzyme quantity used for the perfusion is the hinge step in HSC isolation, without this information it is difficult to reproduce the protocol.

Considering the limitations of our protocol, these include contamination during the procedure due to misuse of material and inconsistencies in cell numbers when younger (< 3 months old) mice are used. Also, excessive peritoneal fat might obscure the visibility of the vena cava and thus limit perfusion and clamping capabilities in this case we recommend to perfuse the portal vein and to incise the inferior vena cava. Another limitation that needs to be addressed is working with mice that have advanced hepatic steatosis, such as after a methionine-choline-deficient diet. The excess of lipids from these mice can decrease hepatocyte density, leading to higher yields of hepatocyte contamination in the HSC fraction. Importantly, it is worthwhile to mention that excessive contact with the digestive enzymes taking place during liver perfusion can alter HSC viability by exposing the cells to high levels of stress. Finally, mice with advanced hepatic fibrosis have excessive ECM deposition, thus requiring a higher degree of liver digestion. This is an important issue which needs to be taken into account when working with fibrotic animals. Enzyme solution volumes need to be adapted, increasing the volumes used by up to 15 mL (30 mL instead of 15 mL) in order to obtain an appropriate number of pure and viable HSCs, keeping in mind that volumes need to be adjusted specifically to the animal used in the procedure.

Conclusions

Our work describes an improved protocol for isolating HSCs from C57/BL6 mice, one of the most common mouse strain used to study HSCs and

their activation during liver fibrosis. Our protocol systematically provides all the information necessary for implementing the method in any laboratory (solution composition and preparation, equipment set-up, complete methodology, as well as subsequent cell experimentation protocols). Compared to the other protocols already published in the literature, ours provides all the necessary details needed in order to obtain a consistently high number of viable HSCs which can be then studied. We detail the modifications applied to our protocol compared to previously published work and the consequences of these modifications to the yield of the isolated HSC fraction. The most important modifications which we implemented were the increase in the temperature of the incubation solutions for the *in situ* digestion, as well as the speed of the liver perfusion step. Furthermore, we added an ultracentrifugation step to isolate the HSCs from the other cell types. All these modifications contributed to the increase observed in the yield of the isolation method.

Acknowledgement

Work in the authors' laboratories is supported by INSERM, CNRS and the Fondation pour la Recherche Médicale (Équipe FRM DEQ20140329504), France. PM is the recipient of a fellowship from the Institute of Cardiometabolism and Nutrition (ICAN), France. AB is supported by a scholarship from the Fondation Jacques Tacussel, France.

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