Down-Regulation of the LXR Transcriptome Provides the Requisite Cholesterol Levels to Proliferating Hepatocytes

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Cholesterol homeostasis is critical for cellular proliferation. Liver X receptor (LXR) α and β are the nuclear receptors responsible for regulation of cholesterol metabolism. In physiological conditions, high intracellular cholesterol levels cause increased synthesis of oxysterols, which activate LXR, thus triggering a transcriptional response for cholesterol secretion and catabolism. Here we employed a mouse model of partial hepatectomy (PH) to dissect the molecular pathways connecting cholesterol homeostasis, cellular proliferation, and LXR. First, we show that hepatic cholesterol content increases after PH, whereas the entire LXR transcriptome is down-regulated. Although LXR messenger RNA (mRNA) levels are unmodified, LXR target genes are significantly down-regulated on day 1 after PH and restored to control levels on day 7, when the liver reaches normal size. The inactivation of LXR following PH is related to the reduced oxysterol availability by way of decreased synthesis, and increased sulfation and secretion. On the contrary, cholesterol synthesis is up-regulated, and extracellular matrix remodeling is enhanced. Second, we show that reactivation of LXR by way of a synthetic ligand determines a negative modulation of hepatocyte proliferation. This effect is sustained by the reactivation of hepatic cholesterol catabolic and secretory pathways, coupled with a significant reduction of cholesterol biosynthesis. Our data unveil a previously unrecognized and apparently paradoxical scenario of LXR modulation. During liver regeneration LXR activity is abated in spite of increasing intracellular cholesterol levels. Turning off LXR-transcriptional pathways is crucial to guaranteeing the requisite intracellular cholesterol levels of regenerating hepatocytes. In line with this hypothesis, pharmacological LXR reactivation during PH significantly reduces liver regeneration capacity. (HEPATOLOGY 2010;51:1334-1344.)

Cholesterol is not only the precursor of steroid hormones but also a regulator of embryonic development and cell proliferation, being a key component of the cell membrane.[1,2] Thus, there is a need of coordinate tuning of cholesterol metabolism and lipogenic gene expression during membrane synthesis, cellular differentiation, and proliferation. Proliferating cells satisfy their cholesterol demand by increasing their uptake of exogenous cholesterol[3] through an increase in low-density lipoprotein receptor.[4] When cholesterol synthesis is inhibited by HMG-CoA reductase inhibitor, cell growth is reduced,[5] whereas the subsequent addition of choles-

Abbreviations: ABC, ATP-dependent cassette transporters; Cdc25c, cell division cycle 25 homolog C; CYP7A1, cholesterol 7α-hydroxylase; CYP27A, sterol 27 hydroxylase; CYP46A1, cholesterol 24 hydroxylase; FoxM1b, Forkhead Box m1b; HMGCoA Red, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IL, interleukin; LXR, liver X receptor; MMP, matrix metalloproteinase; PCNA, proliferating cell nuclear antigen; PH, partial hepatectomy; RTqPCR, quantitative real-time PCR; SREBP, sterol regulatory element binding proteins; STAT, signal transducers and activators of transcription; SULT, sulfotransferase.

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terol reverses this effect.[6] Recently, it has been demonstrated that cholesterol starvation resulted in cell cycle arrest specifically at G_2-phase, and this arrest is associated with the inhibition of cyclin-dependent kinase 1 activity. Consequently, it can be inferred that cholesterol acts as a regulator of cell cycle progression.[7,8] Interestingly, the liver is the primary organ controlling lipid homeostasis and it is an organ able to regenerate its entire mass after partial hepatectomy (PH) because the hepatocytes are uniquely able to reenter the cellular cycle. This process consists of several well-orchestrated phases controlled by the complex interplay of cytokines, growth factors, and metabolic status. For regeneration to take place, quiescent hepatocytes must gain proliferative capacity and undergo a transition from G_0 to G_1, a process known as priming. The rapid induction of proliferative factors (up-regulation of proliferation target genes) is accompanied by a complex redesign of the lobule (matrix metalloproteinase 9 [MMP-9] metalloproteinase activation) followed by the reestablishment of normal liver size and renewed quiescence.[9-11]

The liver can be an effective model for studying the relationship between cholesterol metabolism and cell proliferation. Field et al.[12] used the post-PH regenerating rat liver as a model to investigate the effect of cell proliferation on plasma and liver lipid metabolism. During liver regeneration, plasma triglyceride and cholesterol levels significantly decrease, whereas there is a dramatic increase of lipids in the liver. Hepatic cholesterol neosynthesis is induced after cell division begins, when preexisting cholesterol has become insufficient to meet the cellular demand. Knowledge of the transcriptional regulation of cholesterol metabolism during liver regeneration is of critical importance.

Liver X receptors (LXR) α (NR1H3) and β (NR1H2), members of the nuclear receptor family of proteins, are critical for the control of lipid homeostasis and respond to physiological concentrations of sterols.[13] LXRα and β form obligate heterodimers with the retinoid X receptor, and preferentially bind to a DNA hormone response element (termed LXRE) consisting of two hexanucleotides repeats separated by four nucleotides.[14] The identification of oxysterols as the physiological ligands for LXRs promoted these nuclear receptors to sterol sensors that enhance gene transcription involved in regulating cholesterol metabolism.[15] 24S hydroxycholesterol, 25 hydroxycholesterol, and 27 hydroxycholesterol are the *in vivo* ligands for LXR.[16] Oxysterols are synthesized from cholesterol through different enzymes like cholesterol 24 hydroxylase (CYP46A1) and sterol 27 hydrolase (CYP27A1). Sulfation of oxysterols by way of the sulfotransferase SULT2B1 and the subsequent export of their sulfation products from the cell (ABCC1) inactivate the LXR transcriptome.[16-18] In the liver, LXR mediates sterol secretion into bile by controlling the expression of ABCG5 and ABCG8, two members of the ABC superfamily of membrane transporters that reside in the canalicular membrane of the hepatocyte and pump cholesterol into bile.[19] Moreover, the activation of LXR in the liver determines the induction of lipogenic pathways, primarily by way of the up-regulation of sterol regulatory element binding protein 1c (SREBP-1c), acetyl-CoA carboxylase, stearoyl-CoA desaturase, and fatty acid synthase.[20-22] In addition to the metabolic effects of LXR activation, previous studies have shown that specific synthetic ligands for LXRs are able to inhibit cell proliferation[23-25]; moreover, T-cell activation is accompanied by the induction of cholesterol synthesis pathways and the down-regulation of LXR target genes involved in cholesterol catabolism and transport.[17] Meanwhile, T-cell activation and the decrease in cholesterol availability driven by LXR activation negatively affect both proliferation and antigen-stimulated immune response.

Here we show that hepatocyte proliferation induced by PH is accompanied by the suppression of LXR-driven pathways, in spite of increasing intracellular cholesterol levels. This apparent discrepancy appears to be of fundamental importance to satisfy the needs of regenerating hepatocytes for cholesterol. Reactivation of LXR pathways by synthetic ligands hampers the liver regenerative capacity by decreasing the hepatic cholesterol content and by a down-regulation of extracellular matrix remodeling.

Materials and Methods

Mice were hosted under a standard 12/12-hour light/dark cycle and fed standard rodent chow diet and water ad libitum. LXR$\alpha\beta^{-/-}$ were previously generated in the laboratory of Dr. Mangelsdorf (UTSW, Dallas, TX).[26] Ten- and 12-week-old male mice were used in each group of experiments. Mice were treated with GW3965 (Sigma) 20 mg/kg/die or 0.5% methylcellulose (vehicle) by way of

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gavage 2 days before the surgery and every day after PH. PH was done according to the method of Higgins and Anderson[11] under ketamin/xylazine anesthesia. Left lateral and median lobes were completely excised. For the sham-operated controls, an excision was made into the peritoneal cavity, and the liver was exteriorized and put back into the peritoneal cavity followed by closure of the incision. Data were normalized to sham-operated controls at each timepoint after PH. To measure the fraction of the hepatectomy, the liver was excised from each group of mice (n = 4-5) and their weights were compared to the initial total liver mass calculated from the total body weight of each animal.[27] The protocol was approved by the Ethical Committee of our institution.

Zymography. Whole liver proteins were subjected to electrophoresis in a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel supplemented with 0.1% gelatinase B (Sigma-Aldrich). Then the gel was incubated for 1 hour at room temperature in a renaturing buffer (2.5% Triton X-100, 50 mM Tris/HCl pH 7.5). Next the gel was incubated at 37°C for 18-24 hours in a developing buffer (0.15M NaCl, 10 mM $CaCl_2$, 50 mM Tris/HCl, pH 7.5, 0.05% Brij 35). The gel was fixed and stained with 0.5% Coomassie blue in 10% acetic acid, 30% isopropanol. After destaining the gel, gelatinase digestion resulting from MMP-9 activity was detected as a nonstained band.[28]

Liver and Plasma Lipid Content. Liver cholesterol and triglycerides were extracted using the Folch method.[29] Briefly, liver tissue (100 mg) was homogenized in 5 mL of chloroform/methanol 2:1 (v/v) and washed twice with 1 mL of 0.36M $CaCl_2$/methanol. Cholesterol and triglycerides were measured enzymatically from organic phase using a colorimetric Diacron (Grosseto, Italy) kit according to the manufacturer's instructions. For plasma analysis, after its separation from the blood, 10 μL were used to measure cholesterol and triglycerides using the Diacron kit according to the manufacturer's instructions.

Analysis of Oxysterols. 22(R)-hydroxycholesterol, 25-hydroxycholesterol and 25(R),26-hydroxycholesterol ("27"-hydroxycholesterol) were purchased from Steraloids (Newport, RI), 24(S)-hydroxycholesterol and 24(S)-25-epoxycholesterol were purchased from Biomol International (Plymouth Meeting, PA) and the deuterated internal standard (IS) 25-hydroxycholesterol-D_6 was from CDN Isotopes (Point-Claire, Quebec, Canada). Pyridine was from Sigma-Aldrich (St. Louis, MO), *N,O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was from Pierce (Rockford, IL), and all other chemicals were from Carlo Erba Reagenti (Rodano, Milan, Italy). Oxysterols from mouse liver were extracted and purified as described[30] using Strata Si-1 columns (500 mg/6 mL) (Phenomenex, Torrance, CA) in the solid phase extraction step. Dried extracted samples were converted to trimethylsilyl ethers by adding 250 μL pyridine/BSTFA (1:1) and incubating for 30 minutes at 60°C. After drying under a nitrogen flow, samples were resuspended with 50 μL hexane and analyzed by GC-MS using an Agilent 6890/5973 system equipped with the same column and using the same conditions described.[30] Analyses were performed in selected ion monitoring (SIM) mode using the following target ions: *m/z* 173 for 22(R)-hydroxycholesterol, *m/z* 145 for 24(S)-hydroxycholesterol, *m/z* 131 for 25-hydroxycholesterol, *m/z* 129 for both 25(R),26-hydroxycholesterol and 24,25-epoxycholesterol, and *m/z* 137 for the IS (25-hydroxycholesterol-D_6). Different retention times in the column allowed separation of oxysterols with the same target ion.

RNA Extraction. Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. To avoid possible DNA contamination, RNA was treated with DNAase-1 (Ambion, Foster City, CA). RNA purity was checked by spectrophotometer and RNA integrity by examination on agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized retrotranscribing 4 μg of total RNA in a total volume of 100 μL using the High Capacity DNA Archive Kit (Applied Biosystems, Foster City, CA) and following the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction (RTqPCR). RTqPCR primers were designed using Primer Express software. PCR assays were performed in 96-well optical reaction plates using the ABI 7500HT machine (Applied Biosystems). PCR assays were conducted in triplicate wells for each sample. Baseline values of amplification plots were set automatically and threshold values were kept constant to obtain normalized cycle times and linear regression data. The following reaction mixture per well was used: 10 μL Power Syber Green (Applied Biosystems), 2.4 μL primer at the final concentration of 150 nM, 4.6 μL RNase free water, 3 μL cDNA (60 ng). For all experiments the following PCR conditions were used: denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, then at 60°C for 60 seconds. Quantitative normalization of cDNA in each sample was performed using cyclophilin as an internal control. Relative quantification was performed using the $\Delta\Delta C_T$ method. Validated primers for real-time PCR are reported in Supporting Table 1.

Statistical Analysis. Results are expressed as means ± SEM. The comparison of continuous variables among groups was performed with unpaired Student's *t* test or the Mann-Whitney rank sum test when appropriate. The comparison between multiple groups was assessed using

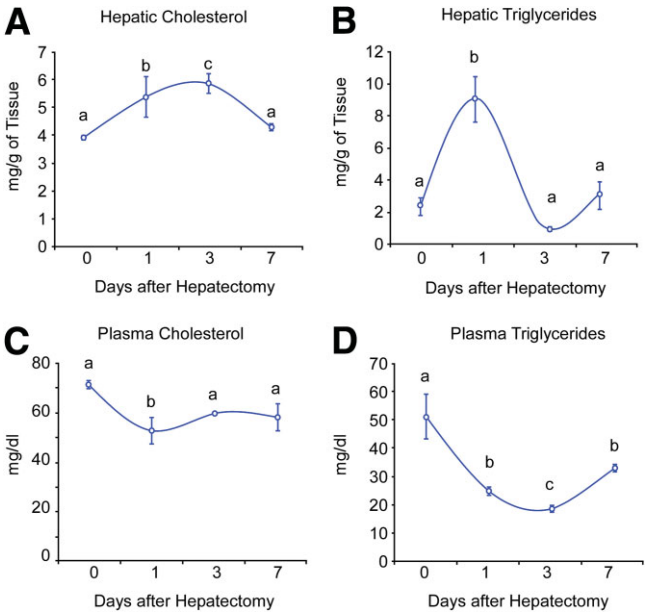

Fig. 1. Hepatic and plasma lipid content during PH-induced liver regeneration in wildtype mice. (A,B) Total cholesterol and triglyceride content of livers from LXR$\alpha\beta^{+/+}$ mice at the indicated timepoints after PH. The data analysis shows a significant increase in total amount of liver lipids. (C,D) Total plasma lipid content from mice at the indicated timepoints after PH. Both cholesterol and triglyceride plasma levels decrease during liver regeneration. The results are shown as mean ± SEM (n = 4-5 at each timepoint). Lowercase letters indicate statistical significance ($P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

one-way analysis of variance (ANOVA) or the Kruskall-Wallis nonparametric ANOVA on ranks. Post-hoc multiple pairwise comparisons were calculated with the Fisher's LSD test. All statistical calculations were performed with the NCSS 2007 software (Kaysville, UT). Statistical tests were conducted as a two-sided alpha level of 0.05.

Detailed methods for liver histology and western blot analysis are reported as Supporting Materials.

Results

Cholesterol and Triglyceride Metabolism During Liver Regeneration. The first step in our study was to monitor lipid metabolism after PH in mice. We found significant changes in both plasma and hepatic cholesterol and triglyceride levels during liver regeneration. Triglycerides and cholesterol accumulated in hepatocytes during the first (24 hours) and the second (72 hours) waves of hepatocyte proliferation (Fig. 1A,B). Most likely, these lipid surges provided the energy for cell proliferation required for tissue regeneration.[12,31] Conversely, under the same conditions, plasma cholesterol and triglyceride levels decreased (Fig. 1C,D), underlining the importance of hepatic lipid accumulation during the proliferative state. On day 7 following PH, we observed a complete return to baseline of plasma and liver lipid levels. Thus, after PH the liver accumulates lipids to satisfy the need of proliferating hepatocytes.

Expression of Genes Involved in Proliferation, ECM Modulation, and Cholesterol Metabolism During Liver Regeneration. Two of the most significant events that sustain liver regeneration are hepatocyte proliferation and lobule remodeling. The proliferation state of the liver was assessed by PCNA immunostaining (Fig. 2A) performed on section obtained at various timepoints after PH. Thus, we investigated the level of gene expression related to cell cycle progression, using *cMyc*, Forkhead Box m1b (*FoxM1b*), and its target gene *Cdc25b*, which control the cell progression through G_1/S and G_2/M phases.[32,33] By real-time PCR (Fig. 2B) we found increased expression of these genes after the 24-hour and 72-hour timepoints, confirming the beginning of liver regeneration. We also studied lobule remodeling throughout regeneration by following the expression of the MMP-9, a zinc endopeptidase involved in the regenerative response to PH as pointed by studies in mice deficient for this MMP.[34] The expression levels of *MMP-9* are significantly induced 24 hours after PH and progressively return to basal levels on day 7 (Fig. 2B).

We then monitored the expression of genes involved in cholesterol biosynthesis, transport, and catabolism. In mice, the most important cholesterol catabolic pathway in the liver is driven by oxysterol-sensing nuclear receptors, namely, LXR. Real-time PCR showed that LXR target genes *Abcg8*, *Srebp1c* (Fig. 2B), and *Abcg5* (Fig. S1), which play key roles in cholesterol secretion and fatty acid synthesis, were dramatically down-regulated after PH. We then hypothesized that the down-regulation of LXR target genes is probably due to the reduction of LXR endogenous ligands. This hypothesis was supported by the induction of both oxysterol-metabolizing enzyme *Sult2b1* and sulfated product excretion pump, *Abcc1*, during liver regeneration (Fig. 2B). We also observed the reduction of oxysterol synthesis pathways in the down-regulation of *Cyp27a1* and *Cyp46a1* (Fig. 2B). Moreover, according to previous studies, our results indicated that during liver regeneration cholesterol biosynthesis is stimulated (increased *HMG-CoA Reductase*[12]), whereas cholesterol catabolism into bile acids is repressed (decreased *Cyp7a1*[27]) (Fig. 2B). Taken together, these data show mutual but opposite regulation of cholesterol biosynthetic and catabolic pathways during liver regeneration after PH. In this scenario, the down-regulation of the tran-

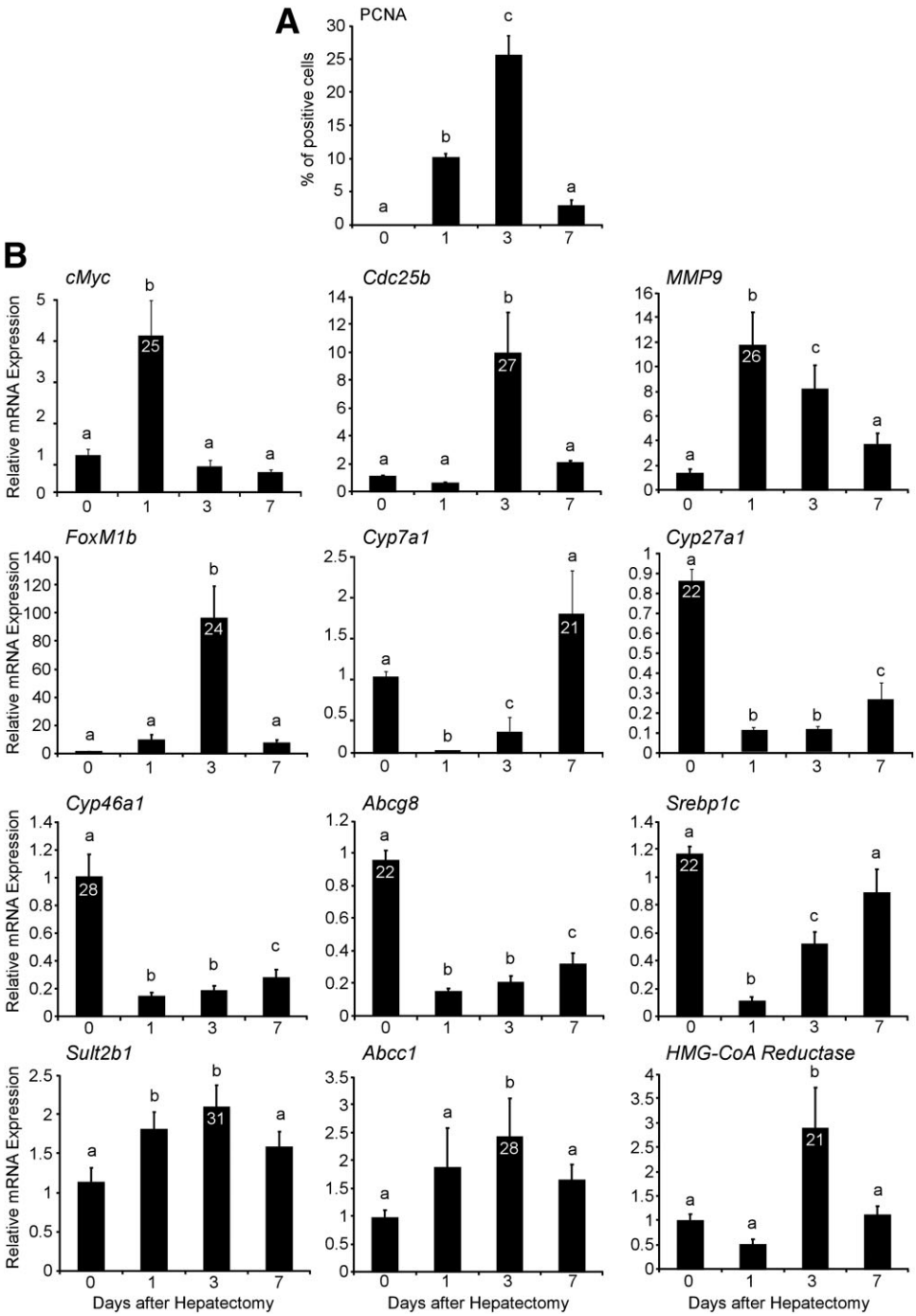

Fig. 2. Expression levels of genes involved in cell proliferation, extracellular matrix remodeling, and lipid metabolism during liver regeneration in LXR$\alpha\beta^{+/+}$ mice. (A) Percentage number of PCNA-positive cells obtained from liver sections at the indicated timepoints. (B) Relative mRNA expression levels of different genes measured by quantitative real-time PCR. Proliferative genes *cMyc, FoxM1b, Cdc25b,* and the extracellular matrix remodeling gene *MMP-9* show a strong transcriptional activation during the first waves of liver regeneration. Genes involved in cholesterol catabolism *(Cyp7a1)*, secretion *(Abcg8)*, and lipogenesis (*Srebp1c)* are strongly down-regulated; genes involved in oxysterol catabolism *(Sult2B1, ABCC1)* are transcriptionally up-regulated; *HMG-CoA Reductase*, involved in cholesterol biosynthesis, is up-regulated, whereas *Cyp27A1* and *Cyp46A1,* involved in oxysterol biosynthesis are down-regulated. The specific transcriptional modulation of these genes highlights the importance of cholesterol availability during cell proliferation. Cyclophilin was used as reference gene and values were normalized to data obtained from sham-operated mice (timepoint 0). The results are shown as mean ± SEM (n = 4-5 at each timepoint). Cycle time numbers at RTqPCR are reported in the bar with highest expression of the specific gene. Lowercase letters indicate statistical significance. ($P < 0.05$).

scriptional LXR system may be due to a reduction of hepatic oxysterols.

Reduction of Hepatic Oxysterol Levels After PH. In order to prove that the down-regulation of the LXR transcriptome is due to reduction of endogenous ligands, we measured hepatic oxysterol levels 2 days after PH. The hepatic amounts of 24(S),25-epoxycholesterol, 24(S)-hydroxycholesterol, and 27-hydroxycholesterol were significantly lower compared to those of control mice, whereas no differences were observed in the hepatic levels of 22-hydroxycholesterol and 25-hydroxycholesterol (Fig. 3). Intriguingly, the reduction in the three oxysterols is lost when LXR is absent or during GW3965 regimen (Fig. S2). These data reveal an unexpected scenario whereby after PH there is a significant accumulation of intracellular cholesterol coupled with a reduced amount of oxysterols, thus explaining the apparent paradoxical down-regulation of the hepatic LXR system in the presence of higher cholesterol concentrations.

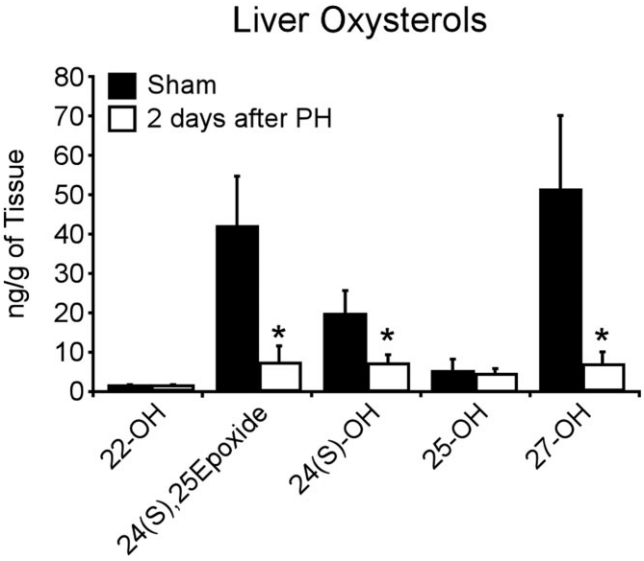

Fig. 3. Hepatic oxysterol concentration after partial hepatectomy. Liver homogenates were prepared from LXR$\alpha\beta^{+/+}$ before and 2 days after PH. Oxysterols were measured after lipid extraction (see SI methods). Data analysis show a significant decrease in total amounts of liver oxysterol during the regenerating phase induced by PH. Significance was reached for 24(S),25Epoxide, 24(S)-OH, and 27-OH. *$P < 0.05$. 22-OH: 22-Hydroxycholesterol; 24(S),25Epoxide: 24(S),25-Epoxycholesterol; 24(S)-OH: 24(S)-Hydroxycholesterol; 25-OH: 25-Hydroxycholesterol; 27-OH: 27-Hydroxycholesterol.

Impact of LXR Activation on Hepatocyte Proliferation During Liver Regeneration. We have shown that the LXR transcriptome is down-regulated during liver regeneration. In order to understand if this is simply an accompanying effect or if the down-regulation of the LXR system is important for the metabolism of proliferating hepatocyte, we treated LXR$\alpha\beta^{+/+}$ and LXR$\alpha\beta^{-/-}$ mice that underwent PH with the LXR-specific synthetic ligand GW3965. Livers were excised from each group of mice and weighed, and their weights were used to calculate liver regrowth.[27] Interestingly, LXR$\alpha\beta^{+/+}$ mice treated with GW3965 exhibited a slower liver regrowth[29] (Fig. 4A). Once LXR is activated, liver growth showed a delay already 24 hours after PH. Seven days after PH, when the liver had reached its original mass in control animals, GW3965-treated mice had regenerated only 75% of their original liver mass. No significant differences were observed in the regrowth index between LXR$\alpha\beta^{+/+}$ and LXR$\alpha\beta^{-/-}$ or LXR$\alpha\beta^{-/-}$ treated with vehicle or with GW3965 (Fig. 4B).

To confirm whether LXR activation exerts an effect on the hepatocyte proliferative response, PCNA immunostaining was performed in liver sections (Fig. 4C,D). At 24 and 72 hours after PH, the number of PCNA-positive hepatocytes in GW3965-treated mice was dramatically reduced compared with vehicle-treated mice (Fig. 4E). On the contrary, LXR$\alpha\beta^{-/-}$ mice did not show significant differences in the number of PCNA-stained cells (Fig. 4F). Cytokine signaling transduced by IL-6 and the Janus-activated kinase/STAT3 pathway promotes liver regeneration through the transcriptional activation of key genes for the activation of G_1 phase.[35,36] To evaluate whether LXR activation might also influence the IL-6-STAT3 signaling pathway, the functional state of STAT3 was analyzed by western blotting (Fig. 4G). Already 24 hours after PH, phosphorylated STAT3 was detected in both GW3965 and vehicle-treated mice but a strong decrease was observed 3 and 7 days after PH in LXR-ligand-activated mice. No differences in the STAT3 phosphoactivation were measured in LXR$\alpha\beta^{-/-}$ mice with or without LXR ligand (Fig. 4G). Thus, the activation of LXR in the regenerating liver reduced hepatocyte proliferative capability.

Impact of LXR Activation on Proliferative and Metalloproteinase Gene Expression. The reduction in hepatocyte proliferative capability after LXR activation during liver regeneration was confirmed also by RTqPCR. GW3965 treatment led to a significant decrease in the expression of two well-known genes involved in the regulation of cell cycle progression, *FoxM1b* and *Cdc25b* (Fig. 5A,C). As previously shown, hepatocytes from LXR$\alpha\beta^{-/-}$ mice treated with GW3965 did not show a decrease in their ability to proliferate. This peculiar behavior was confirmed by gene expression analysis of *FoxM1b* and *Cdc25b*. The transcriptional profile of these genes did not change after LXR activation (Fig. 5B,D). Additionally, Castrillo et al.[37] demonstrated that LXR is a negative regulator of MMP-9. These data and the importance of metalloproteinase modulation during liver regeneration led us to investigate whether LXR activation induces a change in the mRNA expression and protein of MMP-9. Figure 5E shows a strong and significant reduction in the expression of *MMP-9* during liver regeneration in mice treated with the LXR agonist. To confirm that this transcriptional modulation corresponds also to a different protein profile of MMP-9, we performed zymography analysis. As shown in Fig. 5F, when LXR was activated, MMP-9 protein significantly decreased and its induction is delayed. Although vehicle-treated mice show a strong induction of MMP-9 protein 24 and 72 hours after PH, GW3965-treated mice lose this up-regulation at least 24 hours after PH. On the other hand, LXR$\alpha\beta^{-/-}$ mice also had a reduced and delayed induction of MMP-9 protein. This apparent paradoxical molecular regulation of gene transcription has been observed also in the peritoneal macrophages of LXR$\alpha\beta^{-/-}$.[37] These data confirm that LXR acts negatively on liver regeneration, both reducing the hepatocyte proliferation rate and exerting a

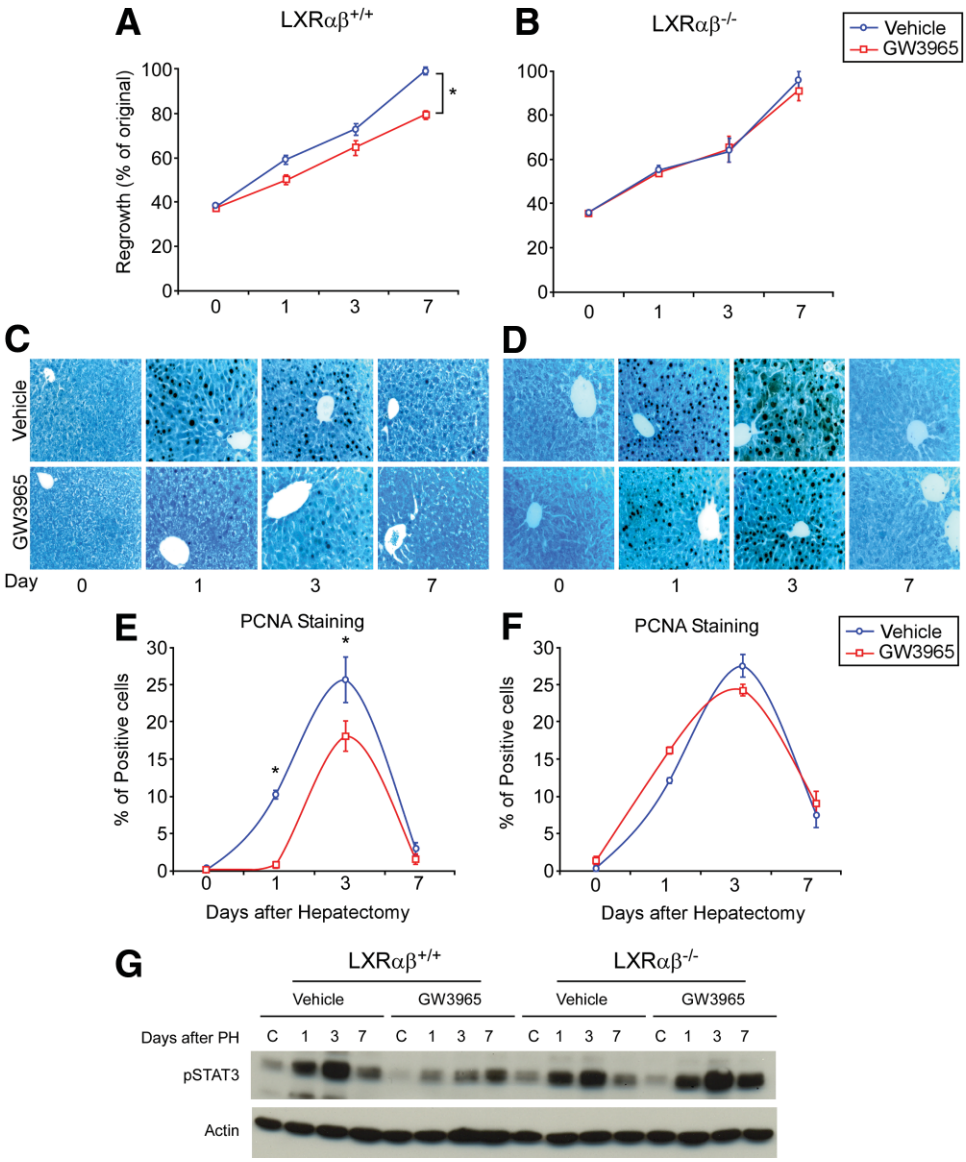

Fig. 4. Hepatocyte proliferation state during liver regeneration after GW3965 treatment. (A,B) Liver regrowth after PH was monitored in LXR$\alpha\beta^{+/+}$ and LXR$\alpha\beta^{-/-}$ mice treated with GW3965 or vehicle. Specific LXR activation resulted in a significant reduction in liver regrowth capacity. (C,D) Immunohistochemistry for PCNA on representative liver sections (20× optical field) from LXR$\alpha\beta^{+/+}$ and LXR$\alpha\beta^{-/-}$ mice are shown. (E,F) Hepatocyte proliferation was determined by the number of the PCNA positive cells 1, 3, and 7 days after PH. A significant decrease in hepatocyte proliferative ability was observed in LXR$\alpha\beta^{+/+}$ mice after LXR activation on day 1 and 3. (G) Hepatic phosphorylated STAT3 was assessed by western blot in LXR$\alpha\beta^{+/+}$ and LXR$\alpha\beta^{-/-}$ mice during GW3965 treatment after PH. A representative sample for each timepoint and genotype is shown. β-Actin was included as loading control. The results are shown as mean ± SEM (n = 4-5 for each genotype at each timepoint). *$P < 0.05$, GW3965 versus vehicle.

specific inhibition on the liver remodeling phase through MMP-9 transcriptional down-regulation.

Modulation of Lipid Metabolism by LXR Activation During Liver Regeneration. To evaluate the effect of LXR activation on lipid homeostasis after PH-induced liver regeneration, plasma and hepatic lipids were analyzed. During the regeneration process, lipid flow is directed from periphery toward the liver. In vehicle-treated mice, plasma cholesterol and triglyceride levels decreased 7 days following PH, whereas GW3965-treated mice displayed different behavior (Fig. 6A-H). Plasma cholesterol levels did not show any significant decrement (Fig. 6A), whereas plasma triglycerides were reduced at 24 hours but rapidly recovered to a basal level at day 7 (Fig. 6B). Indeed, Fig. 5C shows that after GW3965 treatment hepatic cholesterol content was significantly lowered, whereas hepatic triglycerides increased at 24 and 72 hours after PH (Fig. 6D). The loss of significant differences in plasma and hepatic levels of lipids between GW3965 and vehicle-treated LXR$\alpha\beta^{-/-}$ mice (Fig. 6E-H) highlighted the specificity of LXR-mediated changes in lipid metabolic pathways. Taken together, these data show that after PH, LXR activation is combined with a reduction in hepatic cholesterol levels and a reduced proliferative rate.

Expression of Lipid Homeostasis-Related Genes. To confirm that lipid changes were consistent with modulation of LXR-related pathways, gene expression analysis was performed on regenerating livers of LXR$\alpha\beta^{+/+}$ and LXR$\alpha\beta^{-/-}$ mice treated with the LXR ligand. As shown in Fig. 7A-C, LXR target genes that were down-regulated during liver regeneration (Fig. 2B) were transcriptionally activated upon GW3965 treatment. Abcg5 and Abcg8 drive cellular cholesterol secretion, thus their activation (Fig. 7A,B) determined the decrease of cellular cholesterol

concentration. At the same time, induction of *Srebp1c* (Fig. 7C) determined the activation of lipogenic pathways. The activation of LXR together with the resulting changes in lipid homeostasis is also responsible for the modification in the cholesterol biosynthetic pathway. Although under normal conditions liver regeneration went together with an increase in cholesterol biosynthesis (Fig. 2B), LXR activation was also responsible for the absence of HMG-CoA reductase induction after PH (Fig. 7D). The absence of modulation of all these genes (Fig. 7E-H) as well as lipid levels (Fig. 6E-H) in LXR$\alpha\beta^{-/-}$ mice confirmed the specificity of LXR action. A direct statistical comparison of the entire high-throughput RTqPCR expression levels between genotypes and treatment regimen is provided in Supporting Table 2.

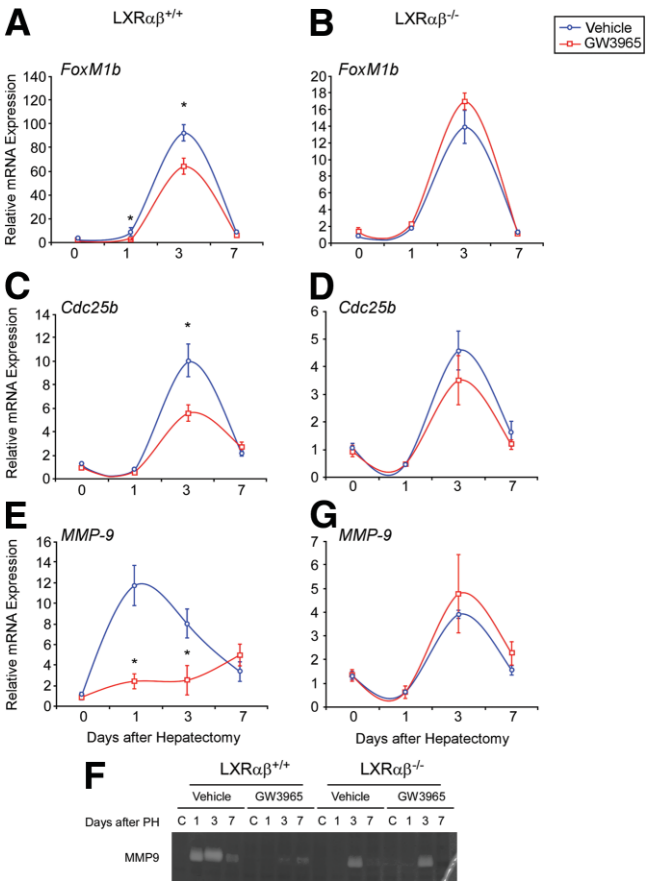

Fig. 5. Expression of genes involved in cell proliferation and extracellular matrix remodeling during liver regeneration after LXR activation. Total RNA was extracted from livers of LXR$\alpha\beta^{+/+}$ and LXR$\alpha\beta^{-/-}$ mice during GW3965 treatment and relative mRNA expression of different genes was measured by RTqPCR. (A-G) LXR activation in LXR$\alpha\beta^{+/+}$ mice negatively modulated expression of proliferative genes *(FoxM1b and Cdc25b)* and extracellular *MMP-9* metalloproteinase gene, confirming the reduction of hepatocyte proliferation capacity and lobule remodeling. LXR$\alpha\beta^{-/-}$ mice treated with GW3965 do not show differences compared to control mice. The results are shown as mean ± SEM (n = 4-5 for each genotype at each timepoint). *$P < 0.05$, GW3965 versus vehicle. (F), MMP-9 gelatinase activity decreases in LXR$\alpha\beta^{+/+}$ mice during GW3965 treatment. Representative samples from each timepoint and genotype are shown.

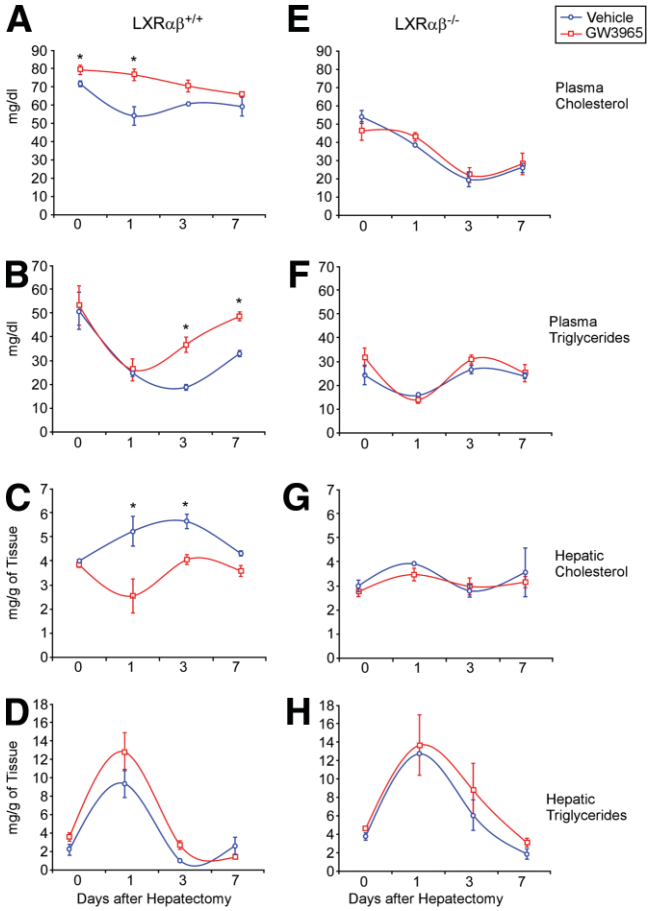

Fig. 6. Hepatic and plasma lipid changes during liver regeneration after LXR activation. LXR$\alpha\beta^{+/+}$ and LXR$\alpha\beta^{-/-}$ mice were treated daily by way of gavage with GW3965 or vehicle 2 days before and after PH at the dose of 20mg/Kg b.w. Liver and plasma from each animal were used to measure total lipid content. (A,B) Plasma cholesterol and triglyceride levels increase after PH in LXR$\alpha\beta^{+/+}$ mice during GW3965 treatment underlining a change in the direction of lipid flow. (C,D) Hepatic cholesterol decreases and triglycerides increase after GW3965 treatment during the regenerating process. (E-H) Plasma and hepatic lipid analysis in liver regenerating LXR$\alpha\beta^{-/-}$ mice does not show any difference between GW3965 and vehicle treated animals confirming the specificity of LXR-mediated changes. The results are shown as mean ± SEM (n = 4-5 for each genotype at each timepoint). *$P < 0.05$, GW3965 versus vehicle.

Discussion

LXR is a cholesterol sensor that up-regulates catabolic pathways during cholesterol intracellular overload. In the present study the regenerating mouse liver was used as a model to investigate the role of the nuclear receptor LXR in the modulation of cholesterol metabolic pathways during cell proliferation. After the first posthepatectomy phase consisting of the initiation of DNA synthesis, the regenerating liver undergoes significant changes in cholesterol homeostasis.[12] PH in mice is coupled with in-

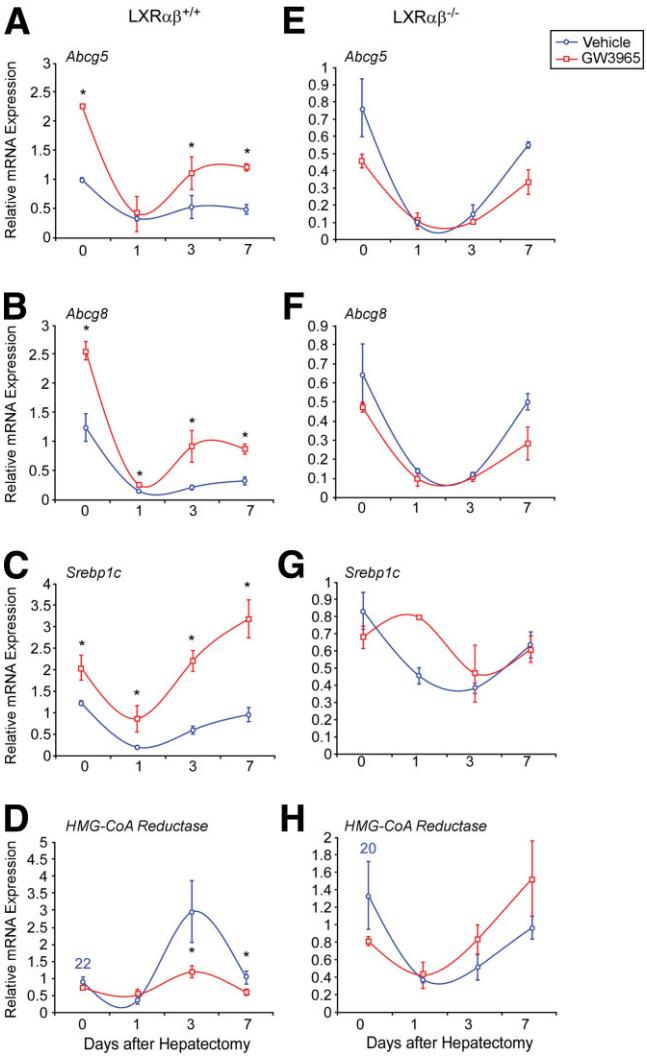

Fig. 7. Expression of genes involved in cholesterol metabolism during liver regeneration after LXR activation. Total RNA was extracted from livers of LXR$\alpha\beta^{+/+}$ and LXR$\alpha\beta^{-/-}$ mice after GW3965 treatment and relative mRNA expression of different genes was measured by quantitative real-time PCR. (A-C) LXR target genes involved in cholesterol secretion *(ABCG5, ABCG8)* and lipogenesis *(SREBP1c)* were up-regulated during GW3965 treatment in LXR$\alpha\beta^{+/+}$ mice after PH. (D) HMG-CoA reductase loses its up-regulation after PH in LXR$\alpha\beta^{+/+}$ GW3965-treated mice, showing that lipid homeostasis changes, driven by LXR activation, determine the failure of cholesterol biosynthesis activation. (E-G) mRNA expression levels of LXR target genes in LXR$\alpha\beta^{-/-}$ mice after GW3965 treatment does not show changes compared to vehicle-treated. The results are shown as mean ± SEM (n = 4-5 for each genotype at each timepoint). *$P < 0.05$, GW3965 versus vehicle.

creased hepatic cholesterol content. This event is explained by an increase in cholesterol hepatic synthesis, decreased catabolism into bile acids, and decrease biliary secretion. Interestingly, a net down-regulation of the entire LXR transcriptome is observed in cholesterol enriched proliferating hepatocytes. mRNA expression of both LXR isoforms is not down-regulated during liver regeneration after PH, whereas there are significant changes in the expression of genes that encode for proteins and enzymes involved in oxysterol synthesis, sulfation, and catabolism. The levels of ligands for nuclear receptors are regulated by catabolic enzymes to ensure that excess signaling does not take place. The expression of these enzymes protects from the action of circulating ligands, and often, with a feed-forward regulatory loop is the same ligand that controls its own catabolism.[38] Our data show a double but opposite regulation of genes involved in oxysterol catabolism and synthesis. Thus, the up-regulation of *Sult2b1* and *Abcc1*, involved in oxysterol catabolism and secretion, and the down-regulation of *Cyp27a1* and *Cyp46a1*, oxysterol biosynthetic enzymes, leads to a net reduction of oxysterol amounts with attenuation of LXR signaling during liver regeneration in order to satisfy a rapidly regenerating hepatocyte demand for cholesterol. In support of this view, mice with constitutive silencing of LXR signaling (LXR$\alpha\beta^{-/-}$) show no difference in their hepatocyte regenerative capacity, because this scenario mimics the ligand inactivation status of the LXR transcriptome normally observed during liver regeneration. Although no direct differences in expression profiles of cytokines and growth factors are observed between LXR$\alpha\beta^{+/+}$ and LXR$\alpha\beta^{-/-}$ mice after PH (Fig. S3), we cannot exclude that other signaling pathways might cooperate with the oxysterol reduction in switching off the LXR system during liver regeneration. Here we present an intriguing *in vivo* model of cell proliferation in which increased intracellular cholesterol levels are uncoupled with the LXR system activation. Our data undoubtedly show that if the LXR system is reactivated after partial hepatectomy by way of administration of an LXR-specific synthetic ligand, there is a significant decrease in hepatic cholesterol content by way of the up-regulation of catabolism and biliary secretion with a concomitant decrease in *de novo* synthesis. The final outcome is a negative modulation of hepatocyte regenerative capacity with concomitant LXR-driven down-regulation of matrix remodeling and lobule structuring (Fig. 8). MMPs are key proteins involved in extracellular matrix remodeling in both normal and pathophysiological processes.[39] MMP activity is regulated at several levels, including gene transcription, cellular secretion, activation of proenzymes, and binding to specific tissue inhibitors. MMP-9 is critical during liver regeneration, due to its direct effect on extracellular matrix remodeling, a process integral to tissue regeneration.[34] Interestingly, ligand activation of LXR has been shown to repress the expression of MMP-9 in macrophages.[37] In our study, LXR ligand activation negatively affects *MMP-9* mRNA expression and protein also in the liver, determining significant aberrations in matrix remodeling or affecting the levels or activation of a variety of

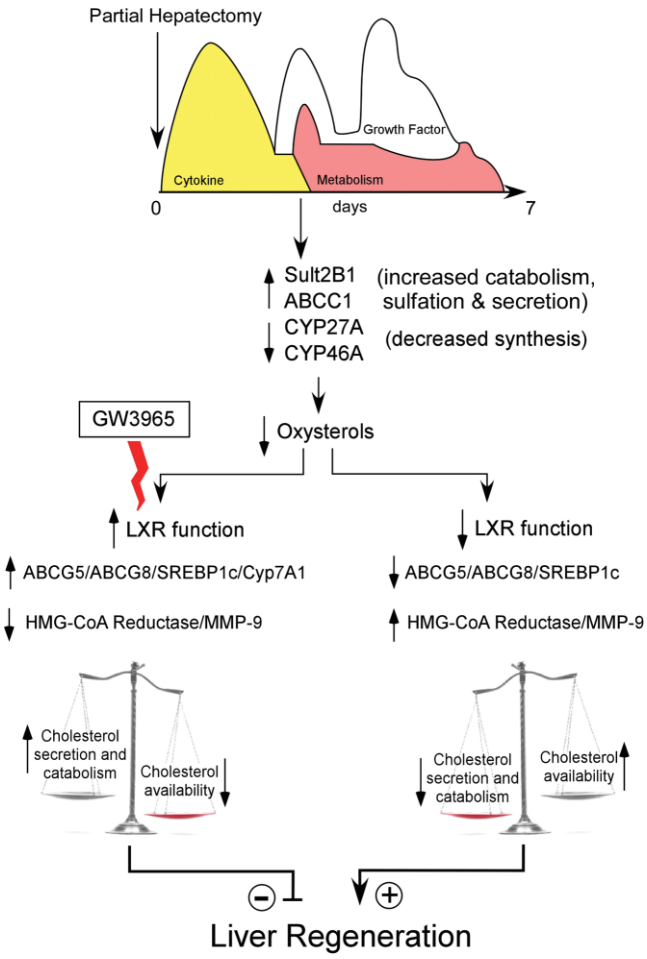

Fig. 8. Schematic representation of oxysterol and cholesterol homeostasis during liver regeneration after PH. After the first phase of liver regeneration (DNA synthesis), there are several modifications in cytokines, growth factors, and metabolic events,[9] with the occurrence of significant changes in oxysterol and cholesterol homeostasis. Double but opposite regulation of genes involved in oxysterol catabolism, secretion (SULT2B1, ABCC1 up-regulation) and biosynthesis (CYP27A1, CYP46A1 down-regulation) determines the reduction in oxysterol amount with the consequent attenuation of LXR-mediated pathways for cholesterol catabolism and secretion (Cyp7A1, ABCG5, ABCG8). The contemporary increase of cholesterol biosynthesis (HMG-CoA reductase) raises cholesterol availability to satisfy proliferating hepatocyte demand for free cholesterol. Reactivation of LXR-driven cholesterol secretion and catabolism pathways through an LXR-specific synthetic ligand and subsequent decrease in cholesterol biosynthesis, reduces cholesterol availability, and negatively modulates hepatocyte regenerative capacity and lobule remodeling by way of MMP9.

inflammatory mediators and growth factors directly linked to the MMP-9 activity.[40]

In closing, our data unveil a previously unrecognized and apparently paradoxical scenario of LXR modulation. During liver regeneration LXR activity is abated in spite of increasing intracellular cholesterol levels. Turning off LXR-transcriptional pathways provides the requisite cholesterol levels to regenerating hepatocytes. In line with this hypothesis, pharmacological LXR reactivation during PH significantly reduces liver regeneration capacity. Thus, strategies aiming at controlling LXR function during hepatocyte proliferation might become useful for the modulation of conditions with continuous hyperplastic deleterious stimuli in the liver such as during viral and alcoholic hepatitis or in hepatocarcinomas.

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