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Early activation of hepatic stellate cells induces rapid initiation of retinyl ester breakdown while maintaining lecithin:retinol acyltransferase (LRAT) activity

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ABSTRACT

Lecithin:retinol acyltransferase (LRAT) is the main enzyme producing retinyl esters (REs) in quiescent hepatic stellate cells (HSCs). When cultured on stiff plastic culture plates, quiescent HSCs activate and lose their RE stores in a process similar to that in the liver following tissue damage, leading to fibrosis. Here we validated HSC cultures in soft gels to study RE metabolism in stable quiescent HSCs and investigated RE synthesis and breakdown in activating HSCs.

HSCs cultured in a soft gel maintained characteristics of quiescent HSCs, including the size, amount and composition of their characteristic large lipid droplets. Quiescent gel-cultured HSCs maintained high expression levels of Lrat and a RE storing phenotype with low levels of RE breakdown. Newly formed REs are highly enriched in retinyl palmitate (RP), similar to freshly isolated quiescent HSCs, which is associated with high LRAT activity. Comparison of these quiescent gel-cultured HSCs with activated plastic-cultured HSCs showed that although during early activation the total RE levels and RP-enrichment are reduced, levels of RE formation are maintained and mediated by LRAT. Loss of REs was caused by enhanced RE breakdown in activating HSCs. Upon prolonged culturing, activated HSCs have lost their LRAT activity and produce small amounts of REs by DGAT1. This study reveals unexpected dynamics in RE metabolism during early HSC activation, which might be important in liver disease as early stages are reversible. Soft gel cultures provide a promising model to study RE metabolism in quiescent HSCs, allowing detailed molecular investigations on the mechanisms for storage and release.

1. Introduction

Vitamin A, also called retinol, is a fat soluble vitamin required for normal development and growth, reproduction and vision [1–3]. Retinol and its metabolites, including retinoic acid, are collectively termed retinoids. They are involved in cellular signaling, influencing cell proliferation and differentiation during embryonic development [2]. Retinoids have been reported to have immunoregulatory functions [4] and are associated with several diseases including cancers [5] and metabolic disorders [6,7]. Since vitamin A cannot be synthesized by mammals but must be absorbed from the diet, storage and release is regulated to maintain a steady supply that is independent of alimentary availability for longer periods of time. The main storage site of vitamin A is the liver, more specifically the hepatic stellate cell (HSC). Here, vitamin A is stored as retinyl esters (REs) in characteristic large lipid droplets of so-called quiescent HSCs [8]. The main enzyme producing REs is lecithin: retinol acyltransferase (LRAT) [9–12]. LRAT receives retinol via cellular retinol binding protein 1 (CRBP1) [13] and produces retinyl esters by transferring the fatty acid from preferentially the sn-1 position of phosphatidylcholine (PC) to retinol [14]. This mechanism has been suggested to result in the observed enrichment of retinyl palmitate in the liver [15,16]. Next to LRAT, Diacylglycerol O-Acyltransferase 1 (DGAT1) has been shown to be able to synthesize RE, but uses an acyl-CoA derived from free fatty acids. REs produced by DGAT1...
lack therefore an enrichment of retinyl palmitate \cite{10,17,18}. How vitamin A is transported within the liver between different cell types and how it is released from the HSC in response to diet shortage is still largely unknown \cite{8,19}.

When the liver is damaged, quiescent HSCs activate and change into myo-fibroblastic cells playing a key role in the development of liver fibrosis \cite{20}. During activation RE storage is reduced \cite{21,22}. The large lipid droplets have disappeared and are replaced by multiple small and dynamic lipid droplets with an enrichment of poly-unsaturated fatty acids (PUFAs) in the triacylglycerol (TAG) species \cite{22–25}. Activation of HSCs is mediated by many molecular factors \cite{20}.

Most research on RE metabolism has been performed in HSCs cultured on plastic. Under these conditions, HSC activation has been shown to start already after half a day of culture based on gene expression levels \cite{25}. It was found that Lrat expression is quickly lost upon activation and RE species profile is different in activated HSCs as compared to quiescent HSCs \cite{10,26,27}. Based on this research, it has been suggested that synthesis of REs might be different in activated HSCs compared to quiescent HSCs. Furthermore, RE breakdown in activated HSCs was shown to involve autophagy, a process that is hardly present in quiescent HSCs \cite{28}.

A major factor that influences the activation status of the HSC is the stiffness of its surroundings. Conventional culture of primary quiescent HSCs on plastic plates results in HSC activation within 7 days. Friedman et al. were the first to obtain evidence that culture of freshly isolated quiescent HSCs on the basement membrane-like matrix Matrigel prevented their transdifferentiation \cite{29}. Others have confirmed these findings by showing increased Acta2 expression and disappearance of the characteristic large lipid droplets with increasing stiffness of the matrix \cite{30–32}.

Here we first validated the capacity of HSCs to maintain a quiescent phenotype in soft gel culture focusing on lipid and RE characteristics. RE metabolism was then studied in quiescent HSCs that could be maintained for prolonged periods of time and compared to activated HSCs. This allowed us to reevaluate our knowledge on the enzymes involved in RE metabolism in both quiescent and activated HSCs.

2. Methods

2.1. HSC isolation

Hepatic stellate cells were isolated from healthy BALB/c mice minimally 3 months old, consisting of a mixture of male and female mice according to availability from breeding. The isolation protocol of Mederacke et al. was used \cite{33}. In short, livers were perfused with pronase and collagenase solutions, after which HSCs were separated from other cells by Nycodenz gradient centrifugation. Nycodenz pronase and collagenase solutions, after which HSCs were separated from other cells by Nycodenz gradient centrifugation. Nycodenz gradient centrifugation is based on the large lipid droplets in the HSCs. Since Lrat−/− mice lack these large lipid droplets a different method was used for the experiments using Lrat+/− mice and their WT controls. Here, HSC isolation followed the protocol described by Riccalton-Banks et al. \cite{34}, as previously described \cite{10}. In short, livers were perfused with collagenase solution, after which HSCs were isolated by differential centrifugation. Lrat−/− mice have a CS7BL/6 J background \cite{11}. Comparison of the Riccalton-Banks and the Nycodenz method showed no differences in loss of Lrat expression and gain of gene expression of activation markers such as Acta2 using plastic activated WT HSCs. Similarly, HSCs isolated mice from WT and Lrat−/− genetic backgrounds showed similar characteristics for activation markers upon culture on plastic plates. Mice experiments were approved by the Dutch Animal Experimental Licensing Committee (DEC) (DEC number: AVD1080020174484).

2.2. Cell culture

Primary HSCs were cultured on standard plastic culture plates or in a matrix by embedding the cells in a droplet of soft gel, surrounding the cell on all sides. All experiments were performed in Matrigel (Corning) or in Cultrex Reduced Growth Factor Basement Membrane Extract, Type 2, Select (R&D Systems Europe Ltd. t/a Bio-Tech). Both gel-types gave similar results (for an example, see Suppl. Fig. 1). PIC-LEC (only used in Suppl. Fig. 1) consists of Noviozel (from Sopachem) a synthetic hydrogel based on polyisocyanopeptides (PIC) at a concentration of 1 mg/ml and laminin-entactin complex (LEC, Corning) used at 3 mg/ml. Cells were cultured in DMEM with 10 % fetal bovine serum, which was replaced weekly. Cells were cultured in a humidified incubator with 5 % CO2 at 37 degrees. For experiments involving RE measurements, cells were kept in the dark at all times. Retinol-D5 was obtained from Toronto Research Chemicals Inc., Canada. Before use the concentration was verified using a DU 720 UV/Vis Spectrophotometer (Beckman Coulter) making a wavelength scan and determining the absorbance at 325 nm. DGAT1 inhibitor (PF-04620110, Sigma) was dissolved in DMSO.

2.3. RNA isolation and rt-qPCR

RNA was isolated using a RNeasy Micro Kit (Qiagen, Hilden, Germany) including an on-column DNase-I treatment to minimize gDNA contamination. cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California). The PCR amplifications were performed using a Bio-Rad detection system with iQ SYBR Green Supermix (Bio-Rad). A 4-fold dilution series from a pool of the samples was used to determine relative gene expression levels, normalized for the average of the levels from the reference genes. Primer sequences and annealing temperatures are listed in Suppl. Table 1.

2.4. Fluorescent imaging

Cells were grown on CELLView slides (Greiner Bio-One BV, Kremmünster, Upper Austria). Lipid droplets were stained using 0.1 μg/ml LD540 (kindly donated by Dr. C. Thiele, Biochemistry and Cell Biology of Lipids, LIMES, University of Bonn, Bonn, Germany; \cite{35}). Nuclei were stained with 30 μg/ml Hoechst 33342 (Molecular Probes, Paisley, UK). Images (z-stacks) were taken with a Leica TCSSPE-II confocal microscope at the Center of Cellular Imaging (Faculty of Veterinary Medicine, Utrecht University, The Netherlands). Images were analyzed using CellProfiler 4.2.6 \cite{36}.

2.5. Lipidomics: retinoids

After incubation, medium was removed and the cells were washed in PBS. Cells cultured on plastic were scraped in PBS, cells cultured in gel were centrifuged (5 min at 500G) in cold PBS. Lipids were extracted according to the method of Bligh and Dyer \cite{37}. All procedures were performed in the dark to prevent photodegradation of retinoids. The method for isolation and measurement of RE has been described in detail \cite{38}. In short: after drying under nitrogen gas samples were dissolved in Methanol/Acetonitrile/Chloroform/Water (46/20/17/17, v/v/v/v) and injected onto a HALO C8 column (2.7 μm, 150 × 3.00 mm; Advanced Materials Technology, Wilmington, DE, USA) with a Kinetex SecurityGuard Ultra C8 column. Column temperature was maintained at 30 °C. Isocratic elution was performed with 90 % acetonitrile/water (95/5, v/v) and 10 % acetonitrile/chloroform (85/15, v/v) for 15 min, followed by gradient elution to 100 % of the latter solvent in an additional 20 min. A 5 min re-equilibration time was used between runs, giving a total run time of 40 min. Flow rate was kept constant at 300 μl/min. Both elution solutions also comprised 0.1 % (v/v) formic acid. The column outlet of the LC was connected to a Q-Exactive HF Orbitrap MS system (Thermo Fisher Scientific, Waltham, United States) equipped with an Atmospheric Pressure Chemical Ionization (APCI) source. The MS parameters included a positive ion spray discharge current of 1.0 μA, an aux sheath gas flow rate of 60 Ar, a gas flow rate of 20 Ar, a sweep gas flow rate of 1 Ar, an ion transfer tube temperature of 325 °C, a
vaporizer temperature of 350 °C, and a scan range of 250–600 m/z with a resolution of 120,000. Peaks were integrated using TraceFinder software (ThermoFisher Scientific, Waltham, United States).

2.6. Lipidomics; neutral lipids (TAG)

Neutral lipids were extracted according to the method of Bligh and Dyer [37] and analyzed with LC-MS [24]. After drying under nitrogen gas samples were dissolved in methanol:chloroform (1:1) and injected onto a HALO C8 column (2.7 μm, 150 × 3.00 mm; Advanced Materials Technology, Wilmington, DE, USA). Column temperature was maintained at 40 °C. Gradient elution was performed from methanol/water (1/1; v/v) to methanol/isopropanol (4/1; v/v) in 2 min, followed by isocratic elution with the latter solvent for an additional 5.5 min. A 2.5 min re-equilibration time was used between runs, flow rate was kept constant at 600 μl/min. The column outlet of the LC was connected to a Q-Exactive HF Orbitrap MS system (ThermoFisher Scientific, Waltham, United States) equipped with an Atmospheric Pressure Chemical Ionization (APCI) source. The MS parameters included a positive ion spray discharge current of 5.0 μA, an aux sheath gas flow rate of 60 Arb, a gas flow rate of 20 Arb, a sweep gas flow rate of 1 Arb, an ion transfer tube temperature of 325 °C, a vaporizer temperature of 450 °C, and a scan range of 200–1100 m/z with a resolution of 120,000. Data was converted to mzML files using mzc convert from the ProteoWizard toolbox (ProteoWizard, Palo Alto, United States) and peaks were integrated using the XCMS package (version 3.14, [39] in R for peak-picking, sample grouping, and retention time-correction. As described previously [24] HPLC provides TAG species with characteristic retention times. Peaks were filtered based on these retention times and having a mass between m/z 800–1000. Non-PUFA TAGs selected: 857.8 (52:3), 859.8 (52:2), 885.8 (54:3). PUFAs-TAGs: 907.9 (56:6), 909.6 (56:5), 935.9 (58:6), 937.9 (58:5), 957.9 (60:9), 959.3 (60:8), 981.5 (62:11), 979 (62:12).

2.7. Lipidomics; phospholipids

Lipids were extracted according to the method of Bligh and Dyer [37]. The phospholipid species composition was determined as described previously [40] by liquid chromatography coupled to mass spectrometry (LC-MS). The extracted lipids were dissolved in methanol:chloroform (1:1; v/v) and were loaded on a HILIC column (2.6 μm HILIC 100 Å, 50 × 4.6 mm, Phenomenex, Torrance, CA, USA). Column temperature was maintained at 25 °C. Gradient elution was performed from acetonitrile/acetone (9:1, v/v) to acetonitrile/H2O (7:3, v/v) with 50 mM ammonium formate in 1 min, followed by isocratic elution with the latter solvent for an additional 2 min. A 1 min re-equilibration time was used between runs, flow rate was kept constant at 1 ml/min. Both elution solutions also comprised 0.1 % (v/v) formic acid. The column outlet of the LC was connected to a Fusion Orbitrap MS system (ThermoFisher Scientific, Waltham, United States) equipped with a heated electrospray ionization (HESI) source. The MS parameters included a negative ion spray voltage of 3.6 kV, an aux sheath gas flow rate of 54 Arb, a gas flow rate of 7 Arb, a sweep gas flow rate of 1 Arb, an ion transfer tube temperature of 350 °C, a vaporizer temperature of 450 °C, and a scan range of 350–1100 m/z with a resolution of 120,000. In parallel, data dependent MS2 analysis was performed. For the conversion of the RAW format to mzML, the open-source tool mzc convert ProteoWizard software (ProteoWizard, Palo Alto, United States) was utilized, with the selection of the “peakPicking filter vendor mzLevel = 1” parameter set. LC/MS peak-picking, sample grouping, and retention time-correction on the mzML files were performed using the XCMS package version 3.14.1 [39,41,42], implemented in R free software version 4.1.2 Team [2020] (The R Foundation for Statistical Computing, Vienna, Austria). The LC/MS peaks were annotated based on retention time, exact m/z-ratio using an in silico generated lipid database. After peak annotation, the acquired MS2 data was used to determine the fatty acid composition.

2.8. Statistics

Graphs were made, including statistics, using GraphPad Prism version 9.3.1. Depending on the amount of groups a one-sample t-test, one-way ANOVA or two-way ANOVA was used. Based on Brown-Forsythe tests and QQ plots normality and equality of group variances was assessed, and if necessary data were log transformed. Specifics for each graph are indicated in the figure legends.

3. Results

3.1. HSCs cultured in a soft gel remain quiescent

In order to assess the activation state of hepatic stellate cells cultured in a soft gel, primary murine HSCs were isolated and cultured for 7 days on either plastic plates or in gel. Subsequently, gene expression levels were determined and compared with the expression in cells obtained directly after isolation (Fig. 1). Gene expression of activation markers α-Smooth Muscle Actin (Acta2) and Collagen-type I α1 (Col1α1) increased to a significant higher level in HSCs grown on plastic compared to cells grown in a soft gel, as has been reported previously in rat HSCs [29–31,43] and mouse HSCs [32]. We next investigated the expression of several genes related to retinoid metabolism. Expression of Lrat, often used as a quiescence marker, was strongly decreased (~63 fold) upon growth on plastic for 7 days (Fig. 1), in agreement with our previous observations [27]. HSCs cultured in gel showed only a ~2.6 fold reduction in Lrat expression. Cellular retinol binding protein 1 (Crbp1) transports retinol in the cytosol and its deficiency leads to enhanced turnover of retinol in the liver [13]. It has been reported to be present in HSCs [44,45]. Crbp1 expression decreased in plastic HSC culture, while it did not change in gel HSC culture (Fig. 1). Expression of aldehyde dehydrogenase 1 family member A1 (Aldh1a1), which can convert retinaldehyde to retinoic acid, slightly increased in plastic HSC culture, as has been reported earlier [46], while expression in gel culture did not increase (Fig. 1). Aldh1a2 showed a similar trend as Aldh1a1, but changes were not significant (data not shown).

A characteristic change in the phenotype that occurs during HSC transdifferentiation is a change in morphology from small quiescent cells with large lipid droplets to large spread-out cells with loss of large lipid droplets [47,48]. Indeed, we observed that HSCs cultured in a soft gel for 7 days remained small, with almost the entire cell filled with large lipid droplets resembling the lipid droplets in quiescent HSCs on day 1 (Fig. 2A). Quantification of lipid droplet diameter showed no decrease in size when HSCs were grown in soft gel for 7 days (Fig. 2B). Also the number of lipid droplets per HSC did not increase in soft gel, contrary to cultures on plastic (Fig. 2C). We previously observed that HSC transdifferentiation is also accompanied by a reduction in RIs and the formation of PUFA-containing TAG species in lipid droplets [22,24,49]. In line with a more quiescent phenotype, HSCs grown in a soft gel did not change their TAG profile and RI levels, in contrast to HSCs grown on plastic (Fig. 2D&E). Our combined results suggest that HSCs cultured in soft gel maintain a quiescent phenotype, identifying next to gene expression of traditional markers additional characteristics related to lipid droplet morphology and content.

3.2. RE dynamics in quiescent and activated HSCs

Maintenance of HSC cultures in soft gel allowed for the first time assessment of RE dynamics in quiescent HSCs. To this end we performed labeling experiments with deuterium-labeled (D5) retinol. Quiescent HSCs on day 1 and day 7 were labeled overnight with retinol-D5 and the incorporation of labeled retinol in retinyl esters was determined by mass spectroscopy. Under these conditions, retinyl esters were readily labeled both on day 1 and day 7 and the labeling on day 7 gel cultures was even...
significantly increased as compared to day 1 gel cultures (Fig. 3A, right bars), consistent with HSCs after 7 days of culture in soft gel maintaining their quiescence. Similar results were obtained with HSCs cultured on plastic, with non-significant changes in RE labeling between day 1 and day 7 (Fig. 3A, left bars). Both with HSCs cultured in soft gel and on plastic, the percentage of labeled REs (Fig. 3B) relative to the total amount of REs (as has been depicted in Fig. 2D) increased. The increase could potentially be explained either by an increase in RE production and/or a decrease of unlabeled REs.

To assess degradation of REs, a pulse-chase experiment was performed in which HSCs were cultured on plastic or soft gel for 7 days and subsequently REs were labeled by overnight incubation with retinol-D5 (similar as performed in Fig. 3A). The next day, the labeled substrate was removed by medium exchange and degradation of labeled REs was assessed after a chase period of 7 days (i.e. 14 days of cell culture). As shown in Fig. 3C, a >50% degradation of newly formed REs was seen in plastic culture, but not in soft gel culture.

These results show that HSCs cultured in soft gel maintain a RE storing phenotype, whereas HSCs grown on plastic develop a RE releasing phenotype.

3.3. Both HSCs cultured in gel and HSCs activated on plastic maintain LRAT activity the first 7 days of culture

We were surprised by the fact that early activating HSCs (day 7, cultured on plastic) maintained their retinyl ester synthesizing capacity, despite concomitantly increasing their RE degradation activity (Fig. 3).

In addition, during these first seven days of activation, gene expression of the main RE synthesizing capacity, LRAT, is almost completely abolished (Fig. 1). Dgat1 expression levels did not change upon plastic culture or gel culture (Suppl. Fig. 2). In order to study the contribution of LRAT and/or DGAT1, HSCs were isolated from both wild type and Lrat−/− mice, and cultured in soft gel (quiescent HSCs) or on plastic (activated HSCs). To measure RE synthesis activity, the cells were subsequently incubated after 7 days of culture with D5-labeled retinol overnight (17 h), in the absence or presence of a DGAT1 inhibitor. RE synthesis in HSCs cultured in soft gel as well as on plastic was not affected by the DGAT1 inhibitor. In addition, RE synthesis was strongly reduced in HSCs without LRAT both in activated HSCs (4.8 % remaining activity) and quiescent HSCs (1.5 % remaining activity) (Fig. 4A). Under both conditions, the low levels of RE-D5 formation in Lrat−/− cells could be inhibited with the DGAT1 inhibitor (Fig. 4A, insert). These results indicate that activating plastic-grown HSC have considerable LRAT activity despite the fact that Lrat gene expression is virtually turned off (Fig. 1). These results suggest that sufficient LRAT protein remains present to be the dominant RE synthesizing protein during early stages of HSC activation, which disappears only later during HSC activation. To investigate this, early activating HSCs were cultured on plastic for several passages (passaged HSCs), which resulted in a complete loss of their RE stores. Their capacity to form REs and the contribution of DGAT1 to this process was investigated in these passaged HSCs as well (Fig. 4B and C). The capacity of RE synthesis is greatly reduced as compared to primary HSCs cultured on plastic (day 7) (Fig. 4B). RE synthesis in passaged HSCs can be inhibited with the DGAT1 inhibitor (Fig. 4C), indicating that LRAT activity is lost in WT cells upon prolonged activation of HSCs.

3.4. Plastic-activated HSCs show a change in retinyl ester species

The contribution of LRAT to RE synthesis at early stages of HSC activation prompted us to determine the RE species present under various conditions (Fig. 5). Freshly isolated HSCs (day 0, quiescent) had a retinyl palmitate (RP) percentage of 77 % and lower amounts of retinyl oleate (19 %) and retinyl stearate (4 %), in agreement with others [10, 50]. HSCs cultured in soft gel indeed maintained this level of RP enrichment up to 14 days of culture (no significant difference compared to day 0, Fig. 5A). This was observed in BME, Matrigel and PIC-LEC, a more synthetic gel (supplementary Fig. 1). In HSCs cultured on plastic the percentage of RP decreased to 52 % (±5.5 %). The decrease in RP was accompanied by an increase in retinyl stearate (RS) (Fig. 5A). This change in RE species distribution did not further change in the next 7 days of culture on plastic (Fig. 5A, day 14 plastic), despite the fact that the total amount of REs decreased by another 60 % (data not shown). Similar changes in RE species distribution were observed when determining the newly synthesized REs, as determined by retinol-D5 incubation (Fig. 5B). Together, our results indicate that LRAT is the main enzyme producing newly formed RE in quiescent and early activated HSCs (Fig. 4), which is also responsible for the shift in RE species that occurs during early HSC activation.

3.5. Changes in PC species in quiescent and activating HSCs

PC is the acyl-donor in the synthesis of retinyl ester species by LRAT by transferring the acyl chain at the sn-1 position to retinol. Therefore, we determined whether the change in fatty acid composition in RE during early activation of HSCs was caused by a change in the fatty acid content of PC using mass spectroscopy. Although our method cannot distinguish between the fatty acids at the sn-1 and sn-2 position of PC, palmitate and stearate are expected at the sn-1 position [16, 51]. Endogenous PC species were measured in quiescent (day 0), early activated (day 7) and passaged HSCs (Fig. 6A, right panel). For retinyl ester species determination, the endogenous RE species composition in quiescent HSCs (day 0) was compared with newly synthesized RE species in early activated HSCs (cultured 7 days on plastic) and passaged HSCs by overnight incubation with retinol-D5 (Fig. 6A, left panel). RE and PC species containing palmitate, oleate or stearate were quantified and their percentages of total amount of respectively RE and PC are shown in Fig. 6A&B. The ratio RP:RS drastically decreased from quiescent HSCs on day 0 (26) to early activating HSCs on day 7 (3), in agreement with our previous observations (Fig. 5B). However, no clear difference was observed in PC species containing palmitate or stearate; the PC palmitate: PC stearate ratios in plastic-cultured HSCs actually
slightly increased from 3.2 on day 0 to 3.8 on day 7. In passaged HSCs, palmitate and oleate make up almost equal amounts of the RE species, which may be related to a DGAT1-mediated incorporation of the main fatty acid species provided to the cells via the medium [52,53].

To investigate whether individual PC species correlated with the observed changes in RE during activation, the 4 most abundant PC species were determined at each timepoint (day 0, day 7, and day 14 of plastic HSC cultures (Fig. 6B)). This resulted in the identification of 6 PC species, which together make up for 60–74% of the total PC amounts under the various conditions. Only one of these species contained stearate (18:0) and the levels of this PC species (PC(18:0_20:4)) did not change significantly over time. The other 5 species contain 16:0 and showed a significant change in levels at early stages of activation (between day 0 and day 7), but not at later stages anymore (day 7 and day 14). Whereas the levels of some PC species decreased (such as PC (16:0_20:4)), others (such as PC(16:0_16:0)) increased. Most notable was the decrease observed in PC(16:0_18:2) (Fig. 6B). Given that some palmitate containing PC species increased and others decreased, the overall ratio of PC species with 16:0 versus 18:0 does not change.

Fig. 2. Lipid droplet analysis and neutral lipid composition of HSCs. 2A: Representative images of HSCs cultured for 1 or 7 days on a glass surface (2D) or in gel. Quantification of LD540 positive lipid droplets was performed on cells from 2 different HSC isolations. Statistics using a one-way ANOVA with Šidák’s multiple comparisons test. 2B: Quantification of lipid droplet diameter from 2A. Statistics using a one-way ANOVA with Šidák’s multiple comparisons test after log transformation of the data. 2C: Quantification of lipid droplet amounts from 2A. 2D: Quantification of retinyl esters (sum of RP, RO, RS) at start (day 1 = 100 %) and end (day 7) of culture in gel or on plastic. Amounts were normalized against cholesterol levels. Average levels of day 1 plastic and day 1 gel are 1.24 (sd 1.59) and 0.77 (sd 1.15) pmol retinyl ester/µmol cholesterol respectively. Culture duplicates or triplicates from 5 different isolations. Statistics using a one-way ANOVA with Šidák’s multiple comparisons test. 2E: Quantification of triacylglycerol species with poly-unsaturated fatty acids (PUFA-TAGs) at start (day 1 = 100 %) and end (day 7) of culture in gel or on plastic. Means of culture quadruplicates from a representative experiment. Statistics using a two-way ANOVA with Šidák’s multiple comparisons test, with culture day (***) and PUFA (***) and interaction (****) as variables. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
Fig. 3. Retinyl ester synthesis and breakdown in quiescent vs activated HSCs. 3A: Comparison of RE-D5 after overnight incubation with retinol-D5 in HSCs 1 day in culture and 7 days in culture. Amounts are normalized to cholesterol levels and shown as percentage of RE-D5 compared to day 1. Samples came from 4 different HSC isolations, all cultured in duplicate. Statistics using a two-way ANOVA with Šidák’s multiple comparisons test, with culture method (ns), culture day (**) and interaction (ns) as variables, after log transformation of the data. Average levels of day 1 plastic and day 1 gel are 0.12 (sd 0.15) and 0.04 (sd 0.04) pmol retinyl ester-D5/pmol cholesterol respectively. 3B: Percentage of RE-D5 compared to total RE levels of samples shown in A. Statistics using a two-way ANOVA with Šidák’s multiple comparisons test, with culture method (*), culture day (****) and interaction (ns) as variables. 3C: percentage of D5 labeled retinyl esters (sum RP, RO, RS) at day 14 of culture compared to levels at day 7 (day 7 = 100 %). HSCs were incubated overnight with retinol-D5 at day 7, after which medium was replaced. Normalized to cholesterol levels. Average levels of day 7 plastic and day 7 gel are 0.20 (sd 0.10) and 0.09 (sd 0.03) pmol retinyl ester-D5/pmol cholesterol respectively. Statistics using a two-way ANOVA with Šidák’s multiple comparisons test, with culture method (*), culture day (*) and interaction (*) as variables, after log transformation of the data. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Fig. 4. LRAT activity remains high in early activating HSCs. Comparison of RE-D5 levels in HSCs, Lrat−/− HSCs, or passaged HSCs cultured on plastic or in gel as indicated. Newly formed RE was determined after overnight incubation with retinol-D5 (10μM) in WT and Lrat−/− HSC in the absence or presence of DGAT1 inhibitor (5 μM) (A,B) or in passaged HSCs (B,C). 4A: Comparison of RE-D5 levels in WT and Lrat−/− HSCs cultured on plastic or in gel for 7 days. Data are from 3 different HSC isolations, with culture duplicates. Average levels of WT day 7 plastic and day 7 gel are 0.19 (sd 0.18) and 0.16 (sd 0.21) pmol retinyl ester-D5/pmol cholesterol, respectively. Statistics were performed using a one-way ANOVA with Šidák’s multiple comparisons test after log transformation of the data. Insert shows data for Lrat−/− HSCs with RE levels normalized to day 7 without inhibitor. Statistics were performed using a one-way ANOVA with Šidák’s multiple comparisons test. Amounts are normalized to cholesterol levels and shown as percentage of RE-D5 compared to day 7 WT without DGAT1 inhibitor. 4B: Comparison of RE-D5 levels in passaged HSCs with day 7 WT on plastic. Data shown are from a representative experiment with culture triplicates and normalized to cholesterol levels. Amounts are normalized as described for panel A. Statistics using a one sample t-test. 4C: Comparison of RE levels in passaged HSCs with and without DGAT1 inhibitor. Data from 3 different experiments cultured in triplicate and are normalized to cholesterol levels. Average level of passaged + ROH is 0.007 (sd 0.006) pmol retinyl ester-D5/pmol cholesterol respectively. Statistics were performed using a t-test (first bar contains mostly n/a values, so not included). NS = not significant * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
4. Discussion

Here we showed that primary HSC cultures embedded in a soft gel maintain their quiescent phenotype, in agreement with others, who showed that culture on a soft matrix suppressed αSMA and maintained LDs in HSCs [29–32, 43]. We extended these findings by quantifying the amount, size and content of the LDs. We previously reported that PUFAs are enriched in TAGs in plastic-activated HSCs and we now show that this is not observed in gel-cultured HSCs, further confirming their quiescent phenotype. To the best of our knowledge culture in soft matrices has not been used in the study of a prominent function of the quiescent hepatic stellate cell; vitamin A storage and release. We show that HSCs cultured in a soft gel for 7 days have a similar RE storing phenotype as compared to HSCs at the start of culture (day 1). Our results were observed in Matrigel as well as in BME and in PIC-LEC (Ye et al., 2020), the latter of which is a much more defined gel than Matrigel, in agreement with the notion that not the composition but the stiffness of the surface determines the activation status of the HSC [29–31, 54–58]. By obtaining a stable quiescent culture, a comparison between in gel cultured (quiescent) HSCs and plastic cultured (early activating) HSCs was possible, showing steady RE synthesis and increased RE degradation in early activating HSCs.

4.1. Retinyl ester turnover in HSCs

Retinyl ester metabolism was investigated by determining retinyl ester synthesis and breakdown using heavy-isotope labeled retinol and by determining total retinyl ester (species) amounts. The measurement of retinyl esters poses specific challenges. First of all, animal to animal variation, causes batch to batch variations in the initial RE content of primary HSCs. Second, the light-sensitivity of retinoids makes the samples highly sensitive to RE degradation and causes sample to sample variation. Third, differences in cell culture (plastic versus gel) further added to variations in sample recovery (scraping versus centrifugation, including the duration of the sample recovery method, related to the light sensitivity of the REs). For these reasons, we expressed the data as percentage of the starting point of interest. The standard deviation of the data is relatively high due to large variations between different experiments in initial retinyl ester content. The data were normalized by cholesterol. Cholesterol is an intrinsic lipid marker that co-extracts with retinyl esters, which can be analyzed and quantified during the same RE quantification procedure, and which helps in reducing sample variation compared to other normalization methods such as protein levels. It will be interesting to investigate HSCs in gel cultures of varying degrees of stiffness, as this may allow to mimic plastic culture activation of HSCs in gel cultures, thus reducing some of the above mentioned variables in RE analysis.

RE degradation in the activating cells is most likely caused by lysosomal acid lipase (LAL/Lipa) after lipophagy of the lipid droplets [28]. In quiescent HSCs RE breakdown was minimal. We showed that in gel cultures, total RE does not decrease during the first 7 days of incubation.
These results are in agreement with the existence of preexisting “original” lipid droplets with relatively slow turnover, as shown before [23,28]. How exactly retinol mobilization from RE storage is regulated in a healthy liver is still unclear. To our knowledge very few studies address RE breakdown specifically in the quiescent HSC. One study indicates hormone sensitive lipase (HSL) as a possible RE hydrolase in a healthy liver, as hormone sensitive lipase (HSL) was not significantly altered during early activation of HSCs (Suppl. Fig. 2).

Retinyl ester turnover is also determined by retinyl ester synthesis. In vitro, two enzymes have been implicated in retinyl ester synthesis, namely LRAT and DGAT1. In vivo, in three independent Lrat knockout mice [9,11,12], only trace amounts of retinyl esters can be recovered, suggesting that LRAT is the predominant enzyme responsible for the synthesis of retinyl esters. Our results recapitulate the in vivo findings and show that in WT HSCs, retinyl ester synthesis is insensitive to DGAT1 inhibitors. Only very low levels of retinyl ester synthesis can be observed in Lrat knockout HSCs, which is sensitive to DGAT1 inhibitors. Indeed, we find expression of Dgat1 by qPCR in our culture systems and it does not significantly vary between the different culture conditions (Suppl. Fig. 2). It remains to be established whether this DGAT1-sensitive retinyl ester synthesis has biological significance and is responsible for the trace amounts of retinyl esters in the Lrat knockout mice.

4.2. Lrat expression during HSC activation

LRAT activity remains high during the first week of HSC in vitro activation, contrary to the observed decrease in the enrichment of RP and despite the observed decrease in gene expression of Lrat. mRNA and protein levels do not always correlate, as protein levels are also regulated by protein degradation, for example via ubiquitination and autophagy [60]. A lack of correspondence between Lrat mRNA and protein was suggested by others [6]. For example, in HSCs from Fxr-null mice, it has been shown that changes in mRNA level of Lrat do not always correspond to changes in protein level [61]. However, loss of LRAT during culture activation has been shown both on gene and protein level in mice and rat [26,27,59,62]. The observed high enzymatic activity despite decreased mRNA levels might also be explained by post translational modifications, which are able to change protein functionality [63]. Whether this is important for LRAT activity remains to be determined. Our data indicate that the amount of enzyme still present after 7 days of plastic culture activation is sufficient to account for the majority of RE production at that time point. Preliminary western blot analysis of quiescent (D1) and early activated (D7) HSCs indicates that indeed LRAT protein is still present after 7 days of activation (Suppl. Fig. 3), despite a ~63-fold decrease in Lrat mRNA levels during the same time period (Fig. 1). These results require further validation, also in other animal species, but raise the question how LRAT protein is protected from degradation during early stages of HSC activation. RE production from retinol added to the medium also does not decrease in the first 7 days of culture. Only after prolonged culture, the amount of RE production decreases, at which point this is mainly mediated by DGAT1 (Fig. 4C).

4.3. HSC activation

Different stages of HSC activation have been described before (reviews: [19,20], and recently also at the level of lipids [64]). Single cell RNA sequencing revealed the presence of different HSC populations at different time points during plastic culture activation. Markers for these timepoints corresponded with different in vivo HSC populations acquired from mice treated for 3 weeks with CCL4 [65], suggesting that in vivo early and prolonged activated HSCs can coexist during liver damage. Heterogeneity in HSCs has been observed more often [66,67], including heterogeneity in Lrat expression after in vivo activation [67]. Disturbances in retinoid metabolism have been linked in several ways to the development of liver diseases, including metabolic dysfunction-associated steatotic liver disease (MASLD), but mechanistic understanding of these correlations is still largely lacking [67]. This highlights the importance of understanding RE metabolism in the liver during health and disease.
administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2024.159540.

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