Distinct Populations of Hepatic Stellate Cells in the Mouse Liver Have Different Capacities for Retinoid and Lipid Storage

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Abstract

Hepatic stellate cell (HSC) lipid droplets are specialized organelles for the storage of retinoid, accounting for 50–60% of all retinoid present in the body. When HSCs activate, retinyl ester levels progressively decrease and the lipid droplets are lost. The objective of this study was to determine if the HSC population in a healthy, uninjured liver demonstrates heterogeneity in its capacity for retinoid and lipid storage in lipid droplets. To this end, we utilized two methods of HSC isolation, which leverage distinct properties of these cells, including their vitamin A content and collagen expression. HSCs were isolated either from wild type (WT) mice in the C57BL/6 genetic background by flotation in a Nycodenz density gradient, followed by fluorescence activated cell sorting (FACS) based on vitamin A autofluorescence, or from collagen-green fluorescent protein (GFP) mice by FACS based on GFP expression from a GFP transgene driven by the collagen I promoter. We show that GFP-HSCs have: (i) increased expression of typical markers of HSC activation; (ii) decreased retinyl ester levels, accompanied by reduced expression of the enzyme needed for hepatic retinyl ester synthesis (LRAT); (iii) decreased triglyceride levels; (iv) increased expression of genes associated with lipid catabolism; and (v) an increase in expression of the retinoid-catabolizing cytochrome, CYP2S1. Conclusion: Our observations suggest that the HSC population in a healthy, uninjured liver is heterogeneous. One subset of the total HSC population, which expresses early markers of HSC activation, may be “primed” and ready for rapid response to acute liver injury.

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Introduction

Retinoids (vitamin A and its metabolites, both natural and synthetic) are essential to many physiological processes, including reproduction, embryonic development, bone growth, immunity and vision [1–3]. Seventy percent of retinoid in the body is stored in the liver [4,5], and, of this fraction, 90–95% is stored in lipid droplets within hepatic stellate cells (HSCs) [6]. These lipid droplets are a distinguishing feature of HSCs and have been proposed to be specialized organelles for retinoid storage due to their retinoid content and responsiveness to dietary retinoid status [7]. Retinyl esters comprise approximately 40% of the lipids present in these droplets, more than any other single lipid species [8,9]. And while the remaining 60% is all non-retinoid lipid species, including triglyceride, cholesterol ester, cholesterol, phospholipid and free fatty acid, there is considerable data in the literature suggesting that the formation and maintenance of these lipid droplets are retinoid-dependent processes [7].

Moriwaki et al. showed that the lipid composition of HSC lipid droplets is strongly regulated by dietary retinoid status, but not by dietary triglyceride intake [8]. HSC lipid droplet retinoid lipid species are decreased in response to a low retinol diet and both retinoid and non-retinoid lipids are elevated in response to a high retinol diet; however, neither retinoid nor non-retinoid content is affected by low or high fat diets. Other data that are highly suggestive of the role of retinoids in these droplets relates to the enzyme lecithin:retinol acyltransferase (LRAT), which is the only known enzyme in the liver capable of synthesizing retinyl ester. O’Byrne et al. showed that the HSCs of wild type (WT) mice have large, distinct lipid droplets, but the cells of LRAT-null mice have none [10]. Thus, the ability to synthesize and store retinyl ester in HSCs is necessary for the presence of HSC lipid droplets.

It is well-established that when HSCs activate, in response to a hepatic insult or disease, the HSCs loose their lipid droplet content and undergo a simultaneous decrease in retinyl ester levels. Leo and Lieber found that there is a nearly 5-fold decrease in total hepatic retinol levels with the development of alcoholic hepatitis and another approximately 4-fold decrease with the development of cirrhosis [11]. It has also been shown in cultured HSCs that retinyl ester stored in HSC lipid droplets is first hydrolyzed and then released into the media as retinol [12]. As HSCs transition from a quiescent to a myofibroblastic phenotype, they undergo...
increased extracellular matrix production, including increased synthesis of collagen I, and become fibrogenic [13,14]. It has not yet been unequivocally determined whether the loss of HSC lipid droplets is a cause or consequence of activation.

We are interested in understanding the factors that regulate HSC retinoid storage as retinyl esters in lipid droplets and the factors that regulate HSC lipid droplet genesis and dissolution. In this study, we employ two methods of HSC isolation, which leverage distinct properties of these cells. One method relies on HSC lipid droplet vitamin A content and the other on HSC expression of collagen I. It was recently shown that the changes in gene expression that accompany HSC activation and the loss of retinyl ester lipid droplets are regulated differently in the in vitro and in vivo models of activation [15]. Similarly, a goal of this study is to determine whether different methods of HSC isolation will yield phenotypically distinct populations of cells and how this may reflect heterogeneity of HSCs in the liver at a given time. We are particularly interested in how these populations compare with regard to their capacities for retinoid storage and lipid droplet formation. Our findings are presented below.

Materials and Methods

Animals

WT and collagen-green fluorescent protein (GFP) mice were used, with both strains congenic for the C57BL/6 genetic background. The collagen-GFP mice have been previously described [16]. Brieﬂy, a gene construct was made containing 3,122 bp of the α1(I) collagen (Col1α1) gene promoter linked to the GFP reporter gene [16]. Hepatic expression of collagen I is solely in HSCs [17,18], and its expression is increased as the cells become activated [19,20]. Thus, GFP expression from this transgene can be used to identify and isolate HSCs. All mice used in the study were males between 90 and 120 days of age at the time of sacrifice. Animals were allowed access to water and a standard nutritionally complete rodent chow diet (W. F. Fisher and Sons, Inc., Somerville, NJ). All mice were maintained on a 12-h dark-light cycle, with the period of darkness between 7:00 a.m. and 7:00 p.m. in a conventional barrier facility. The animal experiments described in this report were conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals and were approved by the Columbia University Institutional Committee on Animal Care (IACUC Protocol # AC-AAAA9687).

Hepatic fibrosis induction

WT C57BL/6 mice were given weekly injections of either 0.5 μl carbon tetrachloride (CCl4) per gram body weight administered in corn oil or an equivalent volume of corn oil alone for 4 weeks and then sacriﬁced. Liver tissue was collected and stored immediately at −80°C prior to analysis.

Hepatocyte isolations

Livers were perfused in situ with EGTA for 5 min and collagenase D (0.5 mg/ml, Roche Diagnostics, Indianapolis, IN) for 15 min, respectively, at a flow rate of 5 ml/min. After perfusion, the partially digested liver was excised, the digest passed through a 100 μm nylon mesh to remove undigested materials, and resuspended in Dulbecco’s Modiﬁed Eagle Medium (DMEM; Gibco, Grand Island, NY) containing 1% penicillin/streptomycin. Hepatocytes were separated from the non-parenchymal cells and debris by centrifugation at 4°C in the following sequence: twice for 5 min at 20 g, once for 10 min at 50 g, and twice again for five min at 20 g. The supernatant was aspirated and the hepatocytes present in the pellet were resuspended in DMEM. Cell yields were determined by counting on a hemacytometer.

HSC isolations

Primary mouse HSCs were isolated according to established protocols [9,21,22]. Brieﬂy, livers were perfused in situ with EGTA for 5 min, pronase E (0.4 mg/ml, EMD Chemicals Inc., Gibbstown, NJ) for 5 min and collagenase D (0.5 mg/ml, Roche Diagnostics) for 8 min, respectively, at a ﬂow rate of 5 ml/min. After excision of the liver from the body, the liver digest was ﬁltered through a cell strainer and washed with Gey’s Balanced Salt Solution (Sigma, St. Louis, MO) containing DNase I (2 mg/ml, Roche Diagnostics). For the WT C57 mice, HSCs were puriﬁed from the remainder of non-parenchymal cells and hepatocyte-derived debris by ﬂoatation through 9% (w/v) Nycodenz (Axis-­­Shield PoC AS, Oslo, Norway) in Gey’s Balanced Salt Solution (without NaCl). Subsequently, the cells were separated by FACS using FACs Calibur (Becton Dickinson, Franklin Lakes, NJ), and vitamin A auto-ﬂuorescent cells were collected based on their emission at 460 nm. This method of HSC isolation yields approximately 10% of the total HSCs estimated to be in the liver. For the collagen-GFP mice, parenchymal cells and debris were separated from non-parenchymal cells by centrifugation at 50 g for 2 minutes. The remaining non-parenchymal cell suspension was then separated by FACS, and GFP-positive cells were collected based on their emission at 530 nm. This method of HSC isolation yields approximately 10% of the total HSCs estimated to be in the liver.

Isolation of RNA and cDNA synthesis

Total RNA was isolated from HSCs using the Qiagen RNeasy kit (Qiagen, Valencia, CA). cDNA synthesis and ampliﬁcation were performed using the NuGEN WT-Ovation Pico RNA Ampliﬁcation kit (NuGEN, San Carlos, CA). The resulting cDNA was then puriﬁed using the Qiagen QIAquick PCR Puriﬁcation kit (Qiagen). cDNA fragmentation and labeling were performed using the NuGEN FL-Ovation cDNA Biotin Module V2 kit (NuGEN).

DNA microarray analysis

Array analysis was conducted on the Affymetrix Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) and analyzed using the GeneSifter gene expression analysis suite (Geospiza, Seattle, WA). Individual gene expression values were normalized to the median. Significant changes in gene expression between sample sets were identiﬁed by a Student’s t-test and subsequent Benjamini–Hochberg correction, with a p-value of 0.05 as the criterion for statistical signiﬁcance. All microarray data are MIAME compliant and are deposited as ArrayExpress accession E-MEXP-3231.

Quantitative real-time PCR

Differential gene expression was conﬁrmed by quantitative real-time polymerase chain reaction (qRT-PCR) using commercially available primer-probe sets (Table S1, Applied Biosystems, Foster City, CA). qRT-PCR was performed on a LightCycler 480 (Roche Diagnostics).

Immunohistochemistry

Liver tissue samples from collagen-GFP mice were collected into 10% formalin. Immunohistochemistry was conducted by the Herbert Irving Comprehensive Cancer Center Histology Service using standard protocols. Desmin expression was localized using an anti-desmin monoclonal mouse anti-human antibody at a dilution of
Arrows point to a hepatic stellate cell. Insets show magnified view of one HSC.

Taken for further analysis. For HSCs, aliquots of 100,000 cells were

Microscope (Olympus America Inc., Center Valley, PA).

and fluorescent images were captured using an FSX100 Olympus

Ashland, MA) and incubated at 37°C for 6 h. Cells were seeded on 35 mm glass bottom dishes (MatTek Co., Ashland, MA) and incubated at 37°C overnight. Phase contrast and fluorescent images were captured using an FSX100 Olympus Microscope (Olympus America Inc., Center Valley, PA).

Primary HSC culture

HSCs were isolated from WT and collagen-GFP mice as described above. Approximately 0.5 x 10^6 cells were resuspended in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin, seeded on 35 mm glass bottom dishes (MatTek Co., Ashland, MA) and incubated at 37°C overnight. Phase contrast and fluorescent images were captured using an FSX100 Olympus Microscope with Apotome (Zeiss, Göttingen, DE), and images were taken using an AxioCam MRm camera (Zeiss).

Reverse-phase HPLC analysis

Liver and HSC retinyl ester levels were determined by procedures described previously [10]. Briefly, 200 mg liver tissue was homogenized in 2 ml of PBS (10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) and 200 μl aliquots were added immediately after homogenization of the cells and tissues.

Triglyceride analysis

Approximately 200 mg of liver or 0.5 x 10^6 HSCs were homogenized in 1 ml of PBS using a Polytron homogenizer. The lipids in the homogenates were then extracted into chloroform:methanol (2:1 v/v), and the chloroform phase was collected. The upper phase was re-extracted with an additional volume of chloroform:methanol to ensure complete triglyceride recovery, and the pooled chloroform phases were evaporated under N2. One milliliter of 2% Triton X-100 in chloriform for liver or 50 μl for HSCs was then added to the samples, and the chloroform was re-evaporated under N2. For liver, triglycerides were solubilized for colorimetric assay through addition of 1 mL of deionized water into the glass tubes. Employing a Matrix Plus Chemistry Reference Standard (VWR Laboratories Inc., Providence, RI), according to the manufacturer’s instructions, a colorimetric triglyceride assay using the Infinity Triglyceride Reagent (Thermo Fisher Scientific Inc., Middletown, VA) was performed in a 96-well plate for 5 μl of each liver sample. For HSCs, 200 μl of Triglyceride Reagent was added directly to the dried-down samples, and the entire volume was read in the colorimetric assay. Color development was measured on a Multiskan Plus Microtiter Plate Reader at 520 nm.

Western blot protein analysis

HSC proteins were analyzed by Western blot assay. For all proteins analyzed, 5 μg of total protein was separated on 10%
SDS-PAGE gels and transferred onto polyvinylidene fluoride membrane (Millipore Immunobilon-P Transfer Membrane) at 100 V for 1 h at 4°C. The membranes were then incubated with 10% milk blocking buffer for 1 h at room temperature (RT), followed by overnight incubation at 4°C with the following primary antibodies: alpha smooth muscle actin (1:100, rabbit polyclonal, Abcam, Cambridge, MA, ab5694), adipose differentiation related protein (1:500, rabbit polyclonal, Abcam, ab52356), CYP2S1 (1:500, rabbit polyclonal, Abcam, ab69650), CYP2E1 (1:500, rabbit polyclonal, Abcam, ab28146) and LRAT (1:500, mouse monoclonal, gift from Dr. Krzysztof Palczewski, Department of Pharmacology, Case Western Reserve University). The secondary antibody incubations were for 1 h at RT with either a donkey horseradish peroxidase-conjugated anti-rabbit IgG antiserum (1:10,000, GE Healthcare, Piscataway, NJ, cat # NA934V) or goat horseradish peroxidase-conjugated anti-mouse IgG antiserum (1:10,000, EMD Chemicals Inc., Gibbstown, NJ, cat # 401215). Immunoblots were developed using the ECL system (Thermo Scientific, Rockford, IL).

**Table 2. KEGG Pathway Enrichment.**

| z-score | KEGG Pathway                                    | Gene Set | Total Changed | Up    | Down | z-score |
|---------|-------------------------------------------------|----------|---------------|-------|------|---------|
| UP      | Cell adhesion molecules (CAMs)                  | 131      | 51            | 34    | 17   | 5.64    |
|         | Cytokine-cytokine receptor interaction          | 232      | 74            | 48    | 26   | 4.95    |
|         | Leishmaniasis                                   | 66       | 30            | 19    | 11   | 4.72    |
|         | Type 1 diabetes mellitus                        | 44       | 19            | 14    | 5    | 4.50    |
|         | Leukocyte transendothelial migration            | 114      | 44            | 26    | 18   | 4.16    |
|         | ECM-receptor interaction                        | 76       | 27            | 18    | 9    | 3.63    |
|         | Maturity onset diabetes of the young            | 25       | 10            | 8     | 2    | 3.41    |
|         | Steroid biosynthesis                            | 17       | 6             | 6     | 0    | 3.25    |
|         | Glycine, serine and threonine metabolism        | 30       | 9             | 8     | 1    | 2.80    |
| DOWN    | Axon guidance                                   | 127      | 54            | 24    | 30   | 4.76    |
|         | Focal adhesion                                  | 189      | 70            | 33    | 37   | 4.01    |
|         | Gap junction                                    | 83       | 25            | 7     | 18   | 3.25    |
|         | Cell cycle                                      | 123      | 27            | 4     | 23   | 2.89    |
|         | ErbB signaling pathway                          | 84       | 22            | 6     | 16   | 2.48    |
|         | Chemokine signaling pathway                     | 173      | 47            | 20    | 27   | 2.11    |

Tests of KEGG pathway enrichment were conducted on the set of 5363 differentially expressed genes identified by the Student’s t-test as having a p-value equal to or less than 0.05. The tests were performed using the GeneSifter software. A pathway is defined as enriched if it has a positive z-score up or down greater than or equal to 2.

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**Table 3. Markers of HSC Activation.**

| AFFY ID   | Gene Name               | Gene ID | FC   | p-value  |
|-----------|-------------------------|---------|------|----------|
| 1449254_at | Secreted phosphoprotein 1 | Spp1 | 115.5 | 0.000001 |
| 1419123_a_at | Platelet-derived growth factor, C polypeptide | PdgfC | 39.3 | 0.002963 |
| 1449351_s_at | Platelet-derived growth factor, C polypeptide | PdgfC | 32.5 | 0.003461 |
| 1456658_at | Actin, alpha 2, smooth muscle | Acta2 | 5.5 | 0.001718 |
| 1456156_at | Leptin receptor         | Lepr    | 4.2  | 0.001739 |
| 1451924_a_at | Endothelin 1             | Edn1    | 4.2  | 0.000544 |
| 1416454_s_at | Actin, alpha 2, smooth muscle | Acta2 | 3.5  | 0.016302 |
| 1438303_at | Transforming growth factor, beta 2 | Tgfb2 | 2.7  | 0.01149  |
| 1448729_a_at | Septin 4                 | Sept4   | 0.49 | 0.003582 |
| 1443434_s_at | Plexin C1                | Plxnc1  | 0.45 | 0.000309 |
| 1455851_at | Bone morphogenetic protein 5 | Bmp5 | 0.40 | 0.000004 |

Differences in gene expression are reported as fold-change (FC) relative to AF-HSCs.

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Hepatic Stellate Cell Retinoid and Lipid Storage
Nycodenz flotation of cells present in a pronase digest of a liver. Vitamin A autofluoresces at 460 nm upon excitation at 350 nm, so exposure to this excitation wavelength allowed us to collect a population of cells that is enriched in vitamin A. This population of lipid droplet- and vitamin A-enriched HSCs would be referred to subsequently as AF-HSCs. The second method of HSC isolation used in this study capitalizes on transgene GFP expression driven by the Col1a1 promoter in these mice [16] and data indicating that expression of collagen I in the liver occurs exclusively in the HSCs [17,18]. We performed FACS on digests of non-parenchymal cells and collected GFP-positive cells based on their excitation at 488 nm. This GFP-expressing population of HSCs will be referred to as GFP-HSCs. To confirm that GFP expression is confined to HSCs, we performed immunohistochemistry using antibodies to GFP and the HSC marker desmin [23]. We show that GFP expression is found only in the HSCs, with GFP expression colocalizing with desmin expression, and is not found in the other major cell types in the liver (Figure 1A–C). We further analyzed the integrity of these populations by qRT-PCR using desmin mRNA expression as a marker of HSC purity, and we show that the two different methods of HSC isolation yield cell populations with equivalent desmin expression (Figure S1A).

Microarray analysis overview

For this study, we performed gene microarray analysis on populations of AF-HSCs isolated from 6 mice and GFP-HSCs isolated from 5 mice. cDNA purified from these populations was run on Affymetrix Mouse Genome 430 2.0 Arrays, covering over 45,000 transcripts in the mouse genome, and data was analyzed using GeneSifter software. The quality of the samples used is demonstrated by the box plot and scatter plot summaries of the AF and GFP groups, which show that the spread of all the array data points is identical in both groups (Figure S1B and S1C). Five thousand three hundred sixty three probe sets were more than 2-fold up- or down-regulated in the GFP-HSCs compared to AF-HSCs (Table 1). As a second-stage filter and an analysis with biological implications, the list of transcripts with significant differential expression between AF- and GFP-HSCs was screened against >200 known KEGG (Kyoto Encyclopedia of Genes and Genomics) pathways. This analysis identified 15 KEGG pathways where a significant number of genes were either up- or down-regulated (Table 2), Significance at the pathway level was defined as a z-score with an absolute value greater than 2. As described by Walewski et al., a positive z-score greater than or equal to 2 indicates that a significant number of genes in the list of differentially expressed genes are significantly up- or down-regulated in the experimental group in that particular pathway [24]. Notably, a number of the enriched KEGG pathways identified by this study have a direct or indirect relationship with the extracellular matrix (ECM), which is a target of deregulation in HSCs that are in the early stages of activation [13]. These pathways are classified as cellular adhesion molecules (CAMs), ECM-receptor interaction, focal adhesions, and gap junctions (Table 2).

GFP-HSCs are phenotypically similar to HSCs undergoing early activation

As a result of activation, HSCs undergo increased collagen I production and a simultaneous loss of their retinyl ester-containing lipid droplets [18,19]. Because we have selected for a population of HSCs that express Col1a1, we were interested in whether GFP-HSCs would display characteristics of activated HSCs. The array suggests that a number of markers of HSC activation are elevated in the GFP-HSCs, including alpha smooth muscle actin (Acta2), platelet-derived growth factor C (Pdgfc), transforming growth factor beta 2 (Tgfb2), and endothelin (Edn1) (Table 3). Elevated expression of all markers was confirmed by qRT-PCR; however, only Acta2 up-regulation was found to be significant (Figure 2A). Elevated protein levels of ACTA2 are also shown by western blot analysis (Figure 2B).

The elevated levels of markers of HSC activation prompted us to then examine whether the GFP-HSCs have less retinyl ester and triglyceride, which are the two predominant lipid species present in quiescent HSC lipid droplets [8]. We found that, while the livers of the WT and collagen-GFP mice have comparable levels of both lipid species (Figures 3A and 3G), the GFP-HSCs have
significantly less retinyl ester (Figure 3B) and triglyceride (Figure 3D). To further assess lipid droplet content in these cells, AF- and GFP-HSCs were isolated by FACS and cultured overnight on glass plates. Lipid droplet quantity and size were assessed by phase contrast and fluorescent microscopy (Figure 4A and 4B). We found that GFP-HSCs have on average more lipid droplets per cell than AF-HSCs (Figure 4C); however, these droplets are smaller in size, determined by the average diameter of lipid droplets per cell measured in microns (Figure 4D). We also calculated the theoretical lipid droplet volume per cell, assuming a lipid droplet is a perfect sphere. Total lipid droplet volume per cell is the same in both populations (Figure 4E), and furthermore, the
lipid droplet-associated protein adipose differentiation related protein (ADRP) is also expressed at equivalent levels in both AF- and GFP-HSCs (Figure 4F).

Changes in retinoid- and lipid-related gene expression

To better understand the lower levels of retinyl ester and triglyceride in GFP-HSCs, we focused our array analysis on retinoid- and lipid-related gene expression. The retinoid and lipid nuclear receptors were of high interest, as this group of genes represents a major route of gene regulation with many downstream effectors. The array data indicated that levels of the retinoid X receptor alpha (Rxra), retinoic acid receptor alpha and gamma (Rara and Rarg) and the peroxisome proliferator activated receptor alpha (Ppara) are lower in GFP-HSCs (Table 4). However, qRT-PCR analysis did not confirm significant differences in the expression of these genes in this population of cells (Figure 5). Similarly, the array suggested differential expression in the retinol- and fatty acid binding proteins, specifically retinol binding protein (Rbp4), cellular retinol binding

Figure 4. Assessment of lipid droplet content in AF-HSCs and GFP-HSCs after overnight culture. AF-HSCs and GFP-HSCs were isolated by FACS and incubated at 37°C overnight on glass bottom dishes. Phase contrast and fluorescent images were captured at 60× magnification for both AF- and GFP-HSCs (Figure 4F).
protein 1 (Rbp1) and fatty acid binding protein 4 (Fabp4) (Table 5). qRT-PCR analysis of mRNA levels confirmed that Fabp4 has significantly higher expression in the GFP-HSCs (Figure 6). The array additionally suggested that there may be significant gene transcriptional changes relating to lipid metabolism underlying the altered lipid content in the GFP-HSCs. There is elevated expression of two triglyceride lipases, diacylglycerol lipase, beta (Daglb) (Table 6) and arylacetamide deacetylase (Aadac) (Table 7). A number of phospholipases exhibited higher expression in the GFP-HSCs, including various members of the A and C subfamilies (Table 7). Notably, the array showed that carboxylesterase 3 (Ces3) expression is 82-fold higher in the GFP-HSCs (Table 7), and this elevation was confirmed by qRT-PCR of Ces3 mRNA levels (Figure 7). This is interesting in light of the fact that a number of carboxylesterases have been proposed to be hepatic retinyl ester hydrolases [25,26]. Also, we found via qRT-PCR analysis that lipoprotein lipase (LpL) mRNA expression is elevated in the GFP-HSCs (Figure 7).

Cytochrome-mediated retinoid catabolism

Since it is well established that HSC retinoids are lost upon cell activation, we hypothesized that the lower levels of retinyl ester in GFP-HSCs is a consequence of either increased mobilization of retinol from the HSCs or increased retinoid catabolism within the HSCs. Our data suggest that the first hypothesis is unlikely since we observe no differential expression of the retinoid-binding proteins in the GFP-HSCs (Figure 6), which are responsible for inter- and intracellular transport of retinoid in the body. To investigate the second hypothesis, we considered changes in gene expression in cytochrome P450 enzymes. The cytochromes (CYPs) are known to metabolize both exogenous compounds, including chemicals in the environment, carcinogens and drugs, and also endogenous compounds, including retinoids [27]. The array suggests that a number of CYPs are differentially expressed in GFP-HSCs (Table 8). However, we are specifically interested in CYPs that are known to metabolize retinoids and/or are proposed to play a role in HSC activation. Of the twenty five CYPs shown to be differentially expressed on the array, two meet these criteria: Cyp2s1, which has 32-fold higher expression in GFP-HSCs, and Cyp2e1, which has approximately 3-fold higher expression in GFP-HSCs (Table 8). We confirmed higher levels of Cyp2s1 and Cyp2e1 mRNA (Figure 8A) and in CYP2S1 protein (Figure 8B) in GFP-HSCs compared to AF-HSCs. CYP2E1 protein was unchanged (Figure 8B).

### Table 4. Retinoid- and Lipid-related Nuclear Receptors.

| AFFY ID   | Gene Name                  | Gene ID | FC  | p-value     |
|-----------|----------------------------|---------|-----|-------------|
| 1454773_at| Retinoid X receptor alpha   | Rxra    | 0.5 | 0.005977    |
| 1450180_a_at| Retinoic acid receptor, alpha | Rara  | 0.4 | 0.002350    |
| 1419416_a_at| Retinoic acid receptor, gamma | Rarg  | 0.4 | 0.003971    |
| 1425762_a_at| Retinoid X receptor alpha   | Rxra    | 0.4 | 0.000323    |
| 1439675_at| Peroxisome proliferator activated receptor alpha | Ppara | 0.3 | 0.004452    |

Differences in gene expression are reported as fold-change (FC) relative to AF-HSCs.
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CYP2S1 is a recently-identified cytochrome enzyme that has been shown to be expressed highly in the lung, small intestine and spleen and to be inducible by dioxin, which acts via the aryl hydrocarbon receptor [28,29]. It has also been reported to both metabolize and be induced by all-trans-retinoic acid [30]. The literature does not definitively establish the expression of CYP2S1 in the liver; CYP2S1 expression has been detected in the liver, albeit at very low levels [31,32]. More recently, CYP2S1 was shown to be expressed in human HSCs [33]. To further elucidate the expression of CYP2S1 in the mouse liver, we isolated both hepatocytes and HSCs and measured mRNA levels of 6 CYPs, which were chosen based on their known liver expression, roles in retinoid metabolism and/or proposed roles in hepatic disease. We found that while Cyp2e1 is more highly expressed in hepatocytes, Cyp2s1 is highly enriched in HSCs (Figure 9A).

Having shown Cyp2s1 to be highly expressed in HSCs and elevated in collagen-expressing HSCs relative to the non-collagen-expressing population, we became interested in whether this cytochrome might play a role in the early events of hepatic fibrosis, when HSCs activate and lose their lipid droplet content. To address this question, we injected WT C57 mice around 3 months of age with either CCl4 (0.5 μl/g body weight) or corn oil vehicle control once a week for 4 weeks and assessed changes in mRNA expression. A conventional Nycodenz-based method yields a population of cells with equivalent desmin expression to the conventional Nycodenz-based method of HSC isolation yields a population of cells with desmin-negative, lipocyte-like cells in the liver [34] and that these HSCs are vitamin A-deficient [35]. Our study extends these earlier reports and the proposition that not all HSCs in the liver are equivalent by using different methodologies to isolate two distinct populations of HSCs from the liver and to characterize their morphological and biochemical properties. The different methods of cell isolation we used take advantage of distinct properties of these cells: the first method relies on HSC lipid droplet vitamin A content and the second on HSC collagen I gene expression.

The standard method of HSC isolation employs a Nycodenz density gradient to separate non-parenchymal cells from a liver digest where hepatocytes have been destroyed by exposure to pronase [9,21,22]. The low buoyant density of the numerous lipid droplets present in the HSCs (see Figure 4) allows these cells to float to the top of the gradient and thereby be separated from the other less lipid-rich cell types present in the liver. To obtain a vitamin A-enriched population of HSCs, we additionally conduct FACS on the nycodenz-purified cells. Vitamin A autofluoresces upon excitation at 350 nm, so exposure to this wavelength of light allowed us to collect a population of cells that is enriched in vitamin A. We also employed a second FACS-based method to isolate HSCs. This involved the use of collagen-GFP mice in which GFP expression is driven by the Colla1 promoter [16]. Our data confirm that collagen I expression in the liver is confined to the HSCs, with GFP expression co-localizing exclusively with desmin-positive cells. Our data additionally show that the GFP-based method of HSC isolation yields a population of cells with equivalent desmin expression to the conventional Nycodenz-based method. Thus, we demonstrate for the first time a novel method of HSC isolation that yields cells of identical purity to cells isolated by the conventional Nycodenz-based method.

When a liver is confronted with acute injury, a wound-healing response is initiated which ultimately returns the liver to its healthy state; however, when the liver is confronted with chronic injury, the result is often hepatic fibrosis, which is the production of excess fibrous connective tissue as a reparative process to contain the site of injury [13,14]. In the early stages of hepatic fibrosis, the HSCs undergo a process of activation, whereby they transition from a quiescent, lipocyte-like state to a myofibroblastic, contractile phenotype. Activated HSCs express high levels of COL1a1 and ACTA2, which are key components of the extracellular matrix. Other key proliferative, fibrogenic and contractile stimuli in hepatic fibrosis are PDGF-C, TGF-β and EDN1, respectively [36]. Induction of ACTA2 is the single most reliable marker of HSC activation because it is absent from other resident liver cells.

### Table 5. Retinol and Fatty Acid Binding Proteins.

| AFFY ID   | Gene Name       | Gene ID | FC   | p-value |
|-----------|-----------------|---------|------|---------|
| 1451263_a_at | Fatty acid binding protein 4 | Fabp4 | 10.9 | 0.001481 |
| 1417023_a_at | Fatty acid binding protein 4 | Fabp4 | 4.9  | 0.003252 |
| 1416022_at | Fatty acid binding protein 5 | Fabp5 | 3.4  | 0.002208 |
| 1426229_at | Retinol binding protein 4 | Rbp4  | 3.0  | 0.022103 |
| 1416021_a_at | Fatty acid binding protein 5 | Fabp5 | 2.8  | 0.000799 |
| 1450779_at | Retinol binding protein 7 | Fabp7 | 2.1  | 0.014394 |
| 1425105_at | Retinol binding protein 3, interstitial | Rbp3 | 0.5  | 0.024528 |
| 1448754_at | Retinol binding protein 1, cellular | Rbp1 | 0.5  | 0.010727 |

Differences in gene expression are reported as fold-change (FC) relative to AF-HSCs. doi:10.1371/journal.pone.0024993.t005

**Discussion**

The idea that there is heterogeneity and plasticity in the HSC population in the liver is not new, but has not previously been well-studied. Studies in the early 1990s introduced this idea, utilizing the HSC marker desmin to show that there is a population of desmin-negative, lipocyte-like cells in the liver [34] and that these HSCs are vitamin A-deficient [35]. Our study extends these earlier reports and the proposition that not all HSCs in the liver are equivalent by using different methodologies to isolate two distinct populations of HSCs from the liver and to characterize their morphological and biochemical properties. The different methods of cell isolation we used take advantage of distinct properties of these cells: the first method relies on HSC lipid droplet vitamin A content and the second on HSC collagen I gene expression.

The standard method of HSC isolation employs a Nycodenz density gradient to separate non-parenchymal cells from a liver digest where hepatocytes have been destroyed by exposure to pronase [9,21,22]. The low buoyant density of the numerous lipid droplets present in the HSCs (see Figure 4) allows these cells to float to the top of the gradient and thereby be separated from the other less lipid-rich cell types present in the liver. To obtain a vitamin A-enriched population of HSCs, we additionally conduct FACS on the nycodenz-purified cells. Vitamin A autofluoresces upon excitation at 350 nm, so exposure to this wavelength of light allowed us to collect a population of cells that is enriched in vitamin A. We also employed a second FACS-based method to isolate HSCs. This involved the use of collagen-GFP mice in which GFP expression is driven by the Colla1 promoter [16]. Our data confirm that collagen I expression in the liver is confined to the HSCs, with GFP expression co-localizing exclusively with desmin-positive cells. Our data additionally show that the GFP-based method of HSC isolation yields a population of cells with equivalent desmin expression to the conventional Nycodenz-based method. Thus, we demonstrate for the first time a novel method of HSC isolation that yields cells of identical purity to cells isolated by the conventional Nycodenz-based method.

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![Figure 6](https://example.com/fig6.png)  
**Figure 6. mRNA levels of retinol and fatty acid binding proteins.** Relative mRNA expression levels of Fabp4, Rbp4, and Rbp1 in AF-HSCs (solid bars) and GFP-HSCs (striped bars) are shown. Values are normalized to 18S and given as the mean ±1 S.D. for 5 AF and 5 GFP HSC isolations. doi:10.1371/journal.pone.0024993.g006
in either normal or injured liver; furthermore, its expression connotes a contractile phenotype [37]. Our data show that GFP-HSCs have significantly elevated levels of both ACTA2 mRNA and protein, indicating that this is a population of HSCs in early activation. Because the livers from which these cells were isolated were not experimentally injured or chemically induced, these findings suggest that there is a population of pre-activated HSCs in the liver under physiological conditions. This idea supports earlier findings that there is a subset of the population ready to respond to hepatic injury. It is important to emphasize that the GFP-HSCs have not been experimentally injured or chemically induced, so we distinguish these cells as “pre-activated” HSCs: they are not fully activated, but they display characteristics that are consistent with early stages of activation. LRAT expression is low, retinyl ester levels are diminished, these lipid droplets are smaller in size, suggesting that LRAT, which is the only hepatic enzyme capable of retinyl ester synthesis, is not active in these cells. The retinyl ester content is dependent on expression of LRAT, which is the only hepatic enzyme capable of retinyl ester synthesis in vivo [10]. We show that when LRAT expression is diminished, these lipid droplets are smaller in size, suggesting degradation. Importantly, it is also established that as HSCs undergo activation, they lose their retinyl ester-containing lipid droplets; though it is not known if this loss is a cause or consequence of the activation process. Thus, our findings correlate strongly with the events known to occur in the early stages of HSC activation. LRAT expression is low, retinyl ester levels are decreased, and there are marked alterations in the HSC lipid droplets.

Our observations regarding the different lipid droplet content in the GFP-HSCs additionally allows us to hypothesize about the events taking place in the very early stages of HSC activation. It is important to emphasize that the GFP-HSCs have not been experimentally induced, so we distinguish these cells as “pre-activated” HSCs: they are not fully activated, but they display

Table 6. Lipases.

| AFFY ID   | Gene Name                | Gene ID | FC | p-value |
|-----------|--------------------------|---------|----|---------|
| 1430550_at| Lipase, family member M  | Lipm    | 6.1| 0.047718|
| 1452398_at| Phospholipase C, epsilon 1| Plce1   | 3.9| 0.000292|
| 1417785_at| Phospholipase A1 member A| Pla1a   | 3.5| 0.001223|
| 1430700_a_at| Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) | Pla2g7 | 3.5| 0.002280|
| 1457157_at| Phospholipase C-eta1a     | Plch1   | 2.6| 0.007681|
| 1425338_at| Phospholipase C beta 4    | Plcb4   | 2.5| 0.007491|
| 1436821_at| Phosphatidylinositol-specific phospholipase C, X domain containing 3 | Plcd3 | 2.4| 0.031885|
| 1437872_at| NAPE-PLD mRNA for N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D | Napepld | 2.3| 0.017539|
| 1451970_at| Diacylglycerol lipase, beta | Daglb | 2.3| 0.011264|
| 1435771_at| Phospholipase C beta 4    | Plcb4   | 2.1| 0.002450|
| 1448558_a_at| Phospholipase A2, group IVA | Pla2g4a | 2.0| 0.001474|
| 1424259_at| Lipase maturation factor 1| Lmff1   | 0.5| 0.042273|
| 1454619_at| Lipase maturation factor 2| Lmff2   | 0.5| 0.002383|
| 1416013_at| Phospholipase D family, member 3 | Pld3 | 0.4| 0.000290|
| 1421261_at| Lipase, endothelial       | Lipg    | 0.4| 0.008316|
| 1432928_at| Phospholipase C, delta 4  | Plcd4   | 0.4| 0.039780|
| 1450188_s_at| Lipase, endothelial       | Lipg    | 0.4| 0.002089|
| 1455448_at| Diacylglycerol lipase, alpha | Dagla | 0.4| 0.000027|
| 1417433_at| Lysocephospholipase 2     | Lypl2   | 0.2| 0.003292|
| 1433949_x_at| Soluble PLA2-Bb precursor | Pla2g1b | 0.2| 0.018829|
| 1450128_at| Phospholipase A2, group IIA | Pla2g7 | -  | 0.038422|

Differences in gene expression are reported as fold-change (FC) relative to AF-HSCs.
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characteristic features of such cells, including decreased retinyl ester levels and elevated expression of ACTA2 mRNA and protein. A priori, we would expect the early events of HSC activation to follow one of the following three scenarios: (i) lipid droplets progressively decrease in size, eventually leading to the complete loss of droplets; (ii) lipid droplets progressively decrease in number, eventually leading to the complete loss of droplets; or (iii) lipid droplets are initially broken-down into many more, smaller droplets – increase in number, decrease in size - and then they progressively decrease in number until none remain in the cell. Interestingly, our data provide strong evidence for the third, more complex scenario. We show that the GFP-HSCs, a population of uninduced but collagen-expressing cells, have more lipid droplets than the AF-HSCs, and these droplets are smaller in size, as determined by a decrease in average diameter. Our data is in good agreement with other studies investigating lipid droplet dissolution in other cellular systems. It is known that in the first several hours of hormone-stimulated lipolysis in 3T3-L1 adipocytes, the lipid droplets present in these cells are fragmented and dispersed into microlipid droplets, which significantly increases the surface area available for lipase activity [41–43]. A similar pattern of lipid droplet dissolution was reported for rat adipocytes, in which lipolysis was stimulated by electroporation of a constitutively active β-1 adrenergic receptor into the fat pads of the animals [44]. It will be necessary for future studies to confirm this sequence of events in HSCs directly with real-time tracking of lipid droplet remodeling in response to HSC activation. It will also be necessary to define the contribution of the lipid droplet-associated proteins, such as ADRP, to these processes. Our data shows ADRP protein expression is not different in the GFP-HSCs, but its distribution in the cell and on the lipid droplets might be changed as a consequence of the beginning stages of lipolysis.

Our data also establish decreased triglyceride levels, along with the differential expression of many lipid metabolism-related genes, in GFP-HSCs. This is not surprising since triglyceride is the second most predominant lipid species in HSC lipid droplets.

Table 7. Esterases.

| AFFY ID | Gene Name                      | Gene ID | FC    | p-value |
|---------|--------------------------------|---------|-------|---------|
| 1449081_at | Carboxylesterase 3             | Ces3    | 82.2  | 0.000234|
| 1421218_at | Butyrylcholinesterase          | Bche    | 45.9  | 0.001056|
| 1436090_at | Ectonucleotide pyrophosphatase/phosphodiesterase 6 | Enpp6    | 13.7  | 0.014343|
| 1448813_at | Arylacetamide deacetylase (esterase) | Aadac  | 10.9  | 0.003532|
| 1417300_at | Sphingomyelin phosphodiesterase, acid-like 3B | Smrd3b   | 5.3   | 0.000398|
| 1438665_at | Sphingomyelin phosphodiesterase 3, neutral | Smrd3   | 5.1   | 0.002101|
| 1438785_at | Ectonucleotide pyrophosphatase/phosphodiesterase 6 | Enpp6    | 4.8   | 0.004224|
| 1452202_at | Phosphodiesterase 2A, cGMP-stimulated | Pde2a    | 4.5   | 0.006551|
| 1422779_at | Sphingomyelin phosphodiesterase 3, neutral | Smrd3   | 4.3   | 0.041569|
| 1447707_s_at | Phosphodiesterase 2A, cGMP-stimulated | -       | 3.6   | 0.026599|
| 1416913_at | Esterase 1                      | Es1     | 3.4   | 0.044135|
| 1417626_at | Phosphodiesterase 4D interacting protein | -       | 2.8   | 0.001982|
| 1448136_at | Ectonucleotide pyrophosphatase/phosphodiesterase 2 | Enpp2    | 2.8   | 0.004744|
| 1424150_at | Glycerophosphodiester phosphodiesterase domain containing 5 | Gdpd5   | 2.6   | 0.030611|
| 1451857_a_at | Notum pectinacetylerase homolog | Notum   | 2.5   | 0.015403|
| 1417667_a_at | Phosphodiesterase related       | Pter    | 2.4   | 0.000644|
| 1427302_at | Ectonucleotide pyrophosphatase/phosphodiesterase 3 | Enpp3    | 2.3   | 0.000140|
| 1415894_at | Ectonucleotide pyrophosphatase/phosphodiesterase 2 | Enpp2    | 2.2   | 0.001248|
| 1437341_x_at | 2’,3’-cyclic nucleotide 3’ phosphodiesterase | -       | 0.5   | 0.000609|
| 1431913_a_at | Phosphodiesterase 3A, cGMP inhibited | -       | 0.5   | 0.000050|
| 1435096_at | Resistance to inhibitors of cholinesterase 8 homolog B | Ric8b    | 0.5   | 0.001273|
| 1422635_at | Acetylcholinesterase            | Ache    | 0.5   | 0.004425|
| 1437989_at | Phosphodiesterase 8B            | -       | 0.5   | 0.000826|
| 1421353_at | CAMP specific phosphodiesterase 7B | Pde7b    | 0.4   | 0.015231|
| 1423810_at | Protein phosphatase methylesterase 1 | Ppm1    | 0.4   | 0.000273|
| 1418302_at | Palmitoyl-protein thioesterase 2 | Ppt2    | 0.4   | 0.000661|
| 1452897_at | Cell division cycle 2-like 5 (cholinesterase-related cell division controller) | Cdc2l5   | 0.4   | 0.000532|
| 1421535_a_at | Phosphodiesterase 4A, cAMP specific | Pde4a   | 0.4   | 0.002163|
| 1432490_a_at | Phosphodiesterase 10A           | -       | 0.4   | 0.046869|
| 1450453_a_at | CGMP-phosphodiesterase 6 gamma subunit | Pde6g    | 0.4   | 0.035910|
| 1442700_at | Phosphodiesterase 4B, cAMP specific | Pde4b   | 0.4   | 0.000132|
| 1451615_at | Carboxylesterase 8              | Ces8    | 0.3   | 0.012629|

Differences in gene expression are reported as fold-change (FC) relative to AF-HSCs.
doi:10.1371/journal.pone.0024993.t007
**Table 8. Cytochrome P450 enzymes.**

| AFFY ID    | Gene Name                                                                 | Gene ID | FC   | p-value  |
|------------|----------------------------------------------------------------------------|---------|------|----------|
| 1416612_at | Cytochrome P450, family 1, subfamily b, polypeptide 1                      | Cyp1b1  | 100.9| 0.000175 |
| 1428283_at | Cytochrome P450, family 2, subfamily s, polypeptide 1                      | Cyp2s1  | 32.0 | 0.004925 |
| 1460604_at | Cytochrome b reductase 1                                                  | Cybrd1  | 23.6 | 0.000016 |
| 1421075_s_at| Cytochrome P450, family 7, subfamily b, polypeptide 1                      | Cyp7b1  | 17.1 | 0.00044  |
| 1417507_at | Cytochrome b-561                                                          | Cyb561  | 13.2 | 0.000421 |
| 1421074_at | Cytochrome P450, family 7, subfamily b, polypeptide 1                      | Cyp7b1  | 10.2 | 0.000009 |
| 1425040_at | Cytochrome b reductase 1                                                  | Cybrd1  | 9.2  | 0.000095 |
| 1416613_at | Cytochrome P450, family 1, subfamily b, polypeptide 1                      | Cyp1b1  | 8.9  | 0.000029 |
| 1419559_at | Cytochrome P450, family 4, subfamily f, polypeptide 14                     | Cyp4f14 | 8.4  | 0.000002 |
| 1422533_at | Cytochrome P450, family 51                                                | Cyp51   | 4.7  | 0.000374 |
| 1436778_at | Cytochrome b-245, beta polypeptide                                         | Cybb    | 4.4  | 0.000743 |
| 1422978_at | Cytochrome b-245, beta polypeptide                                         | Cybb    | 4.0  | 0.001173 |
| 1444138_at | Cytochrome P450, family 2, subfamily r, polypeptide 1                      | Cyp2r1  | 3.9  | 0.016331 |
| 1436779_at | Cytochrome b-245, beta polypeptide                                         | Cybb    | 3.8  | 0.003131 |
| 1450646_at | Cytochrome P450, family 51                                                | Cyp51   | 3.4  | 0.001651 |
| 1415994_at | Cytochrome P450, family 2, subfamily e, polypeptide 1                      | Cyp2e1  | 2.9  | 0.006021 |
| 1422534_at | Cytochrome P450, family 51                                                | Cyp51   | 2.8  | 0.010709 |
| 1418780_at | Cytochrome P450, family 39, subfamily a, polypeptide 1                     | Cyp39a1 | 2.2  | 0.038740 |
| 1418709_at | Cytochrome c oxidase, subunit VIII 1                                      | Cox7a1  | 2.1  | 0.017825 |
| 1440211_at | Cytochrome P450, family 2, subfamily j, polypeptide 11                     | Cyp2j11 | 0.5  | 0.026833 |
| 1416933_at | P450 (cytochrome) oxidoreductase                                          | Por     | 0.5  | 0.002868 |
| 1430452_at | Cytochrome P450, family 20, subfamily A, polypeptide 1                     | Cyp20a1 | 0.5  | 0.000408 |
| 1416112_at | Cytochrome c oxidase, subunit VIII                                         | Cox8a  | 0.4  | 0.000037 |
| 1450752_at | Cytochrome c, testis                                                      | Cct     | 0.3  | 0.011154 |
| 1425645_s_at| Cyp2b10-like pseudogene                                                   | Cyp2b10 | 0.3  | 0.040338 |

Differences in gene expression are reported as fold-change (FC) relative to AF-HSCs.

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(31.7%), only slightly less than retinyl ester (39.5%) [8,9]. It is likely that when retinyl esters are lost as a result of activation, the HSC lipid droplet is targeted and signaled to breakdown, and thus, synthesis of the non-retinoid lipids is down-regulated. This is analogous to the idea discussed above that when the predominant lipid found in a particular droplet is not synthesized, the droplet will not form. In the case of the GFP-HSCs we are studying, some retinyl ester is still being made since LRAT is still expressed; thus, the droplets are present, but they are smaller in size. The decrease in HSC triglyceride levels could be a consequence of either decreased triglyceride synthesis or increased triglyceride catabolism. The array data suggest that triglyceride synthesis is not markedly different in the GFP-HSCs. There are no genes involved in triglyceride biosynthesis with significant differential expression in the GFP-HSCs. DGAT1 and DGAT2 are known triglyceride synthesizing enzymes in the liver [45,46], but expression levels of both are the same in the two HSC populations (data not shown). There are, however, many differences in expression in genes involved with triglyceride breakdown, classified as either lipases or esterases. We observe increased expression of two triglyceride lipases and a number of phospholipases. Phospholipids comprise approximately 6% of lipids in HSC lipid droplets [8,9]; thus, the increase in phospholipid catabolism supports the idea that when retinyl ester is low, the non-retinoid lipids are degraded, as well. We also observe a large increase in expression of Ces3. This is interesting in light of the fact that other carboxylesterases have recently been proposed as potential retinyl ester hydrolases in HSCs, including ES-4 and -10 [47,48]. Our findings suggest that Ces3 might be a retinyl ester hydrolase in activated HSCs, but more work will need to be done to confirm its HSC expression and role in retinyl ester hydrolysis. Similarly, we find elevated levels of LpL in the GFP-HSC population. Mello et al. have shown that LpL is expressed at very low levels in hepatocytes and HSCs, but its expression is induced 32-fold in activated HSCs [47]. Thus, the elevated levels of LpL provide further evidence that the GFP population are pre-activated HSCs. Overall, the highly altered lipid metabolism in the GFP-HSCs is very interesting since we still do not know much about the specific lipid species that populate HSC lipid droplets, particularly with regard to the exact fatty acyl compositions of the lipids in these cells. All we currently have is a fairly crude understanding of the breakdown of retinyl ester, triglyceride, cholesterol, phospholipids, and free fatty acids [8,9]. Thus, future work in this area will prove to be very exciting with the potential of using the newly-emerging field of lipidomics and the use of LC/MS/MS for these characterizations.

The cytochrome P450’s are a superfamily of enzymes known to metabolize both exogenous compounds, including chemicals in the environment, carcinogens and drugs, and also endogenous compounds, including retinoids [27]. We were interested in studying six cytochromes in particular, based on either their expression in the liver and/or proposed roles in different types of liver disease. The CYP26s (including CYP26A1, B1 and C1) are thought to be the major retinoid-catabolizing CYPs in the body [49–52]. They are known to metabolize all-trans-retinoic acid and additionally, to be retinoic acid-inducible. CYP26A1 and B1 are expressed in the mouse liver, but C1 is not. CYP2E1 is notable for its role in alcoholic liver disease and for its release of reactive oxygen species, which are known to promote fibrogenesis in HSCs [53,54]. CYP2S1 is a relatively newly-identified CYP and thus is not well-studied; however, it has been reported to metabolize retinoic acid [28–30]. The last two, CYPs 2C37 and 2C39, have liver-specific expression [55]. Additionally, 2C39 has been reported to metabolize retinoic acid [56]. Of these six CYPs, only CYP2S1 was shown on the array, by qRT-PCR and by western blot to be elevated in the GFP-HSCs. Previous reports in the literature suggest that CYP2S1 has very low expression in the liver [31,32]. Our data show that Cyp2s1 is highly enriched in the HSCs, which comprise only 6–8% of cells in the liver and contain 1% of hepatic protein [6,37]. Thus, expression in whole liver homogenates will be masked by the presence of hepatocytes,
Kupffer cells and endothelial cells. Furthermore, we show that Cyp2s1 expression is elevated in vivo by carbon tetrachloride-mediated induction of hepatic fibrosis. Thus, we propose that CYP2S1-mediated retinoid catabolism may play a role in the loss of lipid droplets and retinyl ester content in HSCs undergoing activation in the early stages of hepatic fibrosis.

In conclusion, using FACS-based methods of HSC isolation, we show that there are distinct populations of HSCs in a normal mouse.
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Author Contributions
Conceived and designed the experiments: DND WSB. Performed the experiments: DND RDC. Analyzed the data: DND JLW RDC PDB RAR. Contributed reagents/materials/analysis tools: JLW RAR WSB. Performed the experiments: DND RDC. Analyzed the data: DND JLW RDC PDB RAR. Contributed reagents/materials/analysis tools: JLW RAR WSB. Contributed reagents/materials/analysis tools: JLW RAR WSB. Contributed reagents/materials/analysis tools: JLW RAR WSB. Contributed reagents/materials/analysis tools: JLW RAR WSB.

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Figure S2 Total retinol levels in the liver of mice treated with CCl4. WT C57 male mice were given weekly injections of either corn oil or 0.5 ul CCl4/g B.W. administered in corn oil for 4 weeks and then sacrificed. Total retinol (retinol+retinyl ester) levels are shown, expressed as nmol total retinol per gram of liver. Significance was determined by a Student’s t-test, p-value<0.05. (TIF)

Table S1 ABI primers used for qRT-PCR analysis. Commercially available primers purchased from Applied Biosystems (ABI) are shown with the ABI accession number for all genes qRT-PCR analysis was conducted on. (DOC)
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