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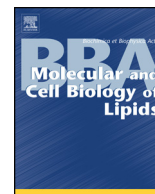
Hormone-sensitive lipase is a retinyl ester hydrolase in human and rat quiescent hepatic stellate cells

Shiva Shajari[a,1], Ali Saeed[a,d,1], Natalia F. Smith-Cortinez[a], Janette Heegsma[a,b], Svenja Sydor[c], Klaas Nico Faber[a,b,*]

[a] Department of Gastroenterology and Hepatology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands
[b] Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands
[c] Department of Gastroenterology, Hepatology and Infectious Diseases, Otto von Guericke University Magdeburg, Magdeburg, Germany
[d] Institute of Molecular Biology & Biotechnology, Bahauddin Zakariya University, Multan, Pakistan

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ABSTRACT

Hepatic stellate cells (HSC) store vitamin A as retinyl esters and control circulating retinol levels. Upon liver injury, quiescent (q)HSC lose their vitamin A and transdifferentiate to myofibroblasts, *e.g.* activated (a)HSC, which promote fibrosis by producing excessive extracellular matrix. Adipose triglyceride lipase/patatin-like phospholipase domain-containing protein 2 (ATGL/*PNPLA2*) and adiponutrin (ADPN/*PNPLA3*) have so far been shown to mobilize retinol from retinyl esters in HSC. Here, we studied the putative role of hormone-sensitive lipase (HSL/*LIPE*) in HSC, as it is the major retinyl ester hydrolase (REH) in adipose tissue.

Lipe/HSL expression was analyzed in rat liver and primary human and rat qHSC and culture-activated aHSC. Retinyl hydrolysis was analyzed after Isoproterenol-mediated phosphorylation/activation of HSL.

Primary human HSC contain 2.5-fold higher *LIPE* mRNA levels compared to hepatocytes. Healthy rat liver contains significant mRNA and protein levels of HSL/*Lipe*, which predominates in qHSC and cells of the portal tree. Q-PCR comparison indicates that *Lipe* mRNA levels in qHSC are dominant over *Pnpla2* and *Pnpla3*. HSL is mostly phosphorylated/activated in qHSC and partly colocalizes with vitamin A-containing lipid droplets. *Lipe*/HSL and *Pnpla3* expression is rapidly lost during HSC culture-activation, while *Pnpla2* expression is maintained. HSL super-activation by isoproterenol accelerates loss of lipid droplets and retinyl palmitate from HSC, which coincided with a small, but significant reduction in HSC proliferation and suppression of *Collagen1A1* mRNA and protein levels.

In conclusion, HSL participates in vitamin A metabolism in qHSC. Equivalent activities of ATGL and ADPN provide the healthy liver with multiple routes to control circulating retinol levels.

1. Introduction

Vitamin A is an essential nutrient important for many physiological functions, including vision, embryogenesis, reproduction, cell differentiation and immune regulation [1]. Animals and humans need to acquire vitamin A from the diet. Carotenes in fruits and vegetables and retinyl esters in meat, fish, eggs and milk are important sources of vitamin A. The liver is the main storage site for vitamin A and controls stable circulating levels of retinol to supply peripheral tissues with vitamin A [1,2].

Dietary carotenes are converted in the intestinal epithelium to retinyl esters and together with animal-originated retinyl esters packed in chylomicrons and transported to the circulation [3]. Chylomicron remnants still contain most of the vitamin A content and are taken up by hepatocytes in the liver. Retinyl esters are hydrolyzed to retinol, which subsequently is exported from the hepatocyte to the circulation bound to retinol binding protein 4 (RBP4). For storage in the liver, retinol is taken up by hepatic stellate cells (HSC) that convert it back to retinyl esters and store it in large cytoplasmic lipid droplets that also contain cholesterol, cholesterol esters, and phospholipids [4,5]. Controlled hydrolysis of retinyl esters to retinol in HSC is believed to maintain stable circulating retinol levels in blood around 2 μmol/L in humans (1–1.5 μmol/L in rodents) [5,6]. Liver injury leads to activation of HSC, which transdifferentiate to migratory and proliferative

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myofibroblasts that produce matrix proteins to promote wound healing. In chronic liver disease, this often leads to scar tissue formation and fibrosis. Importantly, HSC rapidly lose their vitamin A stores in the differentiation process and many chronic liver diseases are associated with vitamin A deficiency as a consequence.

The vitamin A pool in HSC is a resultant of enzymes that esterify retinol and enzymes that hydrolyze retinyl esters. Lecithin retinol acyltransferase (LRAT) and acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) are the main enzymes involved in retinol esterification [7,8]. LRAT appears to be the most relevant retinol esterase in the liver as its absence leads to severe depletion of hepatic vitamin A pools [7]. In line, LRAT expression is rapidly lost in transdifferentiating HSC. Retinyl ester hydrolases (REH) in HSC include adipose triglyceride lipase/patatin-like phospholipase domain containing 2 (ATGL/PNPLA2) and adiponutrin (ADPN/PNPLA3) [9–12]. A single nucleotide polymorphism (SNP) in the *PNPLA3* gene (*PNPLA3-I148M*), the most prominent genetic risk factor associated with non-alcoholic liver disease (NAFLD), leads to accumulation of retinyl esters in the liver in conjunction with reduced serum retinol levels. *Atgl−/−* mice, on the other hand, contain normal hepatic vitamin A levels suggesting that redundancy in HSC-associated REH activity likely exists. Besides the earlier-mentioned retinyl esterases, hormone-sensitive lipase (HSL/*LIPE*) is also expressed in the liver and harbors retinyl ester hydrolase activity [13,14]. A role of HSL in vitamin A metabolism is so far, however, only established in adipose tissue [14,15]. Adipose tissue contains the largest extrahepatic store of vitamin A accounting for up to 15–20% of the total body pool [16]. REH activity is absent in adipose tissue of *Hsl/Lipe−/−* mice and accompanied by a strong increase in retinyl esters and reduction in retinol in this tissue [14]. Hepatic HSL activity has been assigned to hepatocytes where its major substrates are cholesteryl esters [13], while hepatic retinyl ester levels are normal in *Hsl/Lipe−/−* mice, like in the *Atgl−/−* mice [15]. Moreover, *Lipe* mRNA was reported to be absent in freshly-isolated, as well as culture-activated rat HSC [17]. These findings lead to the general assumption that HSL is not involved in hepatic vitamin A metabolism.

Here, we re-evaluated the cell-type specific expression of hepatic HSL in rat and human. We found that HSL is expressed in quiescent rat and human HSC and is rapidly lost when HSC transdifferentiate to myofibroblasts. HSL partly colocalizes with HSC lipid droplets and pharmacological activation of HSL promotes retinyl ester loss from HSC, concomitant with lipid droplet loss. These data support a role for HSL in hepatic vitamin A metabolism, particularly in the healthy liver.

2. Materials and methods

2.1. Animals

Specified pathogen-free male Wistar rats (350–400 g; Charles River Laboratories Inc., Wilmington, MA, USA) were housed under standard laboratory conditions with free access to standard laboratory chow and water. All experiments were carried out according to the Dutch law on the welfare of laboratory animals and guidelines of the ethics committee of University of Groningen for care and use of laboratory animals.

2.2. Hepatic stellate cell isolation and culture

Primary rat hepatic stellate cells were isolated after pronase (Merck; Amsterdam, The Netherlands) and collagenase-P (Roche; Almere, The Netherlands) perfusion of the liver, followed by Nycodenz (Axis-Shield POC; Oslo, Norway) density gradient centrifugation, as described previously [18]. HSC were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with Glutamax (Gibco™ by Life technologies, Thermofisher Scientific, Bleiswijk, The Netherlands) supplemented with 20% heat-inactivated fetal calf serum (FCS), 1 mmol/L sodium-pyruvate, 1× MEM non-essential amino acids, 50 μg/mL gentamycin,

100 U/mL Antibiotic-Antimycotic (all Gibco™) in a humidified incubator at 37 °C with 5% CO_2. After 7 to 10 days of culture-activation cells were harvested for the experiments.

2.3. Fluorescence-activated cell sorting

Following Nycodenz gradient purification, freshly-isolated qHSC were further purified by Side Scatter-Activated Cell Sorting as described before [19] using MoFlo™ XDP (Beckman Coulter, Woerden, The Netherlands). Briefly, two cell populations were sorted with high side scatter (SSC), one with low forward scatter (FSC; R1) and one with high FSC (R2) (Supplementary Fig. S1A). R1 cells were highly pure (> 98% based on vitamin A autofluorescence) single cell HSC. R2 represents clusters of cells containing HSC together with Kupffer cells and/or endothelial cells (Supplementary Fig. S1B). The R1 population was used as highly purified fractions of primary quiescent HSC.

2.4. Hepatocyte isolation and culture

Primary rat hepatocytes were isolated by collagenase (Sigma Aldrich, The Netherlands) perfusion as described before [20]. Cell viability was > 85% as determined by Trypan Blue (Sigma Aldrich). Hepatocytes were cultured on collagen-coated plates in William's E medium (Gibco™) supplemented with 10% FCS, 50 μg/mL gentamycin and 100 U/mL penicillin/strep/amphotericin B. During the 4 h of attachment, 50 nmol/L dexamethasone (Department of Pharmacy UMCG, Groningen, The Netherlands) was supplemented to the medium. Cells were harvested for analysis 4 h or 24 h after isolation. Cells were cultured in a humidified incubator at 37 °C and 5% CO_2.

2.5. Portal myofibroblasts (PMF) isolation and culture

Portal myofibroblasts (PMF) were isolated from the portal tree fraction obtained during the hepatocyte isolation as previously described [21]. Briefly, the portal tree was minced and incubated in a digestion solution containing collagenase, pronase, DNase (Roche, The Netherlands) and hyaluronidase (Sigma Aldrich, The Netherlands) for 1 h at 37 °C. PMF were cultured in IMDM medium supplemented with 20% heat-inactivated fetal calf serum (FCS), 1 mmol/L sodium-pyruvate, 1× MEM non-essential amino acids, 50 μg/mL gentamycin, 100 U/mL Antibiotic-Antimycotic (all GibcoTM) in a humidified incubator at 37 °C and 5% CO_2 for 7 days and harvested for analysis.

2.6. Kupffer cell isolation

Kupffer cells were isolated from the non-parenchymal cell fraction obtained during the hepatocyte isolation. Kupffer cells were purified using a Percoll density cushion at 1800 g for 15 min at 4 °C, as previously described [22]. Kupffer cells were allowed to adhere in culture plates at 37 °C for 30 min in Mg^{2+}- and Ca^{2+}-containing HBSS (Gibco™) supplemented with 10% FCS. Next, remaining hepatocytes were washed away and attached Kupffer cells were further cultured for 24 h in RPMI (Gibco™) supplemented with 10% FCS and Antibiotic-Antimycotic (Gibco™) (100 U/mL) and harvested after 24 h for analysis.

2.7. Tissue specimens

Rat adipose tissue, portal tree and liver specimens were harvested and snap-frozen in liquid nitrogen and stored at −80 °C for further use. The frozen tissue was crushed and dissolved in Tri-reagent (Sigma-Aldrich) for RNA isolation and qPCR analyses.

2.8. The human hepatic stellate cell line (LX-2)

LX-2 cells were kindly provided by Scott Friedman [23]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™)
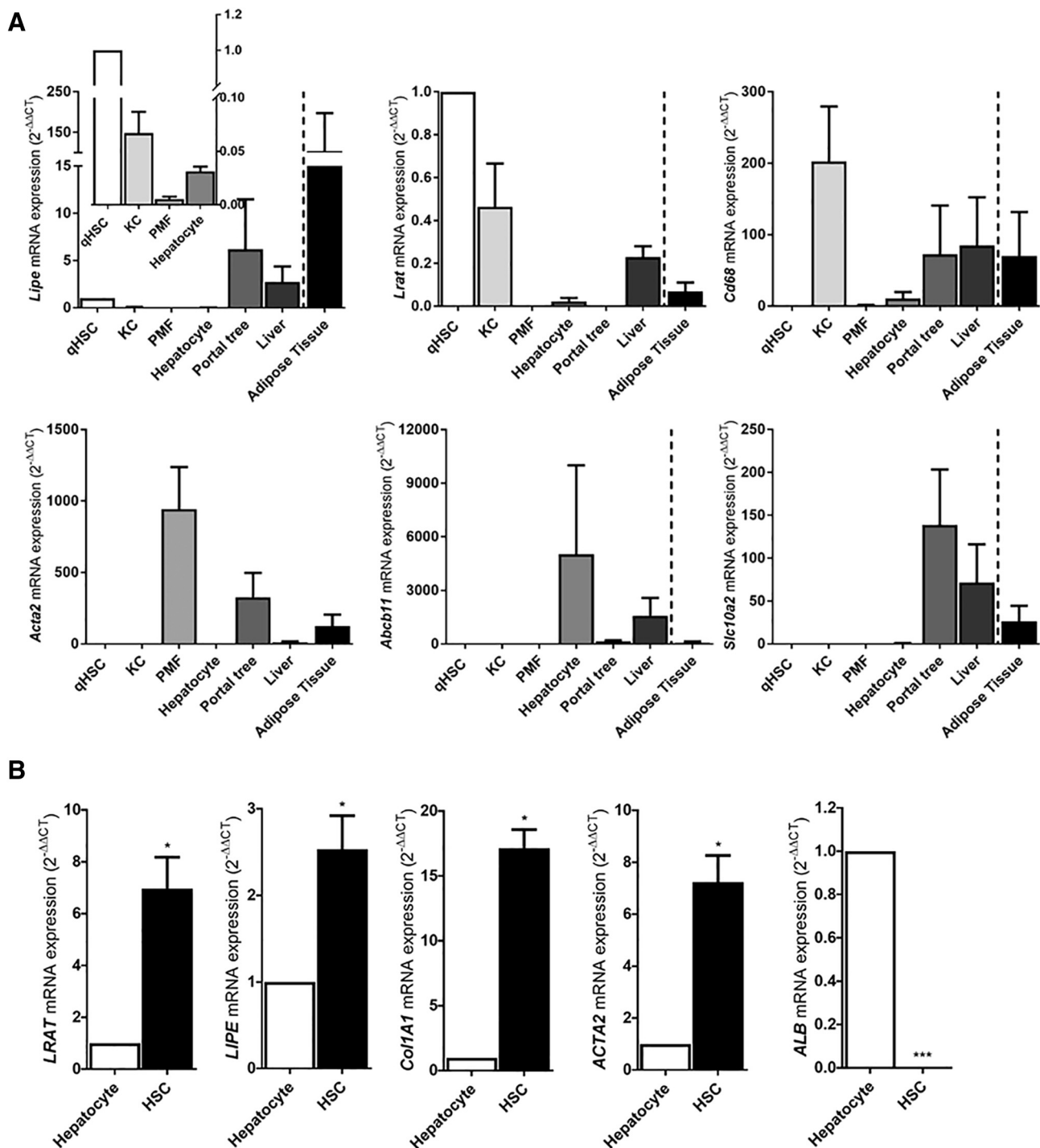
Fig. 1. Rat and human quiescent hepatic stellate cells express hormone sensitive lipase. (A) Quiescent hepatic stellate cells (qHSC), Kupffer cells (KC), Portal myofibroblasts (PMF), hepatocytes and the portal tree fraction were purified form healthy rat liver and mRNA levels of *Lipe* and cell type-specific marker genes (*Lrat*, *Cd68*, *Acta2*, *Abcb11*, *Slc10a2*, respectively) were quantified by Q-PCR and compared to whole liver and adipose tissue. mRNA levels in qHSC (A) and hepatocytes (B) were set to 1. Hepatic *Lipe* mRNA was most abundant in qHSC and in the portal tree that mostly consists of cholangiocytes. (The inset in the left panel shows a zoom of *Lipe* mRNA levels in rat qHSC, KC, PMF and hepatocytes.) (B) Primary human HSC and hepatocytes were purified from healthy tissue in surgical resections. hHSC purification requires short *in vitro* culture to remove contaminating cells, resulting in co-expression of *LRAT*, *COL1A1* and *ACTA2*. *LIPE* mRNA levels were 2.5-fold higher in the hHSC compared to purified human hepatocytes (expressing *ALB*).

supplemented with 10% FCS and Antibiotic-Antimycotic (Gibco™) (100 U/mL).

2.9. Isolation and culture of primary human hepatocytes and HSC

Freshly-isolated primary human hepatocytes and HSC were isolated from macroscopically normal liver specimens obtained from fresh tumor resections by an "all-in-one" liver cell purification procedure as described previously [24]. Primary human hepatocytes were harvested after 3 day *in vitro* culturing. Cultured primary human HSC were harvested approximately 5 days after isolation when they show an intermediate phenotype between quiescent (vitamin A storage) and

A

Fig. 2. HSL expression is lost during culture-activation of hepatic stellate cells. Freshly-isolated and FACS-purified rat HSC were either processed directly (qHSC) or culture-activated for 7–10 days (aHSC) and *Lipe* mRNA and HSL protein levels were analyzed by Q-PCR (A) and Western blotting (B), respectively. qHSC show high expression of *Lipe/*HSL and *Lrat/*LRAT, which are lost in aHSC. Instead, aHSC show high expression of *Acta2/*αSMA. mRNA levels (A) in qHSC were set to 1.

B

Fig. 3. Quiescent hepatic stellate cells contain high *Lipe* mRNA levels in comparison to other retinyl ester hydrolase-encoding genes. Freshly-isolated and FACS-purified rat HSC were either processed directly (qHSC) or culture-activated for 7–10 days (aHSC) and the mRNA levels of three retinyl ester hydrolases, *Lipe*, *Pnpla2* and *Pnpla3* was quantified by q-PCR qHSC contained high mRNA levels of *Lipe*, with lower levels of *Atgl/Pnpla2* and even lower levels of *Adpn/Pnpla3*. Transcript levels of *Lipe* and *Adpn/Pnpla3* sharply decreased with activation of HSC, while *Atgl/Pnpla2* mRNA levels were similar in aHSC and qHSC. mRNA levels of *Lipe* in qHSC were set to 1.

activation (collagen production) [24].

2.10. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed as previously described [25]. Shortly, total RNA was isolated from tissue samples using TRIzol® reagent according to supplier's instruction (ThermoFisher Scientific, The Netherlands). RNA quality and quantity were determined using a Nanodrop 2000c UV–vis spectrophotometer (ThermoFisher Scientific). cDNA was synthesized from 2.5 μg RNA using random nonamers and M-MLV reverse transcriptase (Invitrogen, USA). Taqman primers and probes were designed using Primer Express 3.0.1 and are shown in Supplementary Table S1. All target genes were amplified using the Q-PCR core kit

master mix (Eurogentec, The Netherlands) on a 7900HT Fast Real-Time PCR system (Applied Biosystems Europe, The Netherlands). SDSV2.4.1 (Applied Biosystems Europe, The Netherlands) software was used to analyze the data. Expression of genes was normalized to *18S* and presented as relative levels compared to a control condition set to 1.

2.11. Western blot analysis

Protein samples were prepared for Western blot analysis as described previously [26]. Protein concentrations were quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard. Equal amounts of protein (5–20 μg) were separated on Mini-PROTEAN® TGX™ precast 4–15% gradient gels (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membranes using the Trans-Blot Turbo transfer system, (Bio-Rad). Primary antibodies (against HSL, pHSL, αSMA, Collagen1A1, LRAT and GAPDH; details and dilutions are listed in Supplementary Table S2) and appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000; P0448, DAKO) were used for detection. Proteins were detected using the Pierce ECL Western blotting kit (ThermoFisher Scientific). Images were captured using the chemidoc XRS system and Image Lab version 3.0 (Bio-Rad). The intensity of bands was quantified using ImageJ version 1.51 (NIH, USA). The Ponceau-S staining of the membrane is included as loading control.

2.12. Real-time monitoring of cell proliferation

The proliferation of freshly-isolated rat hepatic stellate cells was monitored for 5 days in presence or absence of Isoproterenol using Real Time Cell Analyzer (RTCA, xCELLigence RTCA DP, ACEA Biosciences, Inc.). Freshly-isolated rat qHSC were plated in E plates having inter-digitated gold microelectrodes to constantly record cell proliferation, according to manufacturer's instructions. Isoproterenol treatment was started at 4 h after the attachment of cells. Results were recorded and analyzed by RTCA Software.
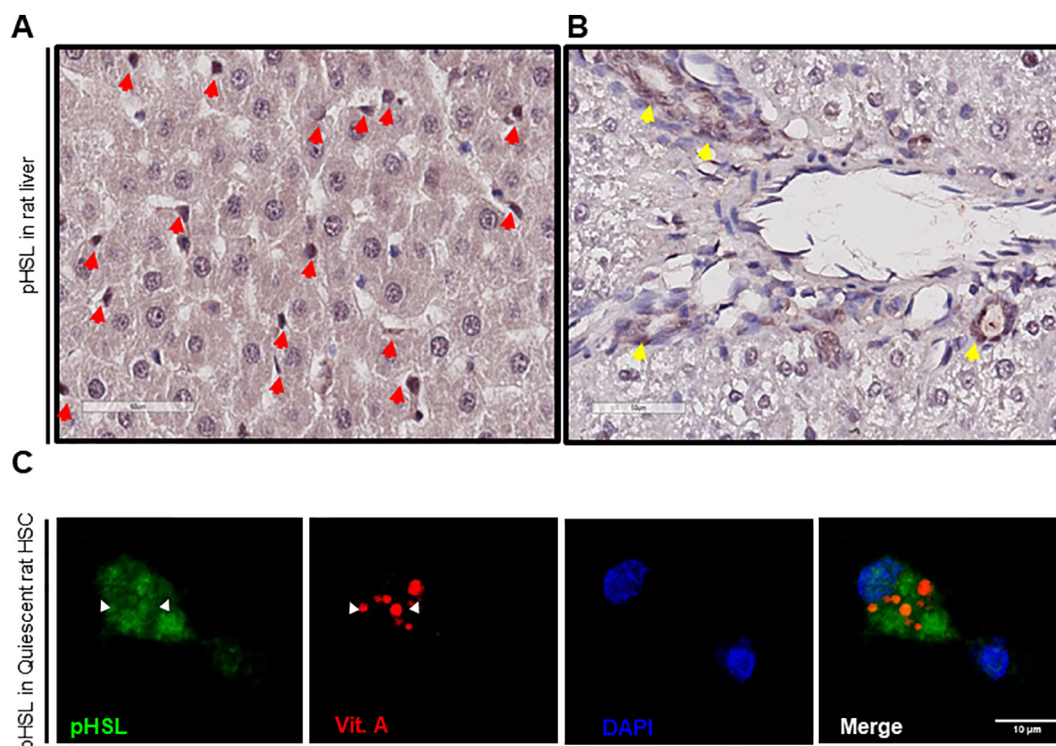
Fig. 4. Quiescent hepatic stellate cells in healthy liver contain phosphorylated, thus active, HSL. Paraffin-sections of healthy rat liver (A, B) and freshly-isolated quiescent hepatic stellate cells (qHSC; C) were stained for phosphorylated-HSL (pHSL) by immunohistochemistry and immunofluorescence microscopy, respectively. pHSL-specific staining was detected in small cells located close to hepatocytes and sinusoids, the typical location for qHSC (indicated by red arrows in A). Additional pHSL-specific staining was detected in the portal areas (yellow arrows in B). Freshly-isolated qHSC showed a pronounced cytoplasmic location of pHSL that partly co-localized with vitamin A-containing lipid droplets, as visualized by the autofluorescence of vitamin A (C).

2.13. Oil red O staining

Rat HSC and LX-2 cells were seeded on coverslips and cultured for the indicated periods, after which they were fixed with 4% paraformaldehyde (Merck Millipore, The Netherlands). LX-2 cells were additionally exposed to BSA-conjugated oleic acid (0.1 mmol/L; Sigma-Aldrich) for 24 h, as previously described [27]. HSC and LX-2 were treated with different concentrations (5, 10, 50 μmol/L) of Iso-proterenol (Iso). Intracellular lipids were stained with Oil Red O solution (Sigma-Aldrich, The Netherlands) and Hematoxylin (Sigma-Aldrich) were stained the nuclei as described before [28]. Slides were scanned using a Nanozoomer 2.0HT (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with Aperio ImageScope (version 11.1, Leica Microsystems, Amsterdam, The Netherlands).

2.14. Immunohistochemistry

Immunohistochemistry for phosphorylated HSL (anti-pHSL; Supplementary Table S2) was performed on paraffin-embedded rat liver sections as previously described [29]. Briefly, after deparaffinization antigen retrieval was performed by microwave irradiation in citrate buffer (10 mmol/L), pH 6.0 and blocking of endogenous peroxidase with 0.3% H_2O_2 for 30 min. After blocking (1% BSA for 30 min), tissue was incubated with polyclonal-rabbit-phospho-HSL (Ser660) primary antibody (1:50 dilution in 1% BSA) overnight at 4 °C in a humidity chamber. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies (1:50; #170-6515, Bio-Rad) was used as secondary antibody. Slides were stained with ImmPact NovaRED Peroxidase (HRP) substrate (cat# SK-4805, Vector Laboratories, The Netherlands) for 11 min, and hematoxylin was used as a counter nuclear stain (2 min at RT). Finally, slides were dehydrated and mounted with Eukitt®, (Sigma-Aldrich). Slides were scanned using a nanozoomer 2.0 HT digital slide scanner (C9600-12, Hamamatsu Photonics, Hamamatsu, Japan) and analyzed

with Aperio ImageScope (version 11.1, Leica Microsystems, Amsterdam, The Netherlands).

2.15. Fluorescence microscopy

Freshly isolated primary rat HSC or LX-2 were seeded in 12-well plates (1 ∗ 10^5 cells/well) containing 18 mm glass coverslips and cultured for the indicated periods. Coverslips were washed with PBS three times, fixed with 4% PFA in PBS at room temperature (RT) for 10 min and permeabilized with 0.1% triton X-100 in PBS for 10 min at RT. Non-specific antibody binding was blocked with 2% BSA in PBS for 30 min. After blocking, coverslips were incubated with anti-pHSL antibody (1:200, see Supplementary Table S2) for 1 h at RT. Coverslips were then washed 3 times with blocking solution and incubated 30 min with secondary antibody (goat-anti-rabbit 488, 1:500 in blocking solution) at RT and covered from light. Next, coverslips were washed 3 times and then mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories). Coverslips were air-dried, sealed using nail polish, stored at 4 °C and covered from light until further use. Images were obtained using a Zeiss LSM 780 NLO two photon Confocal Laser Scan Microscope (Carl Zeiss, Jena, Germany). After double-photon simultaneous excitation, image was obtained for each fluorophore sequentially according to their individual unique emission spectra (emission of autofluorescence at 410 nm, DAPI at 460 nm, pHSL at 530 nm). Images were then processed and analyzed using ImageJ version 1.51 (NIH, Maryland, USA).

2.16. Retinyl palmitate and retinol measurement

Vitamin A content was analyzed by reverse phase HPLC as previously described [30]. Retinol and retinyl esters were extracted and deproteinized twice with n-hexane from tissue and serum with retinol acetate as the internal standard. Samples were diluted in 200 μL ethanol

A

60 min

Isoproterenol (µmol/L)   0   5   50

pHSL   80 kDa

HSL   80 kDa

GAPDH   37 kDa

B

Iso-0µmol/L   Iso-5µmol/L   Iso-10µmol/L   Iso-50µmol/L

ORO

Day1

Day3

C

Iso-0µmol/L   Iso-5µmol/L   Iso-10µmol/L   Iso-50µmol/L

Auto-fluorescence

Day1

Day3

D

QHSC after 24 h

ORO area (µM²) normalized vs control

Auto-fluorescence area (µM²) normalized vs control

Retiny palmitate (µg/mg of protein) normalized vs control

Retinol (µg/mg of protein) normalized vs control

Isoproterenol (µmol/L)   0   50

(caption on next page)

Fig. 5. Hormone-sensitive lipase activation promotes retinyl ester hydrolysis in HSC. Freshly-isolated rat qHSC were allowed to attach for 4 h and subsequently exposed to Isoproterenol (5 and 50 μmol/L) to enhance HSL phosphorylation and thus its enzyme activity. Untreated qHSC contain significant levels of phosphorylated HSL, which could be further increased by Isoproterenol treatment (A, total-HSL and GAPDH are included as controls). Isoproterenol treatment for 1 and 3 days dose-dependently accelerated lipid loss from primary rat qHSC (as shown by oil red O (ORO) staining) (B), which was accompanied by a dose-dependent decrease in vitamin A-autofluorescence (C). ORO staining, vitamin A-autofluorescence and cellular retinyl palmitate and retinol levels were quantified after 24 h exposure with and without 24 h Isoproterenol (D).

and 50 μL was injected for phase separation (150 × 3.0 mm of 5 μM column) and/or measurements (Photo Diode Array Detector at 325 nm) by HPLC.

2.17. Statistical analysis

Data is presented as Mean ± SEM by using the GraphPad Prism 7 software package (GraphPad Software, San Diego, CA, USA). Statistical significance was determined by the unpaired *t*-test. P-values ≤ 0.05*, ≤ 0.01**, ≤ 0.001*** were considered significant.

3. Results

First, we compared mRNA levels of *Lipe* (encoding HSL) in purified cell types from healthy rat livers and related it to its expression in adipose tissue (Fig. 1A). *Lipe* mRNA levels were well-detectable in total rat liver, though were 38-fold lower compared to adipose tissue. Comparing purified hepatic cell fractions, the highest *Lipe* levels were found in freshly-isolated FACS-sorted quiescent hepatic stellate cells (qHSC; marker *Lrat*), with drastically lower levels in purified hepatocytes (marker: *Abcb11*), Kupffer cells (marker: *CD68*) and cultured portal myofibroblasts (marker: *Acta2*) (Fig. 1A inset). In addition, high *Lipe* mRNA levels were detected in the portal tree fractions, that mainly contain cholangiocytes (marker: *Slc10a2*) and portal myofibroblasts. Primary human HSC also contained readily detectable mRNA levels of *LIPE*, which were significantly higher compared to primary human hepatocytes (Fig. 1B). In contrast to quiescent HSC from rat liver, the purification protocol for human HSC included a short *in vitro* cultivation period, in which these cells show early signs of activation (*COL1A1* and *ACTA2*), while a typical marker for quiescent HSC (*LRAT*) is still significantly detected. An earlier study reported the absence of HSL/*Lipe* in (partially activated) rat HSC [17]. Therefore, we next studied whether culture-activation of rat HSC affects *Lipe* expression. Highly-pure FACS-sorted rat HSC were either processed directly (qHSC) or culture-activated for 7–10 days (aHSC). *Lipe* mRNA (Fig. 2A) and HSL protein (Fig. 2B) were readily detected in purified qHSC from healthy rat livers, but were strongly decreased – to undetectable levels – in culture-activated aHSC. A similar profile was observed for the qHSC marker *Lrat*/LRAT, while *Acta2*/αSMA showed the expected low levels in qHSC and a sharp increase in aHSC. In fact, HSL protein had already largely disappeared in 3-day cultured HSC (Supplementary Fig. S2). Thus, HSL expression is rapidly lost during HSC transdifferentiation.

Two enzymes have been characterized to play a role in hydrolyzing retinyl esters in mouse and/or human HSC, *e.g.* ATGL/*PNPLA2* and ADPN/*PNPLA3* [9,10]. HSL could be a third enzyme involved in controlling hepatic vitamin A metabolism, as such role for HSL is already well-established in adipose tissue [14,31,32]. We next analyzed the relative abundance of mRNA levels of these 3 hydrolases in freshly-isolated and purified qHSC from healthy rat liver and after culture-activation (Fig. 3). Quiescent HSC contained high mRNA levels of *Lipe*, with clearly lower levels of *Atgl*/*Pnpla2* and even lower levels of *Adpn*/*Pnpla3*. Transcript levels of *Lipe* and *Adpn*/*Pnpla3* were sharply decreased in aHSC (> 100-fold and > 10-fold, respectively), while *Atgl*/*Pnpla2* mRNA levels were comparable between aHSC and qHSC. HSL enzyme activity is activated by phosphorylation (at serine 563, 565 and 660) and we next analyzed the presence of pHSL in rat liver tissue and purified qHSC by immunohistochemical staining (Fig. 4). pHSL-specific staining was sparsely distributed throughout the liver parenchyma and

detected in small cells located close to hepatocytes and sinusoids (Fig. 4A), the typical location for qHSC. In addition, strong pHSL staining was detected in portal areas (Fig. 4B), in line with the high *Lipe* mRNA levels detected in these cellular fractions (Fig. 1). Immunofluorescence microscopy analysis of pHSL in purified rat qHSC revealed pronounced cytoplasmic staining that partly co-localized with lipid droplets that showed strong vitamin A autofluorescence (Fig. 4C). Together, these data indicate that HSL/*Lipe* is a prominent lipase/hydrolase in rat qHSC.

HSL is best known for its hydrolase activity towards triglycerides and cholesteryl esters. This activity can be enhanced by isoproterenol (Iso) that stimulates HSL phosphorylation at serine 563, 565 and 660 [33–35]. Basel levels of pHSL^{S660} in LX-2 cells (model for human stellate cells) were low, but Iso (5 μmol/L) quickly and strongly induced HSL^{S660} phosphorylation and promoted clearance of lipid droplets from LX-2 cells (Supplementary Fig. S3A–C). LX-2 cells do not store retinyl esters, so a potential role of HSL in hydrolyzing retinyl palmitate was analyzed in primary rat HSC. In contrast to LX-2 cells, primary rat qHSC showed high basal levels of pHSL, which could be further induced by Iso in the early phase of culture-activation of rat HSC (Fig. 5A). Like in LX-2 cells, Iso dose-dependently accelerated lipid loss from primary rat HSC in the first 3 days of culture activation (Fig. 5B, quantification in D: after 24 h with and without 50 μmol/L Iso), which was accompanied by a dose-dependent decrease in vitamin A-autofluorescence in HSC (Fig. 5C, quantification in D: after 24 h with and without 50 μmol/L Iso). In line, Iso treatment (50 μmol/L) lead to a significant reduction in cellular retinyl palmitate levels in 1 day-cultured primary rat HSC, while cellular retinol levels were unchanged (Fig. 5D). These data support a role for HSL in the hydrolysis of retinyl esters, as well as other esterified lipids in HSC.

As "super"-activation of HSL promoted retinoid loss from cultured rat HSC, we analyzed whether this may also affect the activation process of HSC. Freshly-isolated qHSC were seeded in a Real-Time Cell Analyzer (RCTA; xCELLigence) and after the initial 4 h-attachment phase cells were treated with 0, 5, 10 and 50 μmol/L Iso (Fig. 6A). Iso treatment delayed the initial increase in cell index (reflecting cell stretching and/or proliferation) in the first 24 h of culture, after which the cell index increased with similar kinetics as the untreated HSC. A 3-day Iso treatment caused a small, but significant reduction in Collagen 1a1 levels already at 5 μmol/L, both at mRNA and protein level, while only trends in lowering *Acta2*/aSma, *Tgf-β* and *Lrat* levels were detected at that concentration (Fig. 6B and C).

Taken together, our data show that quiescent rat and human HSC contain significant amounts of (phosphorylated, thus active) HSL and promotes retinyl ester hydrolysis in these cells. HSL expression is rapidly lost during transdifferentiation of HSC and thus does not seem to play a role in vitamin A metabolism in aHSC.

4. Discussion

In this study, we show for the first time that hormone sensitive lipase (HSL) is expressed in human and rat quiescent hepatic stellate cells (qHSC) where it plays a role in retinyl ester hydrolysis. HSL is mostly present in it is active phosphorylated state and partly colocalizes with vitamin A-containing lipid droplets in qHSC. HSL expression is rapidly lost when HSC transdifferentiate to myofibroblasts. Pharmacological HSL super-activation accelerated retinyl ester loss from HSC and lead to transient suppression of HSC activation *in vitro*. Thus, HSL is a third
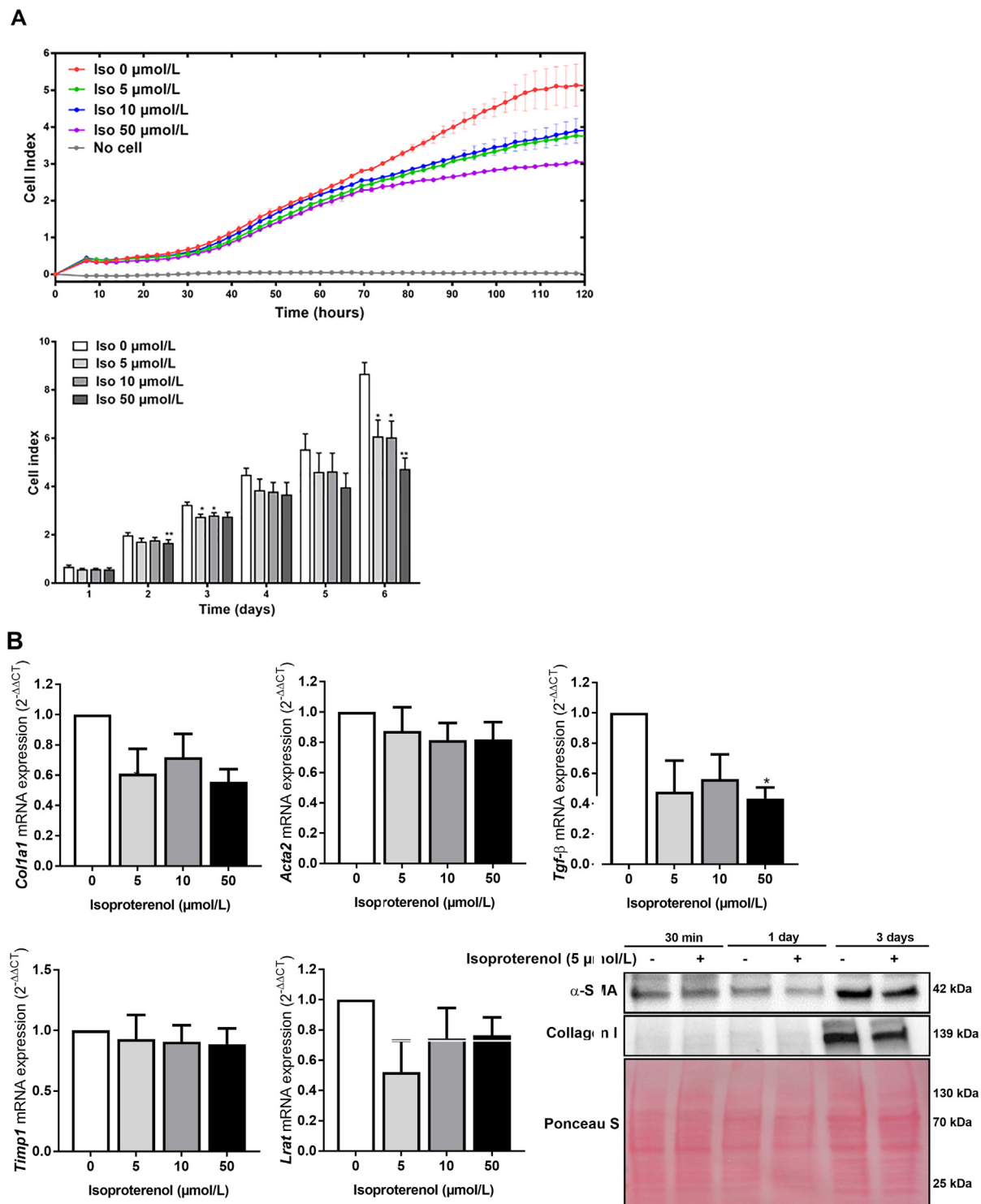
Fig. 6. Super-activation of hormone-sensitive lipase reduces hepatic stellate cell proliferation and activation. Freshly-isolated rat qHSC were allowed to attach for 4 h and subsequently exposed to Isoproterenol (5, 10 and 50 μmol/L). Cell proliferation was monitored for 5 days in a Real-Time Cell Analyzer (RCTA; xCELLigence; A). Markers of HSC activation were quantified after 3 days by Q-PCR (B) and Western blotting after 30 min, 1 day and 3 days (C). Isoproterenol treatment delayed the initial increase in cell index (reflecting cell stretching and/or proliferation) in the first 24 h of culture, after which the cell index increased with similar kinetics as the untreated HSC (A). mRNA levels of *Col1a1* and *Tgf-β* were significantly reduced after 3 days Isoproterenol treatment, while mRNA levels of *Acta2*, *Timp-1* and *Lrat* were not affected. mRNA levels in untreated qHSC were set to 1 (B). Protein levels of COL1A1 were reduced after 3 days of Isoproterenol treatment, while it did not change the aSMA protein level. Ponceau-S staining of total protein was included as loading control.

retinyl ester hydrolase in HSC, where it controls vitamin A metabolism together with ATGL/PNPLA2 and ADPN/PNPLA3.

The fact that HSL contains retinyl ester hydrolase activity is well-known from its role in adipose tissue [14], but it was deemed absent from HSC based on earlier studies [15,17]. The experimental data supporting this is, however, limited. When comparing mRNA levels of various carboxylesterase and lipase genes in different rat liver cell types, no significant mRNA levels of *Lipe* were detected in any of the

cell types analyzed, *e.g.* hepatocytes, endothelial cells, Kupffer cells, qHSC and aHSC [17]. Still, HSL is present in the liver based on other studies [13]. Furthermore, Taschler et al. [15] only tested HSL protein expression in αSMA-positive aHSC of mice and found it to be absent, which is in line with our results with rat aHSC. It remains puzzling to us why no *Lipe* mRNA was detected in any of the rat hepatic cell types analyzed by others [17], in particular in the highly-pure fractions of qHSC, since mRNA levels of *Lipe* in rat qHSC appeared even dominant over *Pnpla2* and *Pnpla3* in our study. Moreover, Western blotting, immunohistochemical staining and immunofluorescence microscopy further confirmed that rat qHSC do express HSL and appears to be mostly in the activate phosphorylated state (pHSL). In addition, and for the first time, we also show that *Lipe*/HSL is expressed in primary human HSC, even more so than in human hepatocytes, as well as in the human HSC cell line LX-2. LX-2 cells have an intermediate transdifferentiation state and most of the HSL appears non-phosphorylated. Upon pharmacological induction of phosphorylation (and activation) by Iso, pHSL immunofluorescent staining becomes apparent and shows a punctate staining pattern, indicative of co-localization with lipid droplets in LX-2 cells, similar as we observed in rat qHSC and as described earlier for pHSL in 3T3L1 adipocytes [36]. Iso treatment accelerated retinyl palmitate loss from rat HSC, implying a role of HSL in retinyl ester hydrolysis in these cells.

So far, 2 enzymes were implicated in retinyl ester hydrolysis in HSC, ATGL/PNPLA2 and ADPN/*PNPLA3*, so HSL/*LIPE* is a third one. Whole body knock-out mice for either *Pnpla2* or *Lipe* do not show altered levels of hepatic retinyl esters [15]. *Pnpla3* knockout mice have not been analyzed for hepatic retinyl ester content yet, but do not show global alterations in lipid homeostasis compared to wild type animals, either on chow or fatty liver-inducing diets [37,38]. This does not necessarily mean that vitamin A metabolism is not disturbed in these mice and thus requires a focused analysis of hepatic vitamin A metabolites in *Pnpla3* −/− mice. Still, it may very well be that like *Pnpla2* −/− and *Lipe* −/− mice also *Pnpla3* −/− mice may not have altered hepatic retinyl ester levels and that these genes can fully compensate each other for retinyl ester hydrolysis in HSC. Both *Lipe* and *Pnpla3* expression are strongly suppressed during HSC transdifferentiation, while *Pnpla2* levels remain stable. This is in line with earlier observations that pharmacological inhibition of ATGL (encoded by *Pnpla2*) leads to accumulation of retinyl esters in activated mouse HSC when exposed to retinol-containing media for 10 days *in vitro*, while this was not observed with HSL inhibitors [15]. Thus, while HSL, ATGL and ADPN have overlapping roles in retinyl ester hydrolysis in qHSC in the healthy liver, ATGL is probably the most important for retinyl ester hydrolysis in aHSC in the chronically injured liver.

Notably, transcriptional regulation of both HSL/*Lipe* and ATGL/*Pnpla2* is under control of PPARγ, which is a well-known marker for qHSC and is lost during transdifferentiation. *Lipe* mRNA levels follow *Pparγ* levels during HSC transdifferentiation [39,40], while *Pnpla2* expression is not affected (our data). This indicates that undefined compensatory mechanisms exist to maintain *Pnpla2* expression in aHSC to secure retinyl ester hydrolase activity in aHSC.

Our work may also have relevance to hepatic HSL functions beyond vitamin A metabolism. HSL is an intracellular neutral lipase that besides retinyl esters hydrolyses cholesterol esters, triglycerides, mono- and di-acylglycerol and other lipids [13]. Hepatic expression was most dominant in the portal tree fractions and purified qHSC, while much lower levels were observed in hepatocytes, both in rat and human liver cells.

The physiological relevance of hepatic HSL is evident from the whole body *Lipe* −/− mouse that is characterized by exaggerated accumulation of cholesteryl esters in *in vivo* models of fatty liver disease [13,41]. Given our data, it will be interesting to analyze which cells in the liver actually accumulate cholesteryl esters in *Lipe* −/− mice. Cells other than hepatocytes may very well be good candidates as hepatocyte-specific deletion of *Lipe* did not replicate the fatty liver phenotype

in mice [42]. While these authors concluded that HSL has little involvement in hepatic lipid metabolism, it may actually be other cell types than hepatocytes that play a crucial role here. It is therefore interesting to determine how much HSC contribute to the accumulation of cholesteryl esters in *Lipe* −/− mice.

Taken together, our data show that HSL is a prominent retinyl ester hydrolase in quiescent rat and human HSC. Retinol release from HSC in the healthy liver is therefore a fine-tuned collaboration between HSL, ATGL/PNAPL2 and ADPN/PNPLA3 to maintain whole body vitamin A homeostasis. HSL expression in qHSC is significantly higher than in hepatocytes, which may point to a broader role of HSC-located HSL in hepatic lipid metabolism. Future work should take the cell type-specific location of hepatic HSL into account when analyzing its role in lipid metabolism.

Author contributions

Study concept and design - S. Shajari, A. Saeed, K.N. Faber; acquisition of data - S. Shajari, A. Saeed, N.F. Smith-Cortinez, K.N. Faber; analysis and interpretation of data – S. Shajari, A. Saeed, N.F. Smith-Cortinez, S. Sydor, K.N. Faber; drafting of the manuscript - A. Saeed, S. Shajari, K.N. Faber; statistical analysis - A. Saeed; obtained funding - S. Shajari, A. Saeed, K.N. Faber; technical assistance - J. Heegsma; study supervision - K.N. Faber.

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Transparency document

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Declaration of Competing Interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials described in this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2019.05.012.

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