Published in final edited form as:

J Hepatol. 2013 October ; 59(4): 731–737. doi:10.1016/j.jhep.2013.05.026.

Impaired liver regeneration in *Ldlr*^{-/-} mice is associated with an altered hepatic profile of cytokines, growth factors and lipids

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Abstract

Background & Aims—It is widely recognized that in the early stages of liver regeneration after partial hepatectomy the hepatocytes accumulate a significant amount of lipids. The functional meaning of this transient steatosis and its effect on hepatocellular proliferation are not well defined. In addition, the basic mechanisms of this lipid accumulation are not well understood although some studies suggest the participation of the Low Density Lipoprotein Receptor (*Ldlr*).

Methods—To address these questions we studied the process of liver regeneration in *Ldlr* null mice and wild-type mice following 75% partial hepatectomy.

Results—*Ldlr* deficiency was associated with a significant decrease in serum albumin concentration, during early stages of liver regeneration, and a delayed hepatic regeneration. Remnant livers of *Ldlr*^{-/-} showed a time-shifted expression of interleukin-6 (IL-6) and a defective activation of tumor necrosis factor-α (TNFα) and hepatocyte growth factor (HGF) expression in

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Conflict of interest: The authors declared that they do not have anything to disclose.

early phases of liver regeneration. Unexpectedly, *Ldlr*^{-/-} showed no significant differences in the content of lipid droplets after partial hepatectomy compared to wild-type mice. However, lipidomic analysis of the regenerating liver from *Ldlr*^{-/-} revealed a lipid profile compatible with liver quiescence: high content of cholesterol esters and ceramide, and low levels of phosphatidylcholine.

Conclusion—Ldlr deficiency is associated with significant changes in the hepatic lipidome that affect cytokine-growth factor signaling and impair liver regeneration. These results suggest that the analysis of the hepatic lipidome may help to predict the success of liver regeneration in the clinical environment, specifically in the context of pre-existing liver steatosis.

Keywords

partial hepatectomy; lipidomics; low-density lipoprotein receptor; liver regeneration; steatosis

INTRODUCTION

The mammalian liver is characterized by its regenerative capacity, which is induced after the loss of parenchymal mass. This regeneration process allows the maintenance of the metabolic homeostasis and, eventually, restores the original hepatic mass [1,2]. The use of the rodent partial hepatectomy (PH) model described originally by Higgins and Anderson [3] has resulted in extensive publications describing the sequential steps leading to liver regeneration. The first step is a priming phase induced by cytokines such as IL-6 and TNF α , which is responsible of priming the quiescent hepatocytes for G0 to G1 transition into the cell cycle. The following step is a proliferative phase in which the coordination of growth factors, transcription factors and metabolic signaling pathways trigger cell division in hepatocytes (approximately 24 h post-hepatectomy) and in non-parenchymal cells shortly after hepatocytes proliferation (36 to 96 h post-hepatectomy) [1,2]. The expression of specific miRNAs (miR-21 and miR-221) [4, 5] and changes in the flow of portal blood per unit liver mass [6] have also been described as necessary in the proliferative phase. Finally, after the liver achieves its original hepatocellular mass, there is a termination phase where cell proliferation is repressed. Although this termination step is poorly understood, several studies have described that transforming growth factor- β (TGF- β) [7], extracellular matrix-mediated signaling via ILK [8] and miR-33 [9] are important candidates for switching off the regenerative response.

Besides the steps described above, it is widely recognized that in the early stages of liver regeneration hepatocytes accumulate a significant amount of lipids in cellular structures called lipid droplets, which are mainly composed of triacylglycerol and cholesteryl esters [10, 11]. The functional meaning of this transient steatosis is not well defined as there are conflicting studies on how alteration of the hepatic lipid contents affects the proliferative response [12–17]. Another point that is not well understood and that needs further investigation is the basic mechanisms through which the hepatocyte accumulate lipids during liver regeneration. There is some evidence suggesting that the transient steatosis is likely due to both an increase of lipogenic activity and altered lipid transport balance into and out of the hepatocyte. In the context of lipid uptake during liver regeneration, some studies suggest the participation of lipid receptors [18–20]. For instance, the hypolipidemia

observed in serum samples from partial hepatectomized rats correlates with the increase in hepatic triglyceride content and supports the idea that lipid transport is affected [18]. In addition, the Ldlr has been shown to be overexpressed in liver after PH [19]. Furthermore, the expression of the *Ldlr* gene is induced as a part of an early growth response in Hep-G2 cells [20]. All these observations suggest a possible involvement of the Ldlr in regulating hepatocyte lipid accumulation and liver regeneration.

The present study was designed in order to address these unanswered questions. Particularly, we hypothesize that *Ldlr* gene deficiency impairs liver regeneration by affecting the hepatic lipidome in hepatectomized mice. To test our hypothesis we studied the hepatic regenerative process in hepatectomized *Ldlr*^{-/-} mice. These mice displayed a 50% increase in hepatic cholesterol levels in the absence of pathological stimuli and dietary manipulation [21]. Therefore, the *Ldlr*^{-/-} gene deficiency is also a good model to study changes occurring in liver regeneration after PH in the context of hepatic hypercholesterolemia.

MATERIALS AND METHODS

Experimental mice

Experiments were performed in seven-old-week male *Ldlr*^{-/-} and WT mice with the C57BL/6 genetic background (Jackson Laboratory, Sacramento, CA, USA). Mice were fed *ad libitum* on a standard pellet diet. All animal experiments were performed with the approval of the Institutional Animal Care Use Committee of New York University Medical Center and the Investigation and Ethics Committees of the Hospital Clinic.

Surgical Procedure

PH was performed according to the technique described previously [3]. Two thirds of the liver (median and left lobes) were removed. After PH, wt mice (n=36) and *Ldlr*^{-/-} mice (n=32) were sacrificed at different time points; 0, 3, 8, 24, 48, 72 and 120 h. Regenerating bottom right lobe was snap-frozen into liquid nitrogen and the upper right lobe was fixed in 4% paraformaldehyde (PFA) at 4°C, cryoprotected overnight in 30% sucrose solution and embedded in OCT medium (Tissue-Tek® O.C.T™ Compound, SAKURA) and frozen for future processing. The percentage of liver regeneration was calculated following the formula: weight of non-removed lobes/Total body weight of mice.

Lipid analysis

Livers (50 mg) were homogenized in 500 µL of PBS, and lipids were extracted from 100 µL of the homogenate in the presence of internal standards for each lipid class [22,23]. The different lipid classes (phospholipids, triglycerides, cholesterol esters and ceramides) were quantified from the chloroform extracts using shotgun lipidomics based on class separation by MS/MS specific methods [24].

Other methods and the statistical analysis description are shown in Supplemental Information.

RESULTS

***Ldlr*^{-/-} mice exhibit hepatic steatosis and elevated levels of circulating cholesterol under basal physiological conditions**

Wild-type and *Ldlr*^{-/-} mice were fed standard diet and mean changes in body weight did not differ significantly among the two experimental groups (Fig. 1A). In addition, gross examination of the livers from wild-type and *Ldlr*^{-/-} mice showed not significant changes in morphology or weight (data not shown). However, the microscopic examination of both hematoxylin/eosin (H&E) and Oil Red O-stained sections from *Ldlr*^{-/-} livers revealed microvesicular steatosis with a non-zonal pattern distribution of lipid droplets, compared with wild-type livers (Fig. 1B). *Ldlr*^{-/-} knockout mice also showed a significant 4-fold increase in serum total cholesterol levels compared to wild-type animals at time 0 h (Fig. 1C).

***Ldlr*^{-/-} mice displayed impaired liver regeneration and worsening of hepatic dysfunction following PH**

To study the role of the *Ldlr* during liver regeneration, we performed 2/3 PH in wild-type and *Ldlr*^{-/-} mice. Mice were sacrificed 3h, 8h, 24h 48h 72h and 120 h after the surgical procedure and wet liver remnant weight, together with the total body weight, was used to calculate the hepatic regenerative rate. In wild-type mice the liver to body weight ratios augmented from 1.5% at 3 h to 3.7% at 120 h after PH (Fig. 2A). In contrast, this upward trend in hepatic remnant weight was only partially reproduced in the *Ldlr*^{-/-} mice. Specifically, *Ldlr* gene deficiency was associated with a delayed regenerative response that resulted in impaired liver regeneration from 48 h to 120 h, compared to wild-type mice. The body weights of *Ldlr*^{-/-} mice plotted in figure 2B reflected to a similar extent the impaired hepatic regenerative rate found in the context of the *Ldlr* gene deficiency. To examine whether the delayed liver regeneration observed in the *Ldlr*^{-/-} mice was associated with further impairment in liver function we measured the serum levels of albumin. Immediately after PH, serum albumin content declined in both wild-type and *Ldlr*^{-/-} mice and remained low 48 h after the surgical procedure. This deficiency was more pronounced in the *Ldlr*^{-/-} mice at 3 and 8 h post hepatectomy, compared with the wild-type condition. At these time points, the differences between both experimental groups were statistically significant.

***Ldlr* gene deficiency impairs hepatocellular cell cycle progression and the expression of IL-6, TNF α and HGF during liver regeneration**

To further investigate the association between *Ldlr* deficiency and impaired liver regeneration, we studied the expression of cell cycle markers (Ki-67, cyclin B1, cyclin D1 and PCNA) in hepatectomized wild-type and *Ldlr*^{-/-} mice. Wild-type mice showed a small but significant population of Ki-67 positive cells at 24 h post-hepatectomy (2% per total nuclei), compared with the pre-hepatectomy time point. This expression peaked at 48 h (50% of Ki-67 positive cells per total nuclei) and thereafter declined markedly (Fig. 3A and Supplemental Figure 1A). In contrast, positive immunostaining for Ki-67 in *Ldlr*^{-/-} mice was barely detectable at 24 h after PH and was significantly reduced by 50% at 48 h post-hepatectomy, compared with that in wild-type mice at the same time point. After 72 hours post-hepatectomy, neither wild-type nor *Ldlr*^{-/-} mice showed significant immunostaining

for Ki-67 (data not shown). Next, the expression of cyclin B1, cyclin D1 and PCNA was analyzed by western blot (Supplemental figure 1B). The livers of wild-type mice showed an increasingly expression of cyclin B1 and cyclin D1 at 24 hours post-hepatectomy. In contrast, the expression of these cell cycle markers in *Ldlr*^{-/-} mice was detected at later stages (72 hours post-hepatectomy) and was lower than the values found in wild-type mice. The expression of PCNA was also significantly reduced in *Ldlr*^{-/-} mice. However, this differential expression of PCNA was evident only at the early stages of liver regeneration.

To understand the mechanism behind the impaired hepatocyte proliferation occurring in *Ldlr*^{-/-} mice, we quantified the hepatic mRNA expression of IL-6, TNF α , HGF and miR-33 in both wild-type and *Ldlr*^{-/-} mice before and at different time points after PH. Before PH, *Ldlr* gene deficiency was associated with a significant repression of the IL-6 gene compared with wild-type mice (0.57 ± 0.03 IL-6 mRNA fold-change in *Ldlr*^{-/-} mice versus 1.00 ± 0.04 IL-6 mRNA in wild-type mice, $P < 0.05$). After PH, we observed a time-shifted expression of IL-6, compared with wild-type mice (Fig. 3B, left panel). In addition, *Ldlr*^{-/-} mice showed a defective activation of TNF α expression in early phases of liver regeneration, compared to wild-type mice (Fig. 3B, middle panel). No significant differences in TNF α expression values were found among the two experimental groups before performing PH. HGF has previously been described as an irreplaceable mitogen for hepatocyte proliferation [25–27]. In agreement with these publications, we found elevated levels of HGF in the 3–8 h liver samples from partially hepatectomized wild-type mice, compared with the pre-hepatectomy samples. In contrast, the basal expression levels of HGF in the liver of *Ldlr*^{-/-} mice did not differ significantly to those found after performing the PH procedure in the same experimental group (Fig. 3B, right panel). We previously described the role of miR-33 in regulating cholesterol homeostasis, fatty acid metabolism and hepatocyte proliferation [28, 9]. Therefore, we assessed the expression of this microRNA in *Ldlr*^{-/-} mice in basal conditions and during liver regeneration. As seen in Supplemental figure 1C, the levels of miR-33 in *Ldlr*^{-/-} knockout mice were decreased by more than 90% compared with wild-type mice at the same time points. These differences were maintained in basal conditions and during liver regeneration.

***Ldlr* gene deficiency does not interfere with lipid droplet formation after PH but is associated with significant changes in the hepatic lipidome**

To explore whether *Ldlr*^{-/-} gene deficiency affects lipid uptake and/or lipid metabolism during liver regeneration we examined the lipidomic profile of livers from wild-type and *Ldlr*^{-/-} mice at different time points following PH. As shown in figure 4, the hepatic lipidome differed significantly between the wild-type and the *Ldlr*^{-/-} mice before and after the PH procedure. Before PH, the major differences found in the liver of *Ldlr*^{-/-} mice were the higher levels of cholesterol esters (2.5-fold increase) and the lower concentration of phosphatidylethanolamine (1.7-fold decrease) and ceramides (2.5-fold decrease), compared with the hepatic lipidome of wild-type mice. During the regenerative process, the intracellular concentration of cholesterol esters in wild-type mice reached a maximum at 48 h after PH and then returned to basal levels. By contrast, the concentration of cholesterol esters remained elevated in the *Ldlr*^{-/-} mice without significant changes throughout the hepatic regeneration. We also detected significant differences in the levels of sphingomyelin

and ceramide after PH. Hence, *Ldlr*^{-/-} mice showed a concentration of hepatic sphingomyelin significantly higher than wild-type at 3 h after PH. In addition, ceramide levels increased significantly after two days in the *Ldlr*^{-/-} mice, compared with wild-type mice where the kinetics of hepatic ceramide content followed a decreasing pattern as the hepatic regeneration progresses. To test the possibility that ceramide content may contribute to cell death in *Ldlr*^{-/-} mice during liver regeneration, we performed TUNEL assay in wild-type and in *Ldlr*^{-/-} mice before PH and at 48 h post-PH, which is when the differences in hepatic ceramide content started to be statistically significant among the two groups. Few TUNEL-positive cells were observed before PH and at 48 h after PH. No statistical differences were found among the two experimental groups (supplemental figure 2).

Another major component of membranes whose concentration was diminished in the liver of *Ldlr*^{-/-} animals during the regenerative process was phosphatidylcholine. In this context, our results showed that the phospholipid profile of the *Ldlr*^{-/-} mice did not change uniformly. For instance, phosphatidylserine was significantly increased in *Ldlr*^{-/-} mice at 8 h and 24 h post-hepatectomy. In contrast to the aforementioned lipid changes, *Ldlr*^{-/-} mice did not showed significant differences in the hepatic levels of triglycerides and free fatty acids before or after PH. Considering what has previously been published regarding liver regeneration [10], the hepatic lipid profile of *Ldlr*^{-/-} mice after PH is compatible with hepatocellular quiescence.

Despite these differences in lipid profiles, hepatectomized wild-type and *Ldlr*^{-/-} mice showed equally spatial and temporal distribution of lipid droplets with no significant differences in their amount (see supplemental results and supplemental figure 3).

DISCUSSION

The most salient results of this study are that *Ldlr* deficiency in mice was associated with: 1) an impaired liver biosynthetic capacity during early stages of liver regeneration, reflected in the decrease in serum albumin concentration; and 2) a delayed volumetric liver regeneration characterized by a temporal shift in the expression of cell cycle markers and impaired hepatocellular proliferation. In general, the functional implications of a delay in hepatic regeneration are not clear. However, it is foreseeable that in the clinical context of impaired liver function, as in the case of patients with hepatocellular carcinoma, associated cirrhosis and pre-existing liver steatosis; a delay in the onset of hepatic regeneration after surgical resection may be associated with increased morbidity and mortality caused by a further decrease of the liver function.

It has been demonstrated that both TNF α and IL-6 act synergistically to prime the G0 to late G1-phase progression into the cell cycle of hepatocytes [29]. Here we showed that *Ldlr* gene deficiency was associated with an impaired overexpression of both IL-6 and TNF α during the early phases of liver regeneration after PH. These abnormalities in the cytokine network may favor the quiescence of hepatocytes in the remnant liver regardless the concentration of growth factors. In this context, after G0 to late G1-phase transition stimulated by cytokines, growth factors function as mitogens for hepatocytes in the remnant liver [1,2]. One irreplaceable mitogen for hepatocyte proliferation is HGF. Conditional hepatic deletion of

the HGF receptor cMet [25, 26] or RNA interference against HGF or cMet in the context of PH [27] results in impaired liver regeneration and high mortality. In our study, the basal expression levels of HGF in *Ldlr*^{-/-} mice did not change significantly after PH, in contrast to the described enhanced HGF expression that we observed in hepatectomized wild-type mice after PH. This interesting finding reinforces the concept that *Ldlr* gene deficiency interferes with the cytokine/growth factor network that regulates hepatocyte proliferation after PH. Another regulator of cell cycle progression and liver regeneration is miR-33 [9]. We previously showed that the inhibition of miR-33 with antisense oligonucleotides significantly increased the liver-to-body mass ratio in hepatectomized wild-type mice. However, in the context of *Ldlr* deficiency the reduced levels of miR-33 expression were not associated with cell proliferation. The explanation for this discrepancy is beyond the scope of this study. However, these results point to the idea that the comprehensive understanding of the hepatic regeneration process requires a more holistic approach that should consider the crosstalk between the signaling pathways that take place during liver regeneration.

It is widely recognized that in the early stages of liver regeneration there is a transient steatosis characterized by the formation of hepatocellular lipid droplets, which are lipid structures enriched in cholesterol esters and triglycerides. However, there is disagreement concerning whether the experimental manipulation of the hepatic lipidome affects the proliferative response in the remnant liver. For example, DeAngelis *et al.* showed that a high-fat diet impairs liver regeneration in mice after PH [30]. These results are in agreement with early studies performed in mice models of steatosis with either ob/ob [14, 19] or db/db [31] gene deficiency. In these animals, an impairment in hepatic regeneration associated with the lack of leptin activity was shown. These results contrast with the observation that liver regeneration is normal or only slightly delayed in other models of diet-induced steatosis, such as the methionine-choline-deficient [15, 17] or the orotic-acid-supplemented diets [14]. Furthermore, a 50% reduction in hepatic triglyceride content, caused by leptin treatment prior PH, was associated with an impairment of hepatocellular proliferation [12]. In contrast, Newberry *et al.* [13] demonstrated that several knockout experimental models with altered lipid metabolism (L-Fabp, MTP, PPAR-alpha and FAS gene deficiency) showed altered hepatic triglyceride content and similar hepatic cholesterol levels without impaired liver regeneration. A plausible explanation for these discrepancies is that different experimental models are associated with different hepatic lipidome composition, which could result in different regenerative phenotypes.

In our study we showed that *Ldlr* deficiency is associated with significant differences in the lipid profile of regenerating liver that are compatible with hepatocellular quiescence. The most relevant difference was the higher levels of cholesterol esters found in the liver of *Ldlr*^{-/-} mice in basal conditions and during the whole regenerative process, compared with wild-type animals. In wild-type mice the intracellular concentration of cholesterol esters reaches a maximum at 48 h after PH and then returns to basal levels. The most plausible functional explanation for this kinetics is that after 48 h post-PH cholesterol esters are metabolized to free cholesterol to allow the formation of new membranes. By contrast, the concentration of cholesterol esters remained elevated in the *Ldlr*^{-/-} mice without significant changes throughout the regenerative process. These results, together with the observation

that high concentration of cholesterol esters are abundant in lipid droplets of quiescent livers, compared with regenerating livers [10], suggest that high levels of cholesterol may favor quiescence in hepatocytes. Another major component of membranes whose concentration is diminished in the liver of *Ldlr*^{-/-} animals is phosphatidylcholine. As in the case of cholesterol esters, the abundance of phosphatidylcholine has been associated with specific populations of lipid droplets. For instance, lipid droplets from regenerating livers contain a high concentration of phosphatidylcholine, while low concentration is a characteristic of quiescent hepatocytes [10]. We also detected significant differences in the levels of sphingomyelin and ceramides. *Ldlr*^{-/-} mice showed a concentration of hepatic sphingomyelin significantly higher than wild-type at 3 h after PH. In addition, ceramide levels are lower at baseline but increased significantly after two days, compared with wild-type mice. The kinetics of hepatic ceramide abundance observed in wild-type mice showed a significant decrease of ceramide levels as the hepatic regeneration progresses, as expected during hepatic regeneration. In summary, our lipidomic study suggest that the analysis of the hepatic lipidome content before liver resection or in early stages of liver regeneration may predict both the success of tissue regeneration and the recovery of liver function. Further studies must be conducted to determine whether lipidomic analysis is useful in the clinical context.

In our study we also showed that *Ldlr* gene deficiency is not associated with quantitative or kinetic differences in the accumulation of hepatic triglyceride content or in lipid droplets formation after PH. These results allow us to conclude that: 1) these two mechanisms are not sufficient to ensure proper hepatic regeneration and 2) the contribution of *Ldlr* to these two features in regenerating liver is not relevant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support: This work was supported by grants from the MINECO-Plan Nacional de I+D+I (SAF2010-19025 to MMR and SAF2012-35979 to WJ) and AGAUR (2009 SGR 1496) and Instituto de Salud Carlos III (FIS-PI10/02114 to MM). The Fernández-Hernando Lab is supported by grants from National Institutes of Health (R01HL1077953 and R01HL106063). The Baldán Lab is supported by a grant from the NIH (R01HL107794). The Ford lab is supported by grants from the NIH (HL074214, HL098907 and RR019232). R.M.A. is the recipient of an AHA Predoctoral Fellowship (11PRE7240026). M.P. was supported by MICINN (contract number BES-2007-16909). N.R. was supported by Ministerio de Educación, Programa Nacional de Movilidad de Recursos Humanos (I+D+i 2008-2011). CIBERehd is financed by Instituto de Salud Carlos III.

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Figure 1. Effect of Ldlr gene deficiency on body weight and lipid homeostasis under basal physiological conditions

In (A), scatter plot showing that mean changes in total body weight between wild-type (WT) and *Ldlr*^{-/-} mice did not differ significantly. The central horizontal line represents the mean SEM. In (B), liver sections from WT and *Ldlr*^{-/-} mice stained with Oil Red O. Inserts: higher magnification. Original magnification x200, insert x400 (n=5). In (C) serum levels of cholesterol. Zero-h cholesterol levels were quantified from serum samples removed during surgery. *P<0.05, compared with WT mice, (n=3).

Figure 2. Impaired liver regeneration and worsening of liver dysfunction after PH in *Ldlr*^{-/-} mice

In (A), liver to body weight ratio of wild-type (WT) and *Ldlr*^{-/-} mice at the indicated time points after PH, (n=5 at each time point). *P<0.05 for WT vs. *Ldlr*^{-/-}. In (B), mean body weights of WT and *Ldlr*^{-/-} mice before and after PH. Error bars indicate standard errors. *P<0.05, compared with WT mice (n=5). In (C), the levels of albumin were measured in serum of WT and *Ldlr*^{-/-} mice pre-PH and post-PH, (n=5). *P<0.05, compared with WT mice.

Figure 3. *Ldlr* gene deficiency impairs cell cycle progression and the expression of IL-6, TNF α and HGF during liver regeneration

(A), immunostaining for the Ki-67 antigen (red) in liver of wild-type (WT) and *Ldlr*^{-/-} mice before and after PH. Original magnification $\times 100$ (n=4). In (B), the mRNA expression of IL-6, TNF α and HGF in liver tissue was quantified by quantitative RT-PCR. Horizontal line with value=1 denotes the baseline level in wild-type mice before PH, (n=4). *P<0.05 versus *Ldlr*^{-/-}; #P<0.005 against the baseline levels of the same experimental condition.

Figure 4. Hepatectomized *Ldlr*^{-/-} mice exhibit a hepatic lipid profile compatible with liver quiescence

The hepatic content of cholesterol ester (CE), phosphatidylethanolamine (PE), ceramide, sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), triglycerides and free fatty acids (FFA) were quantified in liver tissue of wild-type (WT, white bars) and *Ldlr*^{-/-} (grey bars) mice. Zero-h data were obtained prior PH (n=3 in each group). *P<0.05 versus wild-type mice.