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High-yield and high-purity isolation of hepatic stellate cells from normal and fibrotic mouse livers

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Abstract

Hepatic stellate cells (HSCs) have been identified as the main fibrogenic cell type in the liver. Hence, efforts to understand hepatic fibrogenesis and to develop treatment strategies have focused on this cell type. HSC isolation, originally developed in rats, has subsequently been adapted to mice, allowing to study fibrogenesis by genetic approaches in transgenic mice. However, mouse HSC isolation is commonly hampered by low yield and purity. Here we present an easy-to-perform protocol for high-purity and high-yield isolation of quiescent and activated HSCs in mice, based on retrograde pronase-collagenase perfusion of the liver and subsequent density-gradient centrifugation. We describe an optional add-on protocol for ultrapure HSC isolation from normal and fibrotic livers via subsequent flow-cytometric sorting, thus providing a validated method to determine gene expression changes during HSC activation devoid of cell culture artefacts or contamination with other cells. The described isolation procedure takes approximately four hours to complete.

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AUTHOR CONTRIBUTIONS

I.M. performed primary cell isolations and data analysis, recorded and cut the video files and drafted the manuscript. D.H.D. performed *in vivo* injury models, primary cell isolations, data acquisition including RNA isolation and qPCRs, data analysis and drafted the manuscript. S.A. performed primary cell isolations and data acquisition. HU optimized the isolation procedure. R.F.S. designed and oversaw the study, performed data analysis and drafted the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

Supplementary Information

Supplementary Video 1: Cannulation of IVC
Supplementary Video 2: Retrograde perfusion
Supplementary Video 3: Removal and mincing of the liver
Supplementary Video 4: Nycodenz gradient
Supplementary Video 5: Harvest HSCs after centrifugation

INTRODUCTION

Fibrosis, defined as replacement of healthy parenchyma with extracellular matrix (ECM)- and myofibroblasts-rich scar tissue, has been estimated to contribute to up to 45% of deaths in the developed world [1]. In the liver, fibrosis develops in response to hepatic injury, and is common to virtually all liver diseases with hepatocellular damage [2]. Of note, liver fibrosis has been implicated in many of the grave complications of liver disease, such as the development of portal hypertension, progression to liver cirrhosis and the associated occurrence of hepatic failure, as well as the development of hepatocellular carcinoma [2,3]. On a cellular level, multiple cell populations have been considered as contributors to fibrosis including hepatic stellate cells (HSCs), portal fibroblasts, bone marrow-derived fibrocytes and hepatocytes [2,4]. Recent cell fate tracing studies have positively identified HSCs as the dominant contributor to the myofibroblast pool, contributing 82–96% of myofibroblasts in various types of chronic liver diseases [5]. Following liver injury HSCs undergo a characteristic phenotypic change, and differentiate from vitamin A-storing pericytes into ECM-producing myofibroblasts [6]. While HSCs are the key contributors to liver fibrogenesis, HSC activation is modulated by multiple interactions with other hepatic cell types including hepatocytes, macrophages, endothelial cells, cholangiocytes and NK cells [7–10]. Hence, fibrogenesis is viewed as a multicellular hepatic wound healing response with HSCs in its center, mediating the deposition of ECM and also providing contractile properties that regulate sinusoidal blood flow and portal pressure.

Methods to study HSC biology

The study of HSC biology may provide important mechanistic insights into the pathophysiology of liver fibrosis and hold the key to developing therapeutic approaches that block HSC activation and liver fibrosis [11]. HSC isolation allows studying pathways regulating the activation of this key fibrogenic cell type in a well-defined context, and has led to the identification of relevant regulatory pathways such as TGFβ and PDGF [12–14]. While HSC isolation was pioneered in rats [15,16], studying HSCs has shifted largely to mouse models in recent years due to the availability a wide range of genetic models. Until recently, HSC activation has largely been studied *in vitro* using culture-activation as a model for the activation process that HSCs undergo *in vivo*. Although culture activation upregulates many of the key fibrogenic genes such as *Col1a1* and *Acta2*, it has been suggested that culture activation does not reproduce the gene expression pattern associated with the activation process observed *in vivo* [17,18]. The unphysiological HSC activation process in culture is most likely caused by the lack of cell types that typically contribute to HSC activation as well as the presence of an unphysiological environment in cell culture, such as an absence of cell-cell contact, the presence of fetal bovine serum, and plastic surfaces. Co-culture with other cell types such as macrophages can overcome some, but not all, of these limitations [19]. Moreover, while the culture activation model is useful to test inhibitors of HSC activation, it has limited potential to study signals promoting HSC activation due to the overwhelming effect of culture-induced activation that often overshadows the effect of profibrogenic mediators.

In vivo activation of HSCs provides a more physiological HSC activation model that is largely dictated by the choice of a physiological fibrosis model rather than by inherent limitations of the method. *In vivo* activation is not only helpful to understand gene expression patterns and pathways that contribute to HSC activation, but may also be employed to functionally investigate how pharmacologic or genetic interference with specific pathways affects HSC gene expression and activation status. Moreover, it provides a model to study events occurring during the regression of liver fibrosis, including the recently described ability of HSCs to deactivate and return to a nearly quiescent status [20,21]. To achieve the best possible representation of *in vivo* HSC gene expression patterns, it is important to avoid artefacts by analyzing HSCs without plating and exposure to tissue culture, hence allowing a representative "snapshot" of molecular events that occur in HSCs within the liver.

Application of the protocol

The described HSC isolation protocol provides a basis for studying HSCs for a wide range of applications, including culture activation, co-culture and functional interactions with other cell types, analysis of gene expression and epigenetic regulatory mechanisms, as well as proteomic, other omics and single cell analyses. In comparison to portal vein perfusion procedures, which were originally developed for the isolation of rat HSCs [15], the described retrograde perfusion technique via the inferior vena cava (IVC) not only achieves better hepatic perfusions and improved yield in mice, but also provides a less technically challenging procedure that can be adapted by virtually every laboratory. A major advantage of this protocol is that, when using older mice and strains that yield high number of HSCs, one can isolate sufficient quantities of HSCs, typically in excess of 2 million HSCs per mouse, and therefore avoid the common practice of pooling of mice. High-yield isolations typically also reduce contamination of preparations with other cell types. As described in detail in this protocol, isolation of HSCs from fibrotic livers requires modification of enzyme concentrations and perfusion times.

Characterisation of isolated HSCs

Increases in fibrogenic gene expression such as *Col1a1, Acta2* or *Lox,* or decreases in *Hhip* expression in the whole liver are reflected by similar changes in gene expression in HSC isolates (Fig.1a), hence providing a snapshot of molecular events in this key fibrogenic cell population within the fibrotic liver. Increases in fibrogenic gene expression in the whole liver are the combined result of increased activation on a per cell basis and of the expansion of the entire pool of HSC-derived myofibroblasts (which is not of relevance to the evaluation of mRNA expression in isolated HSCs). Therefore, normalizing gene expression for Pdgfrβ, a marker for HSCs [22] that does not increase with HSC activation in our hands, accounts for the number of HSCs within the liver and provides a better estimate of HSC activation in the liver (Supplementary Fig.1) than simply using 18s for normalization (Fig. 1a). Low-level background expression of fibrogenic genes by other cell types and the fact that HSC mRNA representing only ≈1% of the liver's mRNA, may contribute to a higher baseline level and lower induction (e.g. for *Acta2* – see Fig.1a) in the whole liver than in HSC. Previous studies using single cell qPCR have shown that 95–100% of HSC from CCl_4-treated livers display increased expression of activation marker *Col1a1* [20], suggesting

that virtually all HSCs in the fibrotic liver activate. Although analysis of unplated HSCs from fibrotic livers provides a representative picture of molecular events, contamination with other cell types is observed when isolating HSCs by standard gradient centrifugation, especially in HSCs from fibrotic livers (see Fig.1b). Contamination with other cell types may result in misinterpretation of data, in particular when the degree of contamination varies between different conditions (e.g. normal and fibrotic liver), when contamination is variable between HSC isolates, and when sensitive techniques such as quantitative PCR (qPCR) or microarray are employed. For the investigation of HSC *in vivo* activation, we describe an optional add-on protocol, which allows the isolation of ultrapure unplated HSCs which can be combined with virtually any common fibrosis model in mice. FACS-based purification employs endogenous retinoid fluorescence of HSCs (employing channels commonly used for detection of DAPI, e.g. 405–407 nm laser for excitation and a 450/50-nm bandpass filter for detection) as a selection marker (see Fig.2a). Retinoids are subject to rapid bleaching, and brief exposure of HSCs to UV light completely abrogates HSC fluorescence in the DAPI channel (Supplementary Fig.2a) providing evidence that this FACS-based method indeed relies on HSC-specific retinoid expression. Importantly, FACS purification significantly reduces the contamination with hepatocytes (determined by *Alb* mRNA), endothelial cells (determined by *vWF* mRNA), macrophages (determined by *Emr1* mRNA), and cholangiocytes (determined by *Krt19* mRNA) (Fig.2b). Importantly, FACS purification of HSCs does not significantly alter the expression of HSC activation markers such as *Col1a1, Acta2, Lox* or *Hhip* (Fig.2c). Likewise, there is no significant alteration in gene expression of characteristic HSC-enriched markers genes (*Lhx2, Lrat, Pdgfrb, Hand2*, *Vim*), or genes demonstrating the pronounced proliferation (*Ccnb1, Ccnb2*) of HSCs in fibrotic livers (Supplementary Fig.3). Importantly, the FACS purification procedure itself also does not significantly alter the expression of activation genes and typical HSC-enriched genes as determined by comparison to mock-sorted cells that were subjected to FACS purification without selection (Fig.2c, Supplementary Fig.3). Moreover, comparison of the FACS-sorted retinoid-high HSC population with the entire cell population excluded by this FACS procedure (termed “retinoid-low cell population” – which also includes a minuscule fraction of autofluorescent cells with high retinoid and high FITC fluorescence) reveals that this retinoid-low population consist mainly of contaminating cells and only few HSCs, as demonstrated by 18- to 693-fold higher levels of expression of hepatocyte-, macrophage-, cholangiocyte- and endothelial cell-specific genes, and a profoundly lower expression of characteristic HSC and HSC activation genes such as *Pdgfrb, Lrat, Lhx2* and *Hand2* (Supplementary Fig.4a-c). Similar findings were made in the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet model of biliary fibrosis, showing similar levels of activation markers and HSC-enriched marker genes in FACS-sorted and mock-sorted HSCs, but significantly lower levels of contamination with other cell populations and a high level of non-HSC markers and low-levels of HSC markers such as *Pdgfrb* in the retinoid-low population (Supplementary Fig. 5).

In summary, the presented data confirms that retinoid-based FACS-based ultrapurification of HSCs significantly reduces artefacts due to contamination with other hepatic cell types, while preserving the expression pattern of characteristic HSC genes. We have also successfully applied this ultrapurification technique to other genetic, biliary and toxic

fibrosis models including Mdr2^{ko} mice, mice with hepatocyte-specific TAK1-deletion, and mice undergoing bile duct ligation and thioacetamide-induced liver fibrosis (data not shown). Hence, the described easy-to-perform high-yield retrograde perfusion protocol, in combination with FACS-based ultrapurification, is applicable for HSC isolation in a wide range of experimentally-induced liver fibrosis in mice.

Experimental design

All animal experiments included in this protocol should be performed and reported in adherence to the ARRIVE guidelines [23]. Before planning experiments involving HSC isolations, one should carefully consider different strains of mice and plan experiments to allow the use of aged mice. As such, Balb/c mice yield a significantly higher number of HSCs than C57Bl/6 mice, and older mice generally yield more HSCs than younger mice. When not working with transgenic mice, one can therefore consider ordering retired breeders, which are commonly available from commercial vendors. While mice older than 12 weeks are desirable, advanced age (> 6 months) in combination with previous exposure to breeder chow may lead to increased deposition of visceral fat, which makes the precise cannulation and subsequent retrograde perfusion via the inferior vena cava (IVC), described in this protocol, difficult. However, in retired breeders that are maintained on normal chow diet, localization and perfusion of the IVC does not pose any problems. It is important to include appropriate controls, e.g. when analyzing HSC activation in fibrotic livers one needs to include HSCs isolated from healthy mice as quiescent controls. Likewise, due to the variability in fibrosis induction between individual mice, HSCs need to be isolated from an appropriate number of mice in each group to achieve sufficient power.

HSC Isolation Procedure—The isolation of HSCs from murine livers can be divided into three main sequential stages: (i) *In situ* pronase/collagenase perfusion of mouse liver; (ii) subsequent *in vitro* digestion; (iii) density gradient-based separation of HSCs from other hepatic cell populations. The underlying principle is that HSCs can easily be separated from other cells based on their different density due to their abundance of lipid droplets.

The *in situ* perfusion of the mouse liver is the most critical step of a successful HSC isolation. Temperature and enzyme concentrations are key determinants of enzymatic activity and are highly relevant for successful hepatic digestion. As such, it is important to adjust the temperature of the water bath and/or the length of connecting lines. The set-up should be adjusted to achieve temperature of ≈29.5 °C at the end of the line, i.e. where solutions enter the IVC. Therefore, it is crucial to prewarm the pronase and collagenase solutions to 42° C. To ensure constant temperature during *in vivo* digestion, a heat lamp should be placed above the mouse and switched on once the perfusion with pronase solution is started. A key step in the procedure is successful cannulation of the IVC with an i.v. catheter (Fig.3a, Supplementary Video 1), and to then stabilize the perfusion set-up to avoid dislocation of the catheter during the perfusion. Retrograde liver perfusion is only achieved after cutting the portal vein allowing solutions to perfuse the liver and exit via the portal vein. The portal vein becomes distended after the pump has been started and is therefore easy to locate and cut (Fig.3b, Supplementary Video 2). Finally, clamping the IVC above the diaphragm directs all solutions through the liver and prevents the perfusion of other

organs (Fig.3b, Supplementary Video 2). The initial EGTA solution flushes out blood and thus avoids contamination with red blood cells and clotting of hepatic vessels, which may result in incomplete perfusion and digestion. Successful perfusion can be easily observed by swelling of the liver and a change to a pale color after the portal vein has been severed. Subsequently, perfusion with pronase solution is performed for 5 min, followed by collagenase perfusion for 7 minutes to achieve digestion of the liver (Fig.3c). Air bubbles are caught in a trap, hence switching from one solution to the other does not pose a problem in this regard.

Following the *in situ* digestion, the liver is carefully removed and minced under sterile conditions. (Fig.3d, Supplementary Video 3). The minced liver is further digested *in vitro* with prewarmed pronase/collagenase solution (Fig.3e). The liver cell suspension is then filtered through a 70 μm cell strainer to eliminate undigested tissue remnants and washed to remove excess digestion enzymes (Fig.3f). The final step of the isolation procedure is a density gradient centrifugation including collection and counting of HSCs. After resuspending the liver cell suspension with density gradient medium, it is important to very slowy add the density gradient-free medium on top of the cell-density gradient solution (Fig. 3g, Supplementary Video 4). Likewise, it is important to centrifuge the gradient without brake so that the HSC-containing layer on top of the gradient is not disrupted (Fig.3g). After harvesting HSCs (Fig.3h, Supplementary Video 5) and final centrifugation (Fig.3i), the purity of HSC isolation can easily be assessed by fluorescence microscopy after centrifugation by calculating the ratio between retinoid-positive HSCs and all cells (Fig.1b). As shown above, quantitative real time PCR provides a more sensitive method to assess contamination with specific cell types. It should be noted, that percent RNA contamination does not equate percent cell contamination as non-HSC populations, in particular hepatocytes, often express markedly higher amounts of total RNA and specific RNAs used for normalization of qPCR such as 18s.

Isolating HSCs from Fibrotic Mice—The procedure for isolating HSCs from fibrotic mouse livers (Supplementary Methods) is technically similar to isolating HSCs from untreated mice. The only modification is the amount of collagenase added to the collagenase solution and a prolonged perfusion in models with severely fibrotic livers such as the $Mdr2^{ko}$ mice. As HSCs lose some of their retinoid-containing lipid droplets (loss of all droplets virtually never occurs in experimental models of fibrosis – hence isolation of activated HSCs is therefore possible in almost all models), the relative purity decreases and there may also be a decrease in Vitamin A fluorescence when FACS-sorting cells (see below). Isolation of HSCs from fibrotic livers is usually prone to more contamination from other cell populations, in particular hepatocytes or F4/80-positive Kupffer cells (Figs.1–2). Hence, a second purification via FACS sorting (Fig. 2) is recommended for most applications involving HSCs isolations from fibrotic livers, as discussed below.

Isolating HSCs via FACS—Cell sorting by FACS can be employed to reduce contamination with cell types other than HSCs, and is especially relevant when analyzing gene expression patterns. While it is in principle possible to sort HSCs directly from unpurified liver cell suspensions, it may take several hours as HSCs represent only a small

fraction. In contrast, FACS sorting of gradient-purified HSCs typically allows sorting within ≈20 minutes per mouse, and also results in higher purity due to the application of two subsequent purification methods. For sorting, HSCs are resuspended in phenol red-free medium containing 1% FBS. The characteristic HSC-specific Vitamin A fluorescence allows to purify HSCs to >99% purity, using 405–407 nm laser for excitation and a 450/50-nm bandpass filter for detection (Fig.2a – Supplementary Fig.2b). Enrichment of HSCs with concomitant reduction of contaminating cell populations has been previously reported by a scatter-based FACS sorting method for rat HSCs [24]. HSCs, even when isolated from fibrotic livers, are typically seen as a separate retinoid-positive population in the violet FACS channel (see Fig.2a). Studies in Lrat-deficient mice that lack typical retinoid-rich lipid droplets in HSCs have indeed confirmed that retinoid storage and HSC activation are two functionally separate responses [25], and HSC activation does not necessarily translate into immediate loss of HSC retinoids stores. Only in severe and longstanding fibrosis Vitamin A content is so low that the HSC population overlaps other cell populations that have high autofluorescence. In this case, either a selection of cells with the highest Vitamin A fluorescence can be employed (usually achieving acceptable HSC purity) or additional methods such as genetic labeling of HSCs [5] may be required.

MATERIALS

REAGENTS

Animals—

- >12 week-old mice, preferably retired breeders if high yield is desired. Mice for our studies were fed with standard chow (LabDiet, 5053 PicoLab Rodent Diet 20, USA)

CAUTION: All animal experiments must be performed in accordance with relevant institutional guidelines and regulations. All animal procedures were approved by the Institutional Animal Care and Use Committee at Columbia University.

Enzymes/Chemicals—

- Protease (Sigma-Aldrich, Ref# P5147, USA)
- Collagenase D (Roche, Ref# 11 088 882 001, Germany)
- DNase I (Roche, Ref# 10 104 159 001, Germany)
- Nycodenz (Accurate Chemical, Ref# 1002424, USA)

CRITICAL: We optimized the isolation procedure with the above-mentioned enzymes and Nycodenz from the respective suppliers. We cannot give any recommendations on alternative suppliers.

Solutions—

- Sodium chloride, NaCl (e.g. Sigma-Aldrich, Ref# S3014, USA)
- Potassium chloride, KCl (e.g. Sigma-Aldrich, Ref# P9333, USA)

- Sodium Phosphate monobasic monohydrate, $NaH_2PO_4•H_2O$ (e.g. Fisher, Ref# S369, USA)
- Sodium phosphate dibasic, anhydrous, Na_2HPO_4 (e.g. Acros Organics, Ref#42437-5000, USA)
- HEPES (e.g. Sigma-Aldrich, Ref# H4034, USA)
- Sodium bicarbonate, $NaHCO_3$ (e.g. Fisher, Ref# S233, USA)
- EGTA (e.g. Sigma-Aldrich, Ref# E4378, USA)
- D-(+)-Glucose (e.g. Sigma-Aldrich, Ref# G8270, USA)
- Calcium chloride dihydrate, $CaCl_2•H_2O$ (e.g. Sigma-Aldrich, Ref# C7902, USA)
- Magnesium chloride hexahydrate, $MgCl_2•6H_2O$ (e.g. Malinckrodt Chemicals, Ref# 5958, UK)
- Magnesium sulphate heptahydrate, $MgSO_4•7H_2O$ (e.g. Sigma-Aldrich, Ref#230391, USA)
- Potassium phosphate monobasic, KH_2PO_4 (e.g. Malinckrodt Chemicals, Ref# 7100, UK)
- *Optional:* Gey´s Balanced Salt Solution B (GBSS/B) (Sigma-Aldrich, Ref# G9779, USA)
- ddH_2O

Cell Culture—

- Phenol red-free DMEM (Gibco, Ref # 21063-029, USA)
- DMEM (Gibco, Ref # 11965-092, USA)
- FBS (SAFC Biosciences, Ref# 12203C, USA)
- Gentamycin (Gibco, Ref # 15710-064, USA)
- Antibiotic/Antimycotic (Gibco, Ref# 15240, USA,)

EQUIPMENT

- Perfusion pump (Minipuls 3, Gilson, USA)
- Perfusion line (Baxter Healthcare, Ref# 2C6401s, USA)
- Surflo 22Gx1'' I.V. Catheter (Terumo Medical, Ref# SROX2225CA, USA)
- Blood Vessel Clamp (Fine Science Tools, Ref# 18039-45, Germany)
- 250 ml Media bottles for Enzyme Solutions (VWR, Ref# 89000-236, USA)
- 250 ml Pyrex Erlenmeyer flask and stir bar for *in vitro* digestion (Corning, Ref# 4995-250, USA)
- 0.2 μm 150 ml bottle top filter (Corning, Ref# 431161, USA)

- Heat lamp
- Water bath
- Hot plate with magnetic stirrer
- Syringes: 3 ml, 20 ml
- Sterile filter for syringe (0.22μm)
- Sterile pipets 5 ml, 10 ml, 25 ml, 50 ml
- 15 ml Falcon tubes (BD, Ref# 352099, USA)
- 50 ml Falcon tubes (BD, Ref# 352098, USA)
- Sterile Petri dishes
- Refrigerated Benchtop Centrifuge with swinging bucket rotor (e.g. Beckman Coulter, Allegra X-15R, USA)
- Sterile cell culture hood
- FACS Sorter, *Optional* (e.g. BD FACSAria Cell Sorter, USA)
- Tape for fixation of mice (e.g. Rainbow Laboratory Tape, VWR, USA)

REAGENT SETUP

EGTA Solution—Prepare the solution by dissolving the components of the recipe given below in 1 L of ddH_2O. Adjust pH to 7.35–7.4 and filter through a 0.2 μm bottle top filter. Can be made ahead and stored at 4°C up to six months.

Reagent	Final concentraion
NaCl	8000 mg/L
KCl	400 mg/L
$NaH_2PO_4 . H_2O$	88.17 mg/L
Na_2HPO_4	120.45 mg/L
HEPES	2380 mg/L
$NaHCO_3$	350 mg/L
EGTA	190 mg/L
Glucose	900 mg/L

Enzyme Buffer Solution—Prepare the solution by dissolving the components of the recipe given in **the table below** in 1 L of ddH_2O. Stir the solution for at least 30 minutes prior to slowly adding calcium chloride dihydrate ($CaCl_2$•$2H_2O$). Addition of $CaCl_2$ before the solution is thoroughly mixed will result in precipitation of salts from the solution. Adjust pH to 7.35–7.4 and filter through a 0.2 μm bottle top filter. Can be made ahead and stored at 4°C up to six months.

Reagent	Final concentration
NaCl	8000 mg/L
KCl	400 mg/L
$NaH_2PO_4 . H_2O$	88.17 mg/L
Na_2HPO_4	120.45 mg/L
HEPES	2380 mg/L
$NaHCO_3$	350 mg/L
$CaCl_2 . 2H_2O$	560 mg/L

Gey´s Balanced Salt Solution A (GBSS/A)—Prepare the solution by dissolving the components of the recipe given in **the table below** in 1 L of ddH_2O. Adjust pH to 7.35–7.4 and filter through a 0.2 μm bottle top filter. Can be made ahead and stored at 4°C up to six months.

Reagent	Final concentration
KCl	370 mg/L
$MgCl_2 . 6H_2O$	210 mg/L
$MgSO_4 . 7H_2O$	70 mg/L
Na_2HPO_4	59.6 mg/L
KH_2PO_4	30 mg/L
Glucose	991 mg/L
$NaHCO_3$	227 mg/L
$CaCl_2 . 2H_2O$	225 mg/L

Gey´s Balanced Salt Solution B (GBSS/B)—Prepare the solution by dissolving the components of the recipe given in **the table below** in 1 L of ddH_2O. Alternatively, it can be purchased commercially (Sigma-Aldrich, Ref# G9779, USA). Adjust pH to 7.35–7.4 and filter through a 0.2 μm bottle top filter. Can be made ahead and stored at 4°C up to six months.

Reagent	Final concentration
NaCl	8000 mg/L
KCl	370 mg/L
$MgCl_2 . 6H_2O$	210 mg/L
$MgSO_4 . 7H_2O$	70 mg/L
Na_2HPO_4	59.6 mg/L
KH_2PO_4	30 mg/L

Reagent	Final concentration
Glucose	991 mg/L
$NaHCO_3$	227 mg/L
$CaCl_2.2H_2O$	225 mg/L

DNase I—Dissolve 100 mg of DNAse I in 50 ml GBSS/B by gently pipetting as DNAse I is susceptible to physical denaturation. The prepared DNAse I should be aliquoted and stored at −20°C until use. Avoid repeated freezing and thawing and the aliquots should be stable up to 18 months.

Preparation Enzyme Solutions/Nycodenz

EGTA solution: Aliquot 35 ml of EGTA solution per mouse. CRITICAL This solution must be prepared immediately before the start of the isolation procedure in a sterile bottle.

Pronase solution: Dissolve 14 mg/mouse of pronase in 35 ml enzyme buffer solution. Weigh out the enzyme first and add enzyme buffer solution to pronase by filtering through 0.2 μm bottle top filter under sterile cell culture hood. CRITICAL This solution must be prepared immediately before the start of the isolation procedure in a sterile bottle.

Collagenase solution: Dissolve the appropriate amount of collagenase (3.7 U/mouse) in 40 ml enzyme buffer solution (our current lot has an activity of 0.193 U/mg, we use 19.17 mg/mouse). Weigh out the enzyme first and add enzyme buffer solution by filtering through 0.2 μm bottle top filter under sterile cell culture hood. CRITICAL This solution must be prepared immediately before the start of the isolation procedure in a sterile bottle.

> CRITICAL If using for fibrotic mouse livers, increase collagenase 1.5-fold after chronic CCl_4 treatment and 2-fold after bile duct ligation for two weeks. $Mdr2^{ko}$ mice at an age of 2 months, require 12.5 U/ml in 50 ml enzyme buffer.

Pronase/collagenase solution: Dissolve 25 mg/mouse of pronase and 4.4 U/mouse of collagenase to 50ml of enzyme buffer solution. Add 1% DNAse I immediately before adding the minced liver. CRITICAL This solution must be prepared immediately before the start of the isolation procedure in a sterile bottle.

Nycodenz solution: Dissolve 4.94 g of Nycodenz in 15 ml GBSS/A and place on an orbital shaker to dissolve. Once dissolved adjust volume to 17 ml and filter solution through a 0.2 μm syringe filter. Place solution on ice to cool down before mixing with HSCs. CRITICAL This solution must be prepared immediately before the start of the isolation procedure in a sterile bottle.

EQUIPMENT SETUP

Perfusion pump—Adjust the perfusion pump to a flow rate of 5 ml/min by using ddH_2O. Empty the perfusion line and fill the system with EGTA solution.

PROCEDURE

Before the perfusion of the mouse can be started, EGTA, pronase and collagenase solutions need to be heated up in the water bath (42°C). The pronase/collagenase solution should be placed on the heated stirrer (40°C) at the beginning of the perfusion.

Steps 1–12: *In situ* pronase/collagenase perfusion of mouse liver—Timing: 20 min

1 Anaesthetize the mouse according to the institution's approved animal protocol; we perform anesthesia with the inhalation of isoflurane (1–3%).

CAUTION: All animal experiments must be performed in accordance with relevant institutional guidelines and regulations.

2 Fix mouse by using tape to attach upper and lower extremities on a dissecting board and perform a laparotomy to expose the liver and inferior vena cava (IVC).

3 Move the visceral organs to the right side to expose the IVC, and then cannulate the IVC with a 22G catheter.

4 Connect the catheter to the perfusion line and stabilize the setup to avoid dislocation of the catheter (Fig.3a, Supplementary Video 1).

TROUBLESHOOTING

Critical Step: Proper cannulation of the IVC is crucial for a successful perfusion. Ensure that the catheter is correctly inserted in the IVC by aspirating with a syringe to see blood flow. Make certain that no bubbles are introduced by filling the catheter with EGTA solution prior to connecting the perfusion line. Work quickly to avoid delay in perfusion in order to prevent thrombosis which results in improper digestion.

5 Start the pump to begin perfusion of the mouse liver with EGTA solution.

6 Wait until portal vein becomes distended and then cut the portal vein.

7 Cut through the diaphragm and place the clamp on the IVC above the diaphragm to ensure retrograde perfusion of the liver (Fig.3b, Supplementary Video 2). If puncture and initial perfusion were performed adequately, the liver should be pale after severing the portal vein and clamping the suprahepatic IVC.

TROUBLESHOOTING

8 Perfuse the liver with the EGTA for a total of 1–2 min. Switch on the heat lamp directed toward the mouse liver.

9 Change from the EGTA solution to the pronase solution (Fig.3c), and perfuse the liver for 5 min (Fig.3c).

10 Change from the pronase solution to the collagenase solution and perfuse the liver for 7 min. Remove the perfusion line out of the collagenase solution and continue perfusing the liver until the perfusion line is empty (Fig.3c).

Critical Step: Perfuse for 9 min instead of 7 min when isolation HSCs from two months old Mdr2^{ko} mice.

11 Remove the catheter from the IVC. Explant the liver into a sterile Petri dish containing 5ml of the pronase/collagenase solution (Fig.3d).

12 Gently mince the liver under a sterile cell culture hood with forceps (Fig.3d, Supplemental Video 3). The liver should almost be completely fluid without chunks of tissue after successful *in situ* perfusion.

Steps 13–15: *In vitro* digestion—Timing: 50 min

13 Add 1% DNAse to the prewarmed pronase/collagenase solution. Transfer the minced liver into the prewarmed pronase/collagenase solution containing 1% DNAse and place on the stir plate (40°C, stirring level 1) for 25 min (Fig. 3e).

Critical Step: Do not exceed 25 min to prevent loss of cell viability due to overdigestion. However, if *in situ* enzyme perfusion did not thoroughly digest the liver, prolong the *in vitro* digestion procedure by at most 10 min.

TROUBLESHOOTING

14 Filter the digested mouse liver through a 70 μm cell strainer into a 50 ml Falcon tube (Fig. 3e).

15 Centrifuge the mixture at 580 g for 10 min (4°C). Aspirate the supernatant until 10 ml remains in the tube, and then add 120 μl of DNase I and resuspend with a 10ml pipet. Fill up to 50 ml with GBSS/B and resuspend to wash the cells, and then centrifuge again at 580 g for 10 min (4°C) (Fig. 3f).

Steps 16–20: Density gradient-mediated separation—Timing: 60 min

16 Aspirate the supernatant until 10 ml remains in the tube, add 120 μl of DNase I and resuspend with a 10 ml pipet. Fill up to 32 ml with GBSS/B and then add 16 ml of Nycodenz solution. Mix thoroughly by gently pipetting or inverting the tube and pipet 12 ml of cell-Nycodenz suspension into each of the four 15 ml Falcon tubes. Tilt the tubes to moisten the walls to facilitate the overlay.

17 Gently overlay the cell-Nycodenz suspension with 1.5 ml of GBSS/B using a 3 ml syringe with a 26G needle attached (Fig. 3g, Supplementary Video 4). Centrifuge at 1380 g for 17 min (4°C) without brake (Fig.3g).

Critical Step: Make sure to overlay the GBSS/B very slowly and gently above the cell-Nycodenz suspension to create a discontinuous gradient. A clear separation should be observed between the cell-Nycodenz suspension and GBSS/B.

Critical Step: Ensure that the deceleration setting is set to 0 or “no brake,” otherwise the discontinuous gradient will fail to collect HSCs in the interface.

TROUBLESHOOTING

18 At the end of centrifugation, the HSCs are visible as a thin white layer in the interface between the cell-Nycodenz solution and the overlay with GBSS/B

(Fig. 3h). Use a 5 ml pipet to harvest the cells and transfer into a new 50 ml Falcon tube (Fig. 3h, Supplementary Video 5). Repeat the procedure for all four 15 ml Falcon tubes.

19 Add GBSS/B to fill up the Falcon tube to 50 ml and gently pipet to resuspend the harvested HSCs; centrifuge at 580 g for 10 min (4°C) (Fig. 3i).

20 Aspirate the supernatant and resuspend cell pellet in DMEM with 10%FBS if it is to be used immediately for cell culture. If subsequent FACS sorting is planned, proceed to Steps 21–25.

TROUBLESHOOTING

Steps 21–24: Optional Secondary Purification of Isolated HSCs- FACS Sorting —Timing: 40 min

21 If an ultrapure population of HSCs is desired, collect the cell pellet and resuspend in an appropriate volume of DMEM (without phenol red) with 1% FBS to achieve 10^7 cells per ml (use at least a volume of 300 μL).

Critical Step: Ensure cells are protected from light to prevent photobleaching of retinoid fluorescence.

22 Use a 405–407 nm laser for excitation and a 450/50-nm bandpass filter on cell sorter for retinoid detection. To sort out retinoid-positive HSCs, three gating steps are typically performed. The first gate should be set in the forward (FSC-A) and sideward scatter (SSC-A) (Supplementary Figure 2b, left FACS plots), followed by a gate to exclude cell duplets (Supplementary Figure 2b, right FACS plots). Then, cells are analyzed by the violet A channel in combination with either the FITC channel or the forward-scatter (FSC-A), revealing cells according to their retinoid content (Figure 2a).

23 Sort HSCs into 15ml Falcon tubes containing 2 ml DMEM with 20% FBS. Each sort should take approximately 15–20 minutes depending on the volume.

Critical step: If sorted cells will be used for cell culture, sort at a lower pressure, to ensure cell viability. Keep isolated and FACS-sorted cells on ice all times.

TROUBLESHOOTING

24 Centrifuge the cells at 580 g for 10 min (4°C) to pellet the sorted cells. Cells can then be cultured or stored appropriately at −80°C for future analysis.

TIMING

Preparation of Solutions: 1 h

Steps 1–12: *In situ* perfusion: 20 min

Steps 13–15: *In vitro* digestion and washes: 50 min

Steps 16–20: Gradient-mediated separation: 60 min

Steps 21–24: Optional FACS procedure: 40 min

TROUBLESHOOTING

On rare occasions, yield and purity of isolations will be unexpectedly low and systematic troubleshooting is required. This occurs most commonly when new lots of enzyme are used, and adjustment of enzyme concentrations may be required. Additional problems can result from alterations in water quality or calcium deposits in glassware and perfusion lines, in particular if the lines are not changed on a regular base. Of note, improper preparation of the EGTA and enzyme solutions (sometimes visible as a turbid solution) can result in clotting of hepatic vessels, resulting in partial digestion, and/or affect the activity of collagenase and pronase. Likewise, the length of perfusion lines influences the perfusion temperature and should be kept constant. For a summary of potential problems and troubleshooting, see Table 1.

ANTICIPATED RESULTS

As previously mentioned, isolation from mice of the Balb/c background usually results in higher HSC yield than isolation from other strains including C57Bl/6 and transgenic mice of mixed background. However, beside the genetic background, age is the most critical determinant of yield and purity. From experience, mice younger than 8 weeks of age yield fewer than 0.5–1 million cells per mouse and may require pooling of multiple mice for a single experiment. While isolations of HSCs from mice between 8 and 12 weeks of age typically yield between one to two million HSCs per mouse, mice older than 12 weeks or retired breeders (usually 7–9 months of age when ordered from Taconic or Jackson Laboratories) allow isolation of two to three million HSCs. This protocol achieves about 90–98% purity from isolations from normal livers in retired breeders and 80–90% purity when isolating from injured/fibrotic livers. Consequently, if an ultrapure HSC population is desired from mice with liver injury, the add-on FACS protocol is highly recommended to eliminate contamination from non-HSC hepatic cells. However, it should be noted that FACS sorting will improve purity but decrease yield, depending on the stringency of the gating and selection process. The average yield of RNA from 1 million HSCs ranges from 0.8–1.2 μg, with activated cells generally yielding more RNA.

LIMITATIONS

Although the described protocol typically yields large quantities of pure HSCs, there are limitations to be considered. As discussed yield will be lower in C57Bl/6 mice, especially when younger mice are used. In obese mice, excess peritoneal fat may obscure access to the IVC and make it difficult to cannulate. In this particular setting, perfusion via the portal vein can be considered. Also, excessive hepatic steatosis may result in increased hepatocyte contamination as steatotic hepatocytes acquire a density more similar to HSCs. Furthermore, isolation procedures expose HSCs to significant stress such as enzymatic digestions, centrifugation forces and loss of cell-cell communication. These limitations have to be considered when analyzing rapid signaling events such as immediate early gene expression, transcription factors or kinase activation – which may be activated as a result. Comparison between cell isolates that both underwent the same isolation procedure (e.g. comparing

FACS-purified HSCs from normal and fibrotic livers) will circumvent this problem as most changes will occur in both conditions and therefore not affect the comparison. Likewise, digestion procedures may temporarily decrease the expression of cell surface proteins and hamper flow-cytometric analysis of surface markers. Finally, the described add-on FACS sorting protocol can reduce the ability of HSCs to attach to cell culture plates and to undergo culture-activation, in particular if one does not optimize cell collection and employ low-pressure sorting procedures. As discussed, FACS-based ultrapurification reduces the overall yield. Depending on how stringently the gates are set, one can either minimize or even completely exclude contamination by other cells by very selective gating on HSCs at the cost of reduced yield, or increase yield by less stringent gating at the cost of slightly increased contamination with other cells. Although our data clearly show that retinoid-based HSC sorting maintains HSC expression and activation markers and reduces contamination with other cell types, we cannot completely rule out that the retinoid-low HSC population excluded by this method contains a small subset of HSCs with yet unrecognized functions that are different from the main population of retinoid-high HSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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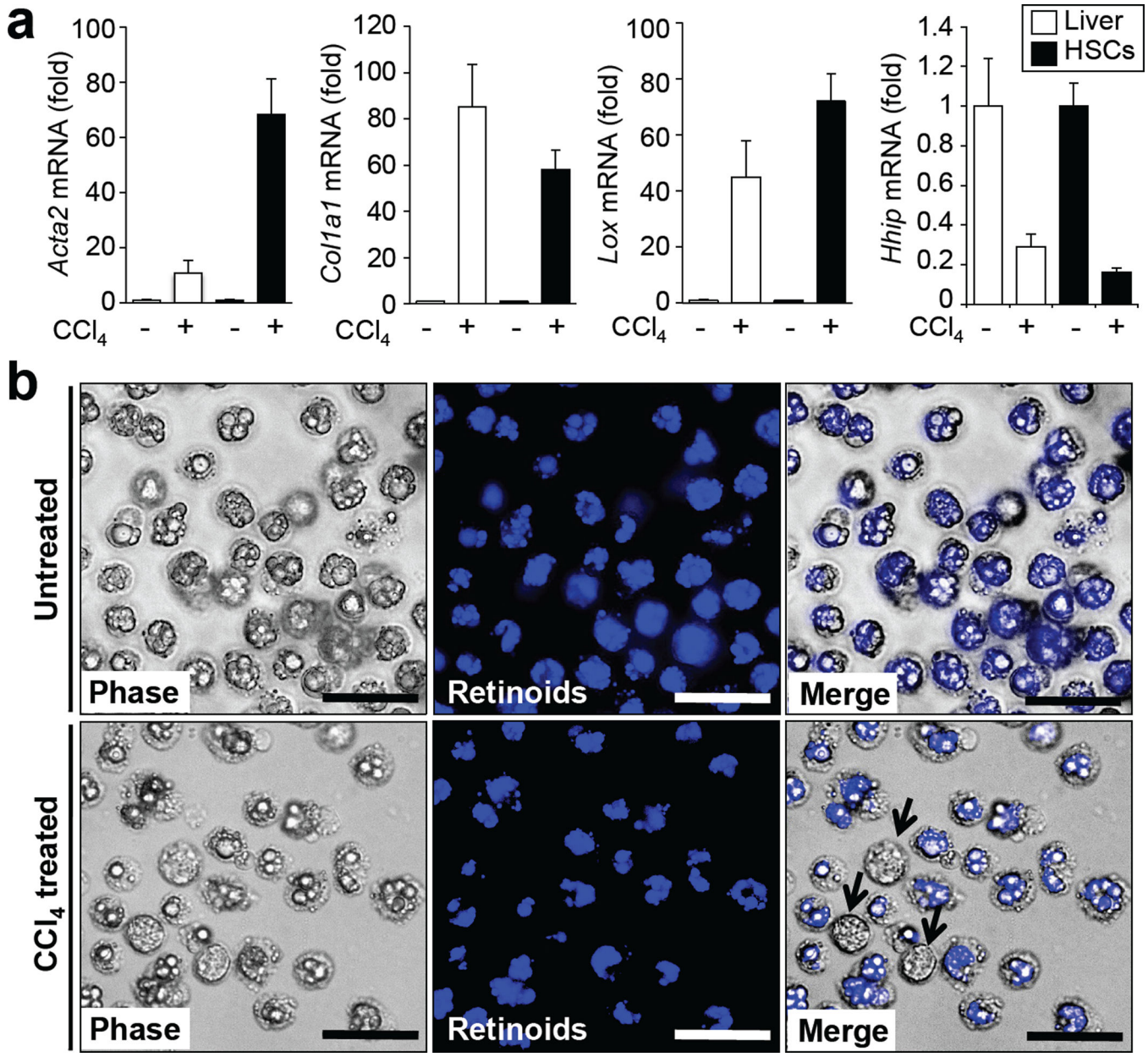

Figure 1. Isolated HSCs reflect gene expression found in fibrotic livers

Mice were treated with four injections of CCl_4 (0.25 μl/g for the first dose and then 0.5 μl/g i.p. for subsequent doses), dissolved in corn oil at a ratio of 1:3, injected every 3 days. **a.** Expression of fibrogenic genes *Acta2* (encoding for αSMA), *Col1a1, Lox* and *Hhip*, (a gene known to be downregulated upon HSC activation), was determined in liver and unplated HSCs isolated by Nycodenz gradient. Gene expression was normalized to *18s*. Data are shown as means ± s.e.m. n=5 for control mice and n=10 for CCl_4-treated mice. **b.** Representative images of freshly isolated HSCs from a control mouse (upper panel) and CCl_4-treated mouse (lower panel) visualized using phase contrast microscopy (left) and retinoid fluorescence (center). A merge (right) of the retinoid fluorescence with the phase contrast image shows complete overlap of retinoid signal with characteristic lipid droplets.

Arrows indicate contamination with non-HSC cell types. Scale bars, 50 μm. All animal procedures were approved by the Institutional Animal Care and Use Committee at Columbia University.

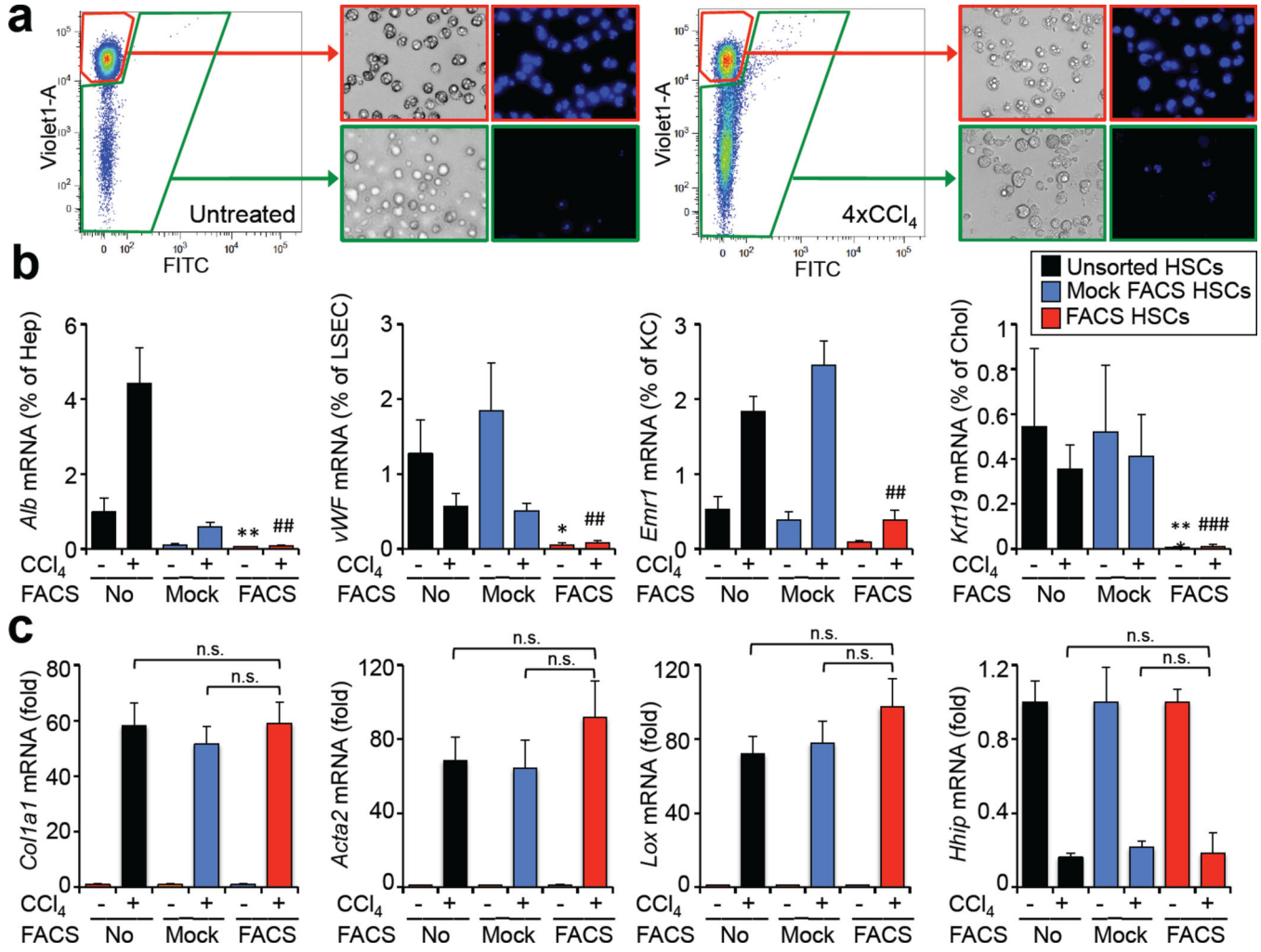

Figure 2. Retinoid-based FACS sorting improves purity of HSC isolates without impairing expression of HSC activation markers

a. Retinoid-based ultrapurification of Nycodenz-gradient purified HSCs from untreated (left panel) and CCl_4-treated (right panel) via FACS. **b.** Gene expression of non-HSC cell contamination markers (*Alb* for hepatocytes ["Hep"], *vWF for* liver sinusoid endothelial cells ["LSEC"], *Emr1* for liver macrophages ["KC"] and *Krt19* for cholangiocytes ["Chol"]) in unsorted, mock-sorted and FACS-ultrapurified HSCs. Relative contamination was determined by comparing the different fractions to pure isolates of hepatocytes, cholangiocytes, liver sinusoidal endothelial cells and liver macrophages (each set as 100% value), respectively. **c.** HSC activation was determined by qPCR for *Col1a1, Acta2, Lox* and *Hhip* in unsorted, mock-sorted and FACS-ultrapurified HSCs. Data are presented as fold induction in comparison to HSCs from control liver. All data are shown as means ± s.e.m. n=5 control mice and n=10 CCl_4-treated mice. All animal procedures were approved by the Institutional Animal Care and Use Committee at Columbia University. $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. control mock- and unsorted HSCs; * $p<0.05$ and ** $p<0.01$ vs. CCl_4-treated mock- and unsorted HSCs; n.s., non-significant.

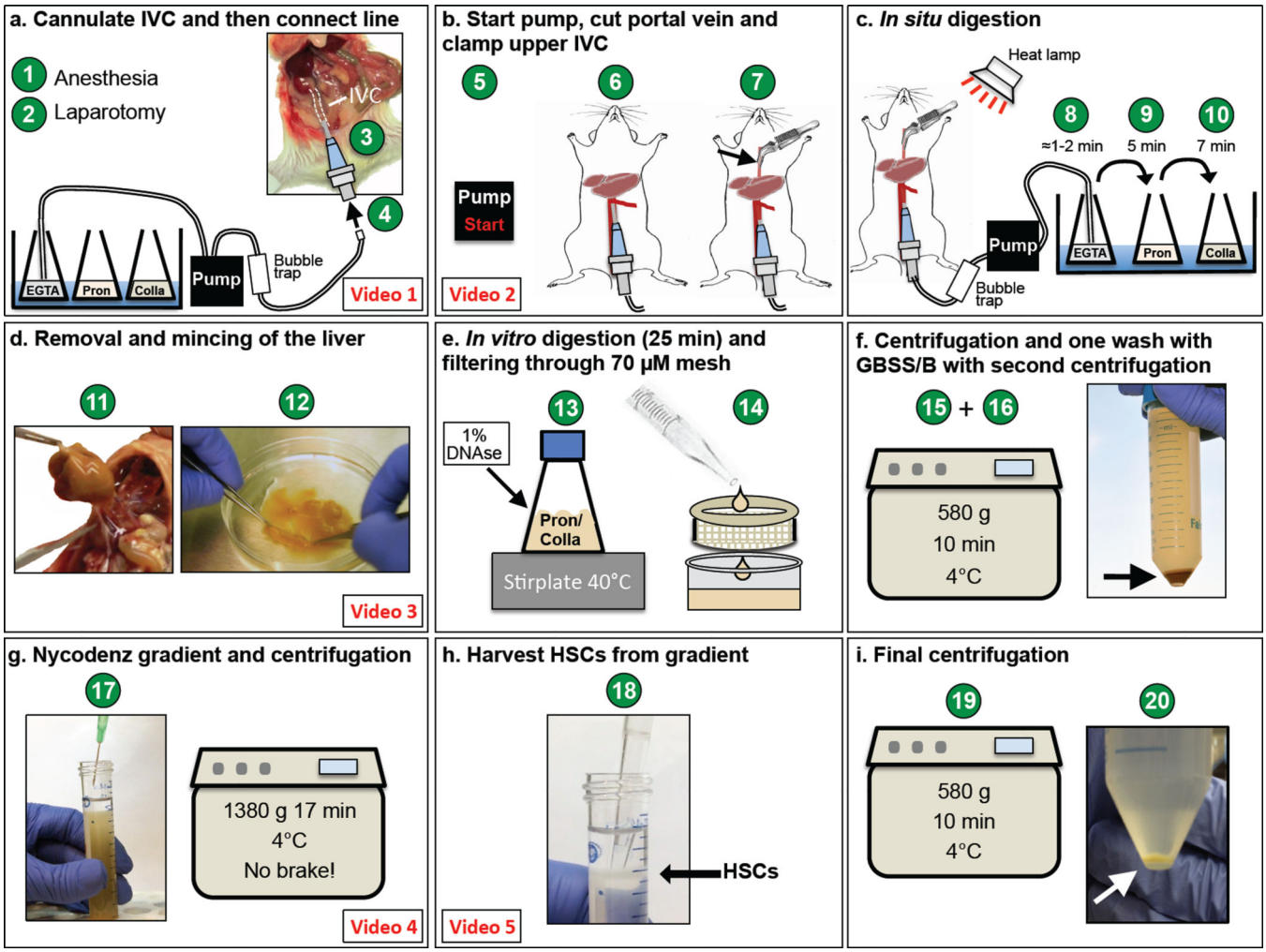

Figure 3. Overview of HSC isolation procedure

a. Cannulation of anesthetized mouse via IVC (Video 1) and experimental set-up. **b.** The pump is started, the portal vein is severed and the suprahepatic IVC is clamped (Video 2). **c.** Sequential perfusion with EGTA, pronase and collagenase solutions. **d.** The digested liver is excised and minced thoroughly on a Petri dish (Video 3). **e.** The liver is further digested *in vitro* and cell-suspension is filtered to remove undigested debris. **f.** Cells are centrifuged and cell pellet is washed with GBSS/B. **g.** GBSS/B is overlayed on cell-Nycodenz mixture to create discontinuous gradient (Video 4). **h.** HSCs are harvested by removing cell layer from the gradient interface (Video 5). **i.** Final centrifugation to pellet and collect HSCs. All animal procedures were approved by the Institutional Animal Care and Use Committee at Columbia University.

Table 1

Troubleshooting Table

Steps	Problem	Possible Reason	Solution
3, 4	Obscured IVC	Obese or old mice	Perfuse from the portal vein
	Discoloration of liver not homogenous after EGTA perfusion	Catheter inserted too deep	Pull back catheter carefully, but do not pull out of IVC and resume perfusion
		Venous air embolism	Try to avoid any air bubbles in the catheter before connecting to perfusion line
		Thrombosis	Try to avoid any delay during cannulation and the start of perfusion as blood may clot vessels preventing successful perfusion
	Swelling close to IVC immediately after starting perfusion	Catheter not placed in IVC	Stop perfusion and keep catheter in place (if catheter is removed bleeding will start which makes another cannulation almost impossible), use a new catheter and try to insert in IVC 1–2 mm cranial of initial attempt
7, 10, 13	Incomplete digestion	Enzymes	Ensure enzyme concentrations are correct, longer digestion is required for fibrotic livers
		Water Quality	Use a different water source and ensure to pH the solutions are to 7.35–7.4
		Calcium deposits in glassware/ perfusion lines	Use only clean glassware in preparing reagents. If calcium deposits are observed in glassware, remove by soaking glassware in a diluted solution of 10% acetic acid and thoroughly washing. Change the perfusion line regularly.
		EGTA/Enzyme Buffer Solutions	Ensure EGTA and enzyme solutions are prepared correctly and pH is 7.35 to 7.4
		Temperature	Ensure that temperature of solutions are 42°C when in vivo digestion is started
		Venous air embolism/Thrombosis	See above
17	Disrupted discontinuous gradient	Pipeting overlay too quickly/roughly	Ensure that the tube walls are moistened to facilitate overlay of GBSS/B. In the case of a disrupted overlay, pipet out GBSS/B layer, resuspend the cell mixture and redo overlay
20	Low yield	Young animal, genetic background	Use mice >12 weeks of age, preferably Balb/C background Pool cells from multiple animals
		Incomplete digestion	See above
20	Low purity	Fatty hepatocytes	Proceed with additional FACS sorting procedure to further purify HSCs
		Incorrect gradient concentration	Weigh out correct amount of Nycodenz
		Low yield	Usually low-yield isolations are not pure and require FACS sorting
20, 23	Low viability	Rough handling during isolation	Pipet gently to minimize mechanical stress on cells
		Enzyme overdigestion	Use correct concentration of enzymes, if new lots are used and overdigestion is observed, shorten perfusion time and/or reduce amount of enzyme used
		Temperature of solution during in vitro digestion	Monitor temperature during *in situ* digestion with thermometer to not overheat the solution and thermodamage the HSCs

Steps	Problem	Possible Reason	Solution
		Suboptimal FACS sort conditions	Sort sells with low pressure FACS-settings and ensure to sort cells into 20% FBS containing medium and not into empty FACS or empty 15mL tubes