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Rat hepatocyte culture model of macrosteatosis: Effect of macrosteatosis induction and reversal on viability and liver-specific function

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Abstract

Background & Aims—A common cause of liver donor ineligibility is macrosteatosis. Recovery of such livers could enhance donor availability. Living donor studies have shown diet-induced reduction of macrosteatosis enables transplantation. However, cadaveric liver macrosteatotic reduction must be performed *ex vivo* within hours. Towards this goal, we investigated the effect of accelerated macrosteatosis reduction on hepatocyte viability and function using a novel system of macrosteatotic hepatocytes.

Methods—Hepatocytes isolated from lean Zucker rats were cultured in a collagen sandwich, incubated for 6 days in fatty acid-supplemented medium to induce steatosis, and then switched for 2 days to medium supplemented with lipid metabolism promoting agents. Intracellular lipid droplet size distribution and triglyceride, viability, albumin and urea secretion, and bile canalicular function were measured.

Results—Fatty acid-supplemented medium induced microsteatosis in 3 days and macrosteatosis in 6 days, the latter evidenced by large lipid droplets dislocating the nucleus to the cell periphery. Macrosteatosis significantly impaired all functions tested. Macrosteatosis decreased upon returning hepatocytes to standard medium, and the rate of decrease was 4-fold faster with supplemented agents, yielding 80% reduction in 2 days. Viability of macrosteatosis reduced hepatocytes was similar to control lean cells. Accelerated macrosteatotic reduction led to faster recovery of urea secretion and bile canalicular function, but not of albumin secretion.

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Conclusions—Macrosteatosis reversibly decreases hepatocyte function and supplementary agents accelerate macrosteatosis reduction and some functional restoration with no effect on viability. This *in vitro* model may be useful to screen agents for macrosteatotic reduction in livers before transplantation.

Keywords

Steatosis; Triglyceride; Albumin; Urea; Bile; Liver transplantation; Lipid metabolism

Introduction

Orthotopic liver transplantation is limited by donor scarcity [1–3]. This has motivated the development of strategies to recover livers from deceased donors currently not considered suitable for transplantation [2,4]. Macrosteatosis, defined as the accumulation of triglycerides (TG) in the form of large lipid droplets that displace the nucleus to the cell periphery, when found in more than 30% of the hepatocytes, is a common cause of donor ineligibility [1,3]. Macrosteatotic livers are more sensitive to ischemia/reperfusion (I/R) injury inherent to liver transplantation and more prone to primary non-function, as well as increased morbidity and mortality post-transplantation [1,3]. The incidence of hepatic macrosteatosis is likely to surge due to the obesity epidemic. Thus, techniques to salvage macrosteatotic livers could significantly enhance donor supply in both the short and long terms [4].

Various approaches targeting the downstream effects of macrosteatosis during I/R have shown promise in pre-clinical and clinical settings [4–8]. However, several studies suggest that excessive hepatic lipid storage is a primary cause of the exuberant I/R response, especially when in the macrosteatotic form [7,9,10]. Therefore, an alternative approach could be to eliminate the intracellular lipid droplets, thus decreasing the frequency of macrosteatotic hepatocytes below acceptable levels [4]. Dieting, exercise, and fibrate drugs over several weeks have been shown to decrease macrosteatosis and enable living donor liver transplantation [11,12]. In a rat liver model of macrosteatosis (induced by a choline and methionine-deficient diet; CMDD), switching to a normal diet 3 days prior to transplantation reduced intrahepatic TG content by 35% and increased recipient viability from 0% to >75% post-transplantation [9].

Diet/drug-induced macrosteatosis reduction occurs over days to weeks, a timescale that is not applicable to deceased human donors, which would require macrosteatotic reduction *ex vivo* within a few hours [4,13]. Animal studies have demonstrated the feasibility of macrosteatosis reduction via machine perfusion of explanted steatotic livers. This process was accelerated by introducing agents that promote lipid metabolism [14–16]. However, a thorough exploration of such agents, and combinations thereof, has yet to be performed. Furthermore, there has been little investigation of the impact of accelerated macrosteatosis reduction on the viability and function of hepatocytes, parameters that are critical for the successful outcome of liver transplantation [9–11,13,15,17].

A suitable cell culture model would facilitate the evaluation of these agents with the ultimate goal of developing protocols to promote accelerated macrosteatosis reduction and functional recovery of transplanted steatotic livers. Microsteatotic hepatocyte culture systems have been described in this context in the literature [4,9,15]; however, their relevance is unclear given that clinical evidence suggests that macrosteatotic – and not microsteatotic – livers are hypersensitive to I/R [4]. Herein, we describe a novel macrosteatotic hepatocyte culture system to investigate the effect of macrosteatosis on viability and liver-specific functions in

hepatocytes, and use this system to explore the impact of accelerated macrosteatosis reduction on viability and the recovery of such functions.

Materials and methods

Hepatocyte isolation and culture

Male lean Zucker rats, (Charles River, Wilmington, MA) (310 ± 20 g) were housed in a 12 h light-dark cycle and temperature-controlled environment (25°C) with water and standard chow *ad libitum*. All experimental procedures followed National Research Council guidelines and were approved by the Rutgers University Animal Care and Facilities Committee. Hepatocytes were isolated using a two-step *in situ* collagenase perfusion technique [9,15]. Viability was $90 \pm 4\%$ as determined by trypan-blue exclusion [15]. Six-well culture plates (Beckton-Dickinson, Franklin Lakes, NJ) were pretreated with $50\text{ }\mu\text{g/ml}$ rat type 1 collagen solution (Beckton-Dickinson) in 0.02 M acetic acid (Sigma-Aldrich, St. Louis, MO) overnight at 4°C and washed with phosphate buffered saline (PBS, Invitrogen, Grand Island, NY). Freshly isolated hepatocytes were suspended (10^6 cells/ml) in standard hepatocyte medium and seeded (10^6 cells/well) as previously described [15]. After incubating the cells at 37°C in a 90% air/ 10% CO_2 atmosphere for 24 h, medium was removed and a collagen gelling solution (0.5 ml/well) was added to form a gel overlay [15]. Cultures were maintained in standard hepatocyte medium for 4 days with a fresh medium change every other day. Spent medium was collected (Fig. 1A, experimental days 1–5) for analysis.

Steatosis induction and reversal

Five-day hepatocyte cultures were switched to steatosis-inducing medium. Standard hepatocyte medium was supplemented with $2000\text{ }\mu\text{M}$ oleic acid, $2000\text{ }\mu\text{M}$ linoleic acid, and 4% (weight to volume) bovine serum albumin (Sigma-Aldrich) for 3 days as previously described [9,15]. Medium was replaced with fresh steatosis-inducing medium for another 3 days of steatosis induction and the spent medium was collected (Fig. 1A, experimental days 5–11). These concentrations of free fatty acids (FFA) were chosen based on a dose response study to determine the FFA dose required to induce macrosteatosis, as indicated in Supplementary Fig. 1. Following 6-day steatosis induction (11 days post-seeding), the medium was replaced with fresh hepatocyte medium with no steatosis reduction supplements (NSRS), or with a combination of the following steatosis reduction supplements (SRS): $10\text{ }\mu\text{M}$ forskolin, $1\text{ }\mu\text{M}$ GW7647, $10\text{ }\mu\text{M}$ scoparone (Sigma-Aldrich), $1\text{ }\mu\text{M}$ GW501516, $10\text{ }\mu\text{M}$ hypericin (Enzo, Farmingdale, NY), 0.4 ng/ml visfatin (Biovision, Mountain view, CA) and amino acids (Invitrogen) at final concentrations described in Supplementary Table 1. This cocktail promoted *in vitro* microsteatosis reduction by activating hepatocellular TG metabolism [15]. SRS medium pH was adjusted to match that of NSRS. Cells were incubated in SRS or NSRS medium for 48 h, after which (13 days post-seeding) the spent medium from all cultures was collected and replaced with NSRS medium for another 48 h. On post-seeding day 15, the spent medium from all experimental conditions was collected (Fig. 1A).

Hepatocyte steatosis assessment

A non-destructive quantitative image analysis method was used to quantify lipid droplet size distribution. Hepatocyte cultures were fixed in 4% paraformaldehyde, stained with the lipid-specific Nile red stain (Adipored™, Lonza, Walkersville, MD), and counterstained with $1\text{ }\mu\text{g/ml}$ nuclei-specific Hoechst-33342 stain (Invitrogen), following the manufacturer's recommendations. Confocal fluorescence images were obtained with an Olympus IX-80 microscope and analyzed using an in-house algorithm for edge detection, yielding unbiased measurements of size (cross sectional surface area) and lipid droplet distribution/cell. In

addition, hepatocytes were scraped and sonicated in NSRS medium and using a lipase assay kit (Sigma-Aldrich), the TG content measured by quantification of liberated glycerol [9]. Time lapse bright-field images of hepatocytes undergoing macrosteatotic reduction were acquired every 15 min for 36 h using a temperature and gas controlled (37 °C and 10% CO₂ balanced with air) Olympus IX-80 microscope stage. The size of individual lipid droplets was monitored using SLIDEBOOK™ software (Intelligent Imaging Innovations, Denver, CO) to calculate a rate of change in cross sectional surface area.

Hepatocyte viability assessment

Cells were washed with PBS, and dead cells quantified following incubation with 4.29 µg/ml Ethidium Homodimer-1 (EthD-1, Invitrogen), and 1 µg/ml Hoechst-33342 (Invitrogen) to stain all cells, for 20 min at 37 °C [18]. Five 20× epifluorescence images were obtained in 5 separate wells/experimental condition. Cells containing EthD-1 labeled nuclei were counted as dead, while those with Hoechst-stained nuclei were counted as live (Supplementary Fig. 2). Percentage of viable cells was determined in each well and averaged for 5 wells/condition [18].

Hepatocyte function assessment

Rat albumin was measured by enzyme-linked immunosorbent assay (Bethyl laboratories, Montgomery, TX) and urea nitrogen using a biochemical assay (Stanbio), both using spent media samples and following the manufacturer's recommendations. Concentrations were normalized to the time period between medium changes and the number of viable hepatocytes to convert into specific secretion rates. Bile canaliculi function was assessed by visualization of excreted fluorescent products after incubation with 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA) (Sigma-Aldrich) at a final concentration of 2 µM. This molecule passively enters the cytoplasm of normal hepatocytes, where esterases metabolize it to a fluorescent product excreted into bile canaliculi [19]. The nuclei were counterstained using Hoechst-33342 as described above. Images were captured with an Olympus IX-80 microscope. General intracellular esterase activity was assessed using calcein-acetoxymethylester (calcein-AM, Invitrogen) at 5 µg/ml. Five epifluorescence images were captured using a 20× objective on an Olympus IX-80 microscope in 5 separate wells/experimental condition. The fluorescence levels obtained in each image were quantified using SLIDEBOOK™ software (Intelligent Imaging Innovations) and averaged for the 5 wells/condition. We assessed inducible cytochrome P450 activity as measured by breakdown of resorufin derivatives per established method [19] and found positive activity in all groups (data not shown). The insulin sensitivity was investigated via insulin withdrawal followed by induction as follows: the standard hepatocyte medium, which contains high levels of insulin (5 U/ml, Sigma-Aldrich), was removed and the cultures were washed 3 times in PBS to remove any insulin residue. Insulin-free basal DMEM (Invitrogen) was added to the cells for 4 h. Then, the medium was removed and either fresh insulin-free DMEM or insulin-rich DMEM (5 U/ml, Sigma-Aldrich) was added to the cells for 30 min. Intrahepatocellular protein was extracted and analyzed for phosphorylated AKT (at Thr 308) as well as total AKT by Western blot using the appropriate antibodies (Cell Signaling Technology, Danvers, MA).

Human liver histology

Human liver tissue samples were obtained, stained with hematoxylin and eosin (H&E) and imaged as described elsewhere [13].

Statistical analysis

Results shown in text and figures are means $\pm$ 1 standard error. One-way ANOVA followed by Fisher's LSD *post-hoc* test was performed using KaleidaGraph (Synergy Software, Reading, PA). Values of $p < 0.05$ indicate statistical significance.

Results

Macrosteatosis induction in primary hepatocytes

Incubation of collagen sandwiched hepatocytes with steatosis-inducing medium for 3 days (corresponding to 8 days post-seeding) induced a microsteatotic appearance and increased the TG content ~2.7-fold; consistent with prior studies [9,15] (Fig. 1B and C). Incubation with steatosis-inducing medium for an additional 3 days increased the TG content even further, reaching ~5-fold the level in lean controls (Fig. 1B and C). This level is consistent with two *in vivo* models of liver macrosteatosis (*ob/ob* mouse and obese Zucker rat) where the intrahepatic TG level is also ~5-fold the corresponding lean controls [7,20]. The additional 3 days of steatosis induction also led to a dramatic increase in the lipid droplet size distribution (Fig. 1C). The number of lipid macrodroplets (cross-sectional surface area $> 350 \mu\text{m}^2$, defining a macrosteatotic lipid droplet (Supplementary Fig. 4)) increased 20-fold compared to the 3-day fatty cells (Fig. 1D). The 6-day fatty cells exhibited morphological characteristics found in macrosteatotic human livers, notably large intracellular lipid droplets displacing the nucleus to the cell periphery, as shown in Fig. 1B and E [1]. In addition, lipid droplet cross sectional surface area was comparable to that in macrosteatotic human livers (Fig. 1B and E). Hepatocyte viability was 85–90% after the 3-day and 6-day fattening protocols and was not significantly different from lean controls (Table 1A).

Reduction of macrosteatosis in primary hepatocytes

Using the 6-day macrosteatotic hepatocytes, the effect of macrosteatosis reduction on viability and liver-specific function was assessed. To accelerate macrosteatosis reduction, SRS, previously shown to accelerate steatosis reduction in microsteatotic cultured hepatocytes, were utilized [15]. NSRS or SRS-containing medium was added to the macrosteatotic hepatocytes for 2 days. When SRS medium was used, macrosteatosis reduction was accelerated ~4-fold compared to NSRS medium to yield ~80% reduction within 2 days (Fig. 2A and B). Interestingly, both media compositions were equally effective in reducing TG content by ~30% during that time frame (Fig. 2C).

The size of individual lipid droplets in macrosteatotic hepatocyte cultures during the first 36 h macrosteatosis reduction period decreased linearly as a function of time, while the nucleus returned towards the center of the hepatocyte (Supplementary Fig. 5A and B).

After 2 days in NSRS or SRS medium, all cells were switched to NSRS medium for an additional 2 days (days 13–15 post-seeding). At the end of this observation period, regardless of the medium used to reduce macrosteatosis for the first 2 days, the hepatocytes exhibited steatosis levels comparable to control lean hepatocyte cultures (Fig. 2D). Thus, while macrosteatotic hepatocytes can eventually exhibit lean-like lipid droplet distribution, SRS medium provides accelerated steatosis reduction during the first few hours.

Viability and function assessment

As indicated in Fig. 3A, macrosteatosis reduction was uneven among the hepatocytes. Viability assessment revealed that reduction occurred only in the viable population (EtHD-1 negative), suggesting that active metabolism was critical. Nevertheless, during the first 2 macrosteatosis reduction days and the subsequent 2 days in NSRS medium, both SRS and

NSRS medium treatments maintained hepatocyte viability similar to lean controls on the same experimental day (Table 1B).

Next, cell function was assessed with liver-specific hepatocyte markers used to assess the function of hepatocyte cultures and livers in transplantation settings including albumin and urea secretion rates (Fig. 3B–D), as well as bile canaliculi morphology and function (Fig. 4) [10,11,13,14,17,19,21]. Microsteatotic hepatocytes maintained the albumin secretion rate and exhibited only a 1.7-fold decrease in urea secretion rates compared to lean hepatocyte cultures. On the other hand, macrosteatotic hepatocytes exhibited ~1.5-fold lower albumin secretion levels and a 5-fold reduction in urea secretion levels compared to lean hepatocyte cultures (Fig. 3B and C). Thus, macrosteatosis induction for 6 days had a more deleterious effect on hepatocyte function than microsteatosis induction for 3 days.

In macrosteatosis induction and reversal cycled hepatocytes, albumin secretion remained flat until the end of the observation time (day 15 post-seeding) below lean hepatocyte control levels and did not recover. In this respect, macrosteatotic reduced hepatocytes, treated with SRS or NSRS, behaved similarly (Fig. 3B). On the other hand, the urea secretion rate at the end of the observation period returned to ~50% of the lean hepatocyte culture levels in hepatocytes initially treated with NSRS and to ~100% in hepatocytes initially treated with SRS medium (Fig. 3C). During the last 2 days, all the cultures received NSRS medium. Therefore, the improved urea secretion recovery rate in hepatocytes initially treated with SRS medium at 15 days post-seeding can be attributed to the long-term effects of SRS treatment, such as the improved macrosteatosis reduction (Fig. 3C).

In lean hepatocyte cultures, bile canaliculi and the accumulation of fluorescent carboxy-DCFDA were easily visualized, suggesting a functional bile canaliculi network (Fig. 4A and B), consistent with similar studies [19]. In macrosteatotic hepatocytes, neither fluorescent carboxy-DCFDA nor the bile canaliculi structure was observed (Fig. 4A and B). Because carboxy-DCFDA requires cleavage by intracellular esterases to fluoresce and eventually be transported into the bile, we investigated whether esterase activity was affected by macrosteatosis. Similar cultures were incubated with calcein-AM, which requires the same esterases to fluorescently label the cytoplasm. Calcein staining was similar in lean and macrosteatotic hepatocytes, suggesting no impairment in esterase activity (Fig. 4C). Thus, the lack of carboxy-DCFDA accumulation in macrosteatotic hepatocytes most likely reflects a disruption of the bile canaliculi function.

Immediately following macrosteatosis reduction (13 days post-seeding), the bile canaliculi morphology recovered completely to levels similar to lean controls, but only in hepatocytes treated with SRS medium (Fig. 4A). Bile canaliculi function partially recovered after macrosteatosis reduction using NSRS medium, while it appeared nearly complete and comparable to lean controls after using the SRS medium (15 days post-seeding) (Fig. 4B). Similarly, the recovery in bile canaliculi function was not attributed to levels of esterase activity (Fig. 4D).

To explore the insulin sensitivity of the hepatocyte cultures, the response to insulin withdrawal followed by induction was investigated. A small increase in phosphorylated AKT could be seen after return to insulin-rich medium in both lean and macrosteatotic cultures; however, the high baseline in phosphorylated AKT limited the ability to study insulin responsiveness in this model (Supplementary Fig. 3).

Discussion

We characterized a novel *in vitro* primary rat macrosteatotic hepatocyte system whereby intracellular TG accumulation and macrosteatotic lipid droplet formation were induced by incubation in FFA-rich medium. Macrosteatosis induction did not decrease hepatocyte viability, but significantly decreased urea secretion, disrupted the bile canalicular network, and maintained albumin secretion rates below lean controls. Macrosteatosis was morphologically reversed by switching hepatocytes back to NSRS medium within 4 days. Comparable macrosteatosis reduction was achieved in only 2 days with SRS medium. Hepatocyte viability remained high and did not depend on the macrosteatosis reduction rate. Macrosteatosis reduction led to the recovery of liver-specific functions at different rates. Using the SRS medium, urea secretion and the bile canalicular network fully recovered, while albumin secretion rate remained flat and below control lean hepatocyte levels during the macrosteatosis reduction experimental 4-day timeframe. Since some aspects of hepatocyte functional recovery were improved by accelerated macrosteatosis reduction, this recovery may be dependent upon macrosteatosis reduction time and/or may be sensitive to the composition of the supplements and their target pathways. We also found that albumin secretion does not fully recover even if we extend culture time (data not shown). It is possible that the extent of defatting is not sufficiently complete to recover this particular function (since the data show that triglyceride content was still ~68% of macrosteatotic levels, thus remaining well above the “lean” baseline). In contrast, macrosteatosis had no effect on levels of hepatocyte nuclear factor 1- α (data not shown), a transcription factor involved in the expression of many liver-specific genes, arguing against a de-differentiation of the cells due to excessive fat accumulation [22].

Our data show that hepatocytes induced for macrosteatosis after 6 days exhibited lower albumin and urea secretion rates than those induced for microsteatosis after 3 days. This may be due to the direct effect of the increased lipid storage on these functions or to the difference in exposure times to FFA-rich medium. It is worth noting that in the clinic, albumin and urea blood data suggest that macrosteatotic patients are asymptomatic, although they exhibit elevated liver enzymes [23]. These tests, however, do not equate the hepatocyte production rates, as in our system, since blood levels are also affected by clearance mechanisms.

Our study indicates that macrosteatosis significantly decreased bile canalicular function, consistent with observations made post 70% partial hepatectomy in a CMDD rat macrosteatotic liver model, where the recovery of the bile canalicular network was significantly delayed [17]. During recovery, the animals were given a normal diet, which is expected to reduce the steatosis level to that of lean animals [9,17]. In addition, steatotic rat liver perfusion studies indicate an increased bile secretion rate post-steatosis reduction [15,16]. Therefore, the steatosis reduction process could have contributed to some aspects of functional recovery of macrosteatotic hepatocytes.

Interestingly, the addition of SRS significantly reduced lipid droplet size compared to NSRS, but TG content was reduced to the same level using either medium composition. This contrasts with prior studies using microsteatotic hepatocyte cultures, where SRS promoted a higher degree of TG reduction compared to NSRS medium [15]. The macrosteatotic culture system, which contains 2-fold higher TG levels compared to the microsteatotic system, may have impaired the ability to eliminate lipolysis products from the cytoplasm, allowing their re-esterification to TG stored in small lipid droplets, which may not be detectable using the Nile red stain method [15,24]. If this is the case, further improvement in TG removal may be possible by preventing TG re-esterification, which

would also help to elucidate whether the redistribution of TG from macro- to micro-droplets, or its removal from the cell altogether, is required [4].

Our study suggests that accelerated macrosteatosis reduction strategies may be used and consequently recover some liver-specific function. The macrosteatosis reduction time scale was ~48 h to reduce the number of macrodroplets by ~80%. In order to clinically translate this approach to liver grafts, this would need to be accomplished in a few hours [2,4,25]. Interestingly, one study showed that *ex vivo* perfusion of obese Zucker rat steatotic liver with a similar cocktail could achieve a significant reduction in lipid droplet size as well as 50% TG reduction within 3 h [15]. Thus, it is possible that faster dynamics occur in perfused livers, possibly due to the flow conditions, which likely enhance nutrient and waste transport between cells and the bathing medium [4]. Further improvements to the macrosteatotic hepatocyte culture model could include introducing flow, thus enabling a more rigorous analysis of the combined effects of lipid reduction agents and flow parameters on the macrosteatosis reduction process [4].

In summary, we have established a model system in which macrosteatosis can be induced by culturing hepatocytes in FFA-rich medium. This model is suitable to explore the effect of macrosteatosis reduction approaches on lipid droplet size as well as hepatocyte viability and liver-specific function. Macrosteatotic hepatocyte cultures maintain high viability but decreased function. Accelerated macrosteatosis reduction does not adversely affect viability and accelerates some functional restoration. This system may be useful to improve our understanding of the effect of macrosteatosis on lipid metabolism and storage, liver-specific functions and hepatocellular response to stresses, such as I/R. This model may also serve as platform to screen and test various lipid metabolism promoting agents that could be eventually used to reduce macrosteatosis in human livers, possibly in conjunction with other methods, such as I/R preconditioning and/or anti-oxidant-based methods to expand the liver donor pool [4,6,8,16].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TG	triglycerides
I/R	ischemia/reperfusion
CMDD	choline and methionine-deficient diet
PBS	phosphate buffered saline
NSRS	no steatosis reduction supplements
SRS	steatosis reduction supplements

EthD-1	ethidium homodimer-1
carboxy-DCFDA	5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate
calcein-AM	calcein acetoxymethylester
H&E	hematoxylin and eosin
FFA	free fatty acid

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Fig. 1. Macrosteatosis induction in primary rat hepatocytes

(A) Experimental timeline. (B) Hepatocyte morphology post-FFA-supplemented culture. Bright-field (top) and fluorescent images of Nile red and Hoechst stained hepatocytes (bottom) for lean (day 8), microsteatotic (day 8), and macrosteatotic hepatocytes (day 11). White arrows = lipid droplets and cross-sectional areas. Red arrows = hepatocyte nuclei. (C and D) Intracellular TG content and macrosteatotic ($>350 \mu\text{m}^2$) droplet number as a function of steatosis. Means $\pm$ S.E. N = 6. * $p < 0.002$ vs. D8 Lean, + $p < 0.007$ vs. D8 Fat. (E) H&E-stained human steatotic liver. Arrows, macrovesicular lipid droplets and cross-sectional surface areas. Bars, 50 μ m.

Fig. 2. Hepatocyte morphology and lipid content during macrosteatosis reduction

Macrosteatotic hepatocytes cultures were supplemented (SRS) or not (NSRS) with steatosis reducing agents for 2 days. (A) Bright-field (top) and fluorescent images of Nile red and Hoechst stained macrosteatotic hepatocytes (bottom) after 2 days of culture. (B and C) Macrosteatotic ($>350 \mu\text{m}^2$) droplet number and intracellular TG content for different steatosis reduction media. Means $\pm$ S.E. N = 6. * $p < 0.01$ vs. D13 Lean. + $p < 0.03$ vs. D11 Fat. (D) Bright-field images of initially SRS or NSRS treated macrosteatotic hepatocytes after 2 additional days of culture in NSRS medium compared to lean controls. Bar, $50 \mu\text{m}$.

Fig. 3. Effect of steatosis induction and reversal on hepatocyte viability, albumin and urea secretion

Macrosteatotic hepatocytes were cultured in steatosis reducing medium until day 13 and then in standard medium until day 15. (A) Hepatocyte viability at the end of the steatosis reduction period (15 days post-seeding). Note that nonviable hepatocytes (EtHD-1⁺) did not reduce steatosis. White arrows, EtHD-1⁺ cells. Bar, 50 μm . (B and C) Albumin and urea secretion rates normalized to viable cell number. Means $\pm$ S.E. N = 6. * p < 0.0001 vs. day 5 lean and ⁺ p < 0.01 vs. lean at the same time point.

Fig. 4. Effect of steatosis induction and reversal on bile canaliculi morphology and function
 (A) Bile canaliculi structures visualized by phase contrast appear as bright white hepatocyte borders (white arrows). (B) Bright-field (top) and fluorescent images (bottom) of hepatocytes stained with green carboxy-DCFDA, which accumulates in functional bile canaliculi, and blue Hoechst, which stains nuclei. Bar = 50 μ m. (C and D) Esterase activity, assessed by the accumulation of calcein in calcein-AM-incubated cells, is not significantly different between lean and macrosteatotic hepatocytes. Data shown are means $\pm$ S.E. N = 5.

Table 1**Hepatocyte viability**

Percent viable hepatocytes in various experimental groups at the relevant time points (based on images obtained from the EtHD-1 viability assay; sample viability staining is shown in Supplementary Fig. 2). Means $\pm$ S.E. N = 5. There are no statistically significant differences between treated hepatocytes and corresponding lean controls on the same experimental day.

A		
	Day 8	Day 11
Lean hepatocytes	89.3% $\pm$ 1.0	88.5% $\pm$ 0.8
Steatosis induced hepatocytes	85.4% $\pm$ 1.8	86.4% $\pm$ 2.0
B		
	Day 13	Day 15
Lean hepatocytes	88.6% $\pm$ 1.2	85.6% $\pm$ 2.9
Macrosteatosis hepatocytes + NSRS	82.0% $\pm$ 2.1	76.2% $\pm$ 2.5
Macrosteatosis hepatocytes + SRS	83.5% $\pm$ 1.9	74.0% $\pm$ 3.8