

## mTOR signaling in liver regeneration: Rapamycin combined with growth factor treatment

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### Abstract

**AIM:** To investigate the effects of mammalian target of rapamycin (mTOR) inhibition on liver regeneration and autophagy in a surgical resection model.

**METHODS:** C57BL/6 mice were subjected to a 70% partial hepatectomy (PH) and treated intraperitoneally every 24 h with a combination of the mTOR inhibitor rapamycin (2.5 mg/kg per day) and the steroid dexamethasone (2.0 mg/kg per day) in phosphate buffered

saline (PBS) or with PBS alone as vehicle control. In the immunosuppressant group, part of the group was treated subcutaneously 4 h prior to and 24 h after PH with a combination of human recombinant interleukin 6 (IL-6; 500 µg/kg per day) and hepatocyte growth factor (HGF; 100 µg/kg per day) in PBS. Animals were sacrificed 2, 3 or 5 d after PH and liver tissue and blood were collected for further analysis. Immunohistochemical staining for 5-Bromo-2'-deoxyuridine (BrdU) was used to quantify hepatocyte proliferation. Western blotting was used to detect hepatic microtubule-associated protein 1 light chain 3 (LC3)-II protein expression as a marker for autophagy. Hepatic gene expression levels of proliferation-, inflammation- and angiogenesis-related genes were examined by real-time reverse transcription-polymerase chain reaction and serum bilirubin and transaminase levels were analyzed at the clinical chemical core facility of the Erasmus MC-University Medical Center.

**RESULTS:** mTOR inhibition significantly suppressed regeneration, shown by decreased hepatocyte proliferation (2% vs 12% BrdU positive hepatocyte nuclei at day 2,  $P < 0.01$ ; 0.8% vs 1.4% at day 5,  $P = 0.02$ ) and liver weight reconstitution (63% vs 76% of initial total liver weight at day 3,  $P = 0.04$ ), and furthermore increased serum transaminase levels (aspartate aminotransferase 641 U/L vs 185 U/L at day 2,  $P = 0.02$ ). Expression of the autophagy marker LC3-II, which was reduced during normal liver regeneration, increased after mTOR inhibition (46% increase at day 2,  $P = 0.04$ ). Hepatic gene expression showed an increased inflammation-related response [tumor necrosis factor (TNF)- $\alpha$  3.2-fold upregulation at day 2,  $P = 0.03$ ; IL-1Ra 6.0-fold upregulation at day 2 and 42.3-fold upregulation at day 5,  $P < 0.01$ ] and a reduced expression of cell cycle progression and angiogenesis-related factors (HGF 40% reduction at day 2; vascular endothelial growth factor receptor 2 50% reduction at days 2 and 5; angiopoietin 1 60% reduction at day 2, all  $P \leq 0.01$ ). Treatment

with the regeneration stimulating cytokine IL-6 and growth factor HGF could overcome the inhibitory effect on liver weight (75% of initial total liver weight at day 3,  $P = 0.02$  vs immunosuppression alone and  $P = 0.90$  vs controls) and partially reversed gene expression changes caused by rapamycin (TNF- $\alpha$  and IL-1Ra levels at day 2 were restored to control levels). However, no significant changes in hepatocyte proliferation, serum injury markers or autophagy were found.

**CONCLUSION:** mTOR inhibition severely impairs liver regeneration and increases autophagy after PH. These effects are partly reversed by stimulation of the IL-6 and HGF pathways.

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**Key words:** Hepatocyte proliferation; Autophagy; Microtubule-associated protein 1 light chain 3; Partial hepatectomy; Rapamycin

**Core tip:** Interference of immunosuppressive medication with liver regeneration is a highly relevant issue for transplantation of small-for-size liver grafts. Inhibition of mammalian target of rapamycin (mTOR) represents an important immunosuppressive strategy after transplantation, yet as mTOR regulates cell proliferation and autophagy, concerns remain regarding a negative impact on regeneration. The exact role of mTOR signaling after living-donor liver transplantation is largely unknown. Here we report that mTOR inhibition by rapamycin severely impairs liver regeneration and increases autophagy after liver resection in mice. The most novel finding of this study is that this impaired regeneration can be partly reversed by treatment with exogenous growth factors.

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## INTRODUCTION

The liver has the remarkable ability to regenerate in order to compensate for lost or damaged liver tissue after injury and thereby restore liver function and maintain homeostasis. This process is ultimately required after living-donor liver transplantation, in which a small-for-size graft is subjected to ischemia and reperfusion injury and transplanted into a recipient with urgent metabolic needs. In this situation, both loss of a substantial part of the initial liver mass as well as oxidative stress after reperfusion are central mechanisms of hepatic injury<sup>[1,2]</sup>.

Liver resection triggers release of the cytokines tumor

necrosis factor (TNF) and interleukin 6 (IL-6), crucial priming factors for the initiation of hepatocyte proliferation by activation of the janus activated kinases/signal transducer and activator of transcription (JAK/STAT) pathway<sup>[3-5]</sup>. This priming phase stimulates resting hepatocytes to enter the G<sub>1</sub> phase of the cell cycle. Simultaneously, growth factors including hepatocyte growth factor (HGF), contribute to the passage of hepatocytes from the G<sub>1</sub> into the S phase by activating the phosphoinositide-3 kinase (PI3K)/Akt signal transduction pathway<sup>[6-8]</sup>. PI3K/Akt interacts with the mammalian target of rapamycin (mTOR), involved in the control of protein synthesis, cell size and proliferation<sup>[9,10]</sup>. Both cascades lead to activation of a variety of signaling pathways, including upregulation of several downstream cyclins like cyclin D1, which is associated with the G<sub>1</sub>-S phase transition of hepatocytes<sup>[3,4,6,11,12]</sup>.

Besides being a key regulator of cell growth and proliferation, mTOR was recently identified to play an important role in the control of autophagy<sup>[13-15]</sup>. Autophagy is an evolutionarily conserved lysosomal degradation pathway that plays an important protective role in case of cellular injury by mediating the elimination of damaged cellular components<sup>[13]</sup>. In non-hepatic cells, autophagy has not only been implicated as a survival response, but also as a mediator of cell death during stress conditions<sup>[16,17]</sup>. Autophagy might therefore play a role in liver regeneration, though this has not been thoroughly studied. This is of special interest to the field of liver transplantation as mTOR inhibition, in combination with a short course of steroids, is an attractive alternative for current calcineurin inhibitor based immunosuppressive strategies. Calcineurin inhibitors are neurotoxic, associated with a 20% incidence of chronic kidney dysfunction and carry a cumulative risk for *de novo* malignancy of up to 55% at 15 years after liver transplantation<sup>[18-22]</sup>. mTOR inhibitors like rapamycin therefore represent an important immunosuppressive option, especially in patients with calcineurin inhibitor-induced neurotoxicity, poor renal function and possibly also in patients with hepatocellular carcinoma. However, in the initial phase after liver transplantation, the mTOR inhibitor rapamycin is rarely used, since it is reported to delay liver regeneration<sup>[23-25]</sup>.

Rapamycin inhibits mTOR complex 1 (mTORC1) by complex formation with FK506 binding protein 12, thereby acting on its downstream messengers and abrogating translation initiation and protein synthesis, which results in cell cycle arrest at the G<sub>1</sub> to S phase<sup>[23-25]</sup>. Cyclin D1 as well as p21 are shown to be important downstream messengers of the rapamycin-mediated cell cycle arrest<sup>[26-28]</sup>. The exact underlying cellular and molecular mechanisms by which mTOR inhibition attenuates liver regeneration and the interplay between mTOR inhibition and autophagy in liver regeneration needs to be further characterized.

Both after kidney as well as deceased liver transplantation, mTOR inhibition in combination with steroids has proven an efficient immunosuppressive strategy. Addition of an mTOR inhibitor to steroid treatment might therefore also show beneficial effects after living-donor

liver transplantation, especially in patients with compromised renal function. Aim of this study is to investigate the effects of mTOR inhibition, in combination with the steroid dexamethasone, on liver regeneration and autophagy in a surgical resection model and in particular its involvement in IL-6 and HGF stimulated pathways. Besides mimicking the post-transplant treatment strategy, this combination of immunosuppressants also allowed more specific investigation of the effects of exogenous IL-6 and HGF, since steroids are multi-potent inhibitors of endogenous production of pro-inflammatory cytokines like TNF and IL-6<sup>[29]</sup>. Effects on body and liver weight, hepatocyte proliferation, autophagy and hepatic function and injury were analyzed at specific time points after surgery in a 70% partial hepatectomy (PH) model in mice.

## MATERIALS AND METHODS

### Animals

Male C57Bl/6 mice (age 12-15 wk) were obtained from Charles River (Maastricht, Netherlands) and maintained in the animal facility on a 12/12 h light/dark schedule. The animals had free access to food and drinking water and received care according to the Guide for the Care and Use of Laboratory Animals. All animal experiments were performed with approval of the institutional animal welfare committee.

### PH and treatments

Liver regeneration was induced in C57BL/6 mice by performing a 70% PH as first described by Higgins and Anderson in 1931. Animals were anaesthetized with isoflurane and, after a midline laparotomy, the left lateral and median lobes of the liver were ligated and resected. The peritoneum and skin were sutured separately. All procedures were performed under clean conditions. Animals were treated intraperitoneally every 24 h, starting at time of PH, with a combination of the immunosuppressants rapamycin (2.5 mg/kg per day; sirolimus oral solution, Wyeth Pharmaceuticals, Louvain-la-Neuve, Belgium) and dexamethasone (2.0 mg/kg per day, Organon, Oss, The Netherlands) in phosphate buffered saline (PBS) (Lonza, Verviers, Belgium; total volume 0.5 mL) or with PBS alone as vehicle control. In the immunosuppressant (Rapa-Dex) group, part of the group was treated subcutaneously 4 h prior to and 24 h after PH with a combination of human recombinant IL-6 (500 µg/kg per day; Peprotech, London, United Kingdom) and HGF (100 µg/kg per day; Peprotech) in PBS. Animals ( $n = 5-9$  per group) were sacrificed 2, 3 or 5 d after PH and liver tissue and blood were collected for further analysis. To investigate the effects of dexamethasone alone, an additional group was treated with dexamethasone alone (Dex) as described above and sacrificed at day 2 after PH.

### Weight calculations

Animals were weighed daily prior to treatment and the resected liver mass was weighed after PH. The initial

total liver weight was calculated as follows: resected liver weight/70 × 100 (g).

At time of sacrifice, animals and their regenerated liver mass were weighed and the percentage of reconstitution of the liver was calculated by: regenerated liver weight/initial total liver weight × 100 (%).

### Immunohistochemistry

One hour prior to sacrifice, animals were injected intraperitoneally with 50 mg/kg 5-Bromo-2'-deoxyuridine (BrdU; B5002, Sigma-Aldrich, Zwijndrecht, Netherlands). Livers were harvested and processed to 4 µm thick formalin fixed, paraffin embedded sections. Immunohistochemical staining for BrdU was achieved using monoclonal mouse anti-BrdU (Bu20a; DakoCytomation, Glostrup, Denmark; 1:80 in blocking buffer) as primary antibody and polyclonal anti-mouse IgG/HRP (P0161; DakoCytomation; 1:1000 in blocking buffer) as secondary antibody (see Supplemental Information for a full description of the protocol). Per animal 4 high power fields (HPF; × 400) were analyzed for BrdU positive hepatocytes.

### Real-time quantitative reverse transcription-polymerase chain reaction

At time of sacrifice, liver tissue was stored overnight at 4 °C and thereafter at -80 °C in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) for RNA preservation. After RNA extraction and reverse transcription (see Supplemental Information for the protocol), real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with a SensiMix SYBR and Fluorescein Kit (Bioline, London, United Kingdom) and MyIQ real time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instruction. PCR primers (Table 1) were synthesized by Isogen Life Science (Maarsse, Netherlands) and Biolegio (Nijmegen, Netherlands). Gene expression levels were normalized using the  $\Delta\Delta CT$  method and TATA binding protein as reference gene, because it is shown to be stable during different phases of liver regeneration<sup>[30]</sup>.

### Western blotting

Liver tissue, preserved in Allprotect as described, was assessed for autophagy by investigating hepatic protein levels of microtubule-associated protein 1 light chain 3 (LC3)-II using rabbit polyclonal LC3A/B (1:1000, Cell Signaling Technology, Danvers, United States) and mouse purified IgG C4/actin (1:2500, BD Biosciences, Franklin Lakes, United States) as primary antibodies and goat-anti-mouse IgG IRDye 680 and goat-anti-rabbit IgG IRDye 800CW (both 1:5000; LI-COR Biosciences, Lincoln, United States) as secondary antibodies (See Supplemental Information for a full description of the protocol). Blots were scanned using an Odyssey Infrared Imager (LI-COR Biosciences) and the results were analyzed using Odyssey software.

### Serum analysis of enzyme levels

Blood samples were collected at time of sacrifice in heparin coated microtubes. After collection, samples were

**Table 1** Reverse transcription-polymerase chain reaction primer sequences

Gene	Name	Accession number	Primer (forward/reverse)
CCND1	Cyclin D1	NM_007631	GCGTACCCGACACCAATCTC CTCCTCTTCGCACTTCTGCTC
PCNA	Proliferating cell nuclear antigen	NM_011045	CTTGGTACAGCTTACTCTGCG AGTIGCTCCACATCTAAGTCCAT
TNFA	Tumor necrosis factor alpha	NM_013693	CCCTCACACTCAGATCATCTTCT GCTACGACGTGGGCTACAG
IL1RN	Interleukin 1 receptor antagonist	NM_031167	GCTCATGCTGGGTACTTACAA CCAGACTTGGCACAAGACAGG
IL6	Interleukin 6	NM_031168	TAGTCCTCCTACCCCAATTCC TTGGTCTTAGCCACTCCTTC
HGF	Hepatocyte growth factor	NM_010427	ATGTGGGGGACCAAACCTCTG GGATGGCGACATGAAGCAG
TGFB	Transforming growth factor $\beta$	NM_011577	CTCCCGTGGCTTCTAGTGC GCCTTAGTTGGACAGGATCTG
KDR	Vascular endothelial growth factor receptor 2	NM_010612	TTTGGCAAATACAACCCCTCAGA GCAGAAGATACTGTCACCACC
ANGPT1	Angiopoietin 1	NM_009640	CACATAGGGTGCAGCAACCA CGTCGTTCTGGAAGAATGA
VEGFA	Vascular endothelial growth factor A	NM_009505	GCACATAGAGAGAATGAGCTTCC CTCCGCTCTGAACAAGCT
FLT1	Vascular endothelial growth factor receptor 1	NM_010228	TGGCTCTACGACCTTAGACTG CAGGTTTGACTTGTCTGAGGTT
TBP	TATA binding protein	NM_013684	AGAACAATCCAGACTAGCAGCA GGGAACCTTCACATCACAGCTC

centrifuged (19 min, 1800 r/min) to separate the serum, which was further analyzed at the clinical chemical core facility of the Erasmus MC-University Medical Center to determine bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

### Statistical analysis

Data are presented as mean  $\pm$  SE. Statistical analysis was performed using the Mann-Whitney test or student *t*-test after checking for normal distribution. A *P*-value  $\leq$  0.05 was considered statistically significant.

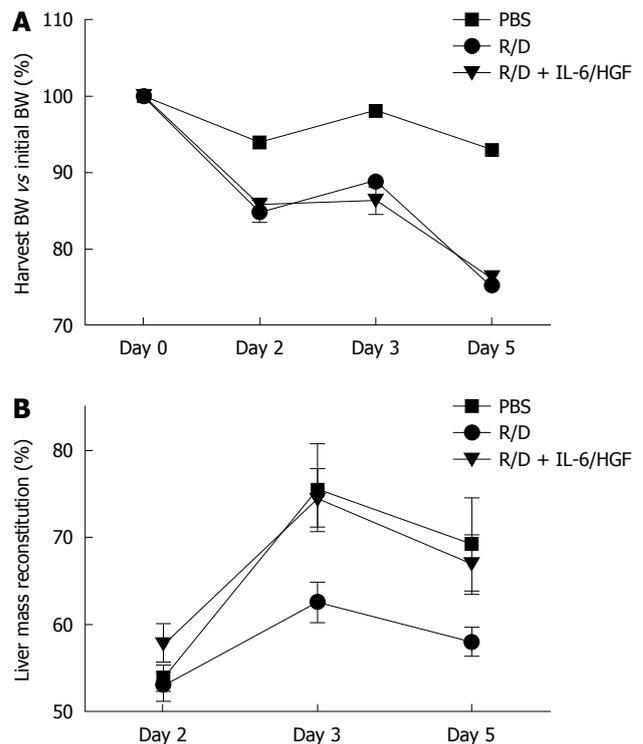
## RESULTS

### Inhibition of mTOR causes progressive body weight loss after liver resection

As shown in Figure 1A, significant and progressive body weight loss was seen after PH in animals treated with Rapa-Dex compared to control treated animals (15% *vs* 6% loss, *P* < 0.01 at day 2; 11% *vs* 2%, *P* = 0.04 at day 3 and 25% *vs* 7%, *P* < 0.01 at day 5). No significant body weight loss was seen in animals treated with Dex alone (9% loss, *P* = 0.11 at day 2; data not shown). Combined treatment with Rapa-Dex and IL-6/HGF could not overcome the progressive weight loss and showed a similar effect on body weight (14% loss, *P* < 0.01 at day 2; 14%, *P* = 0.06 at day 3 and 24%, *P* < 0.01 at day 5).

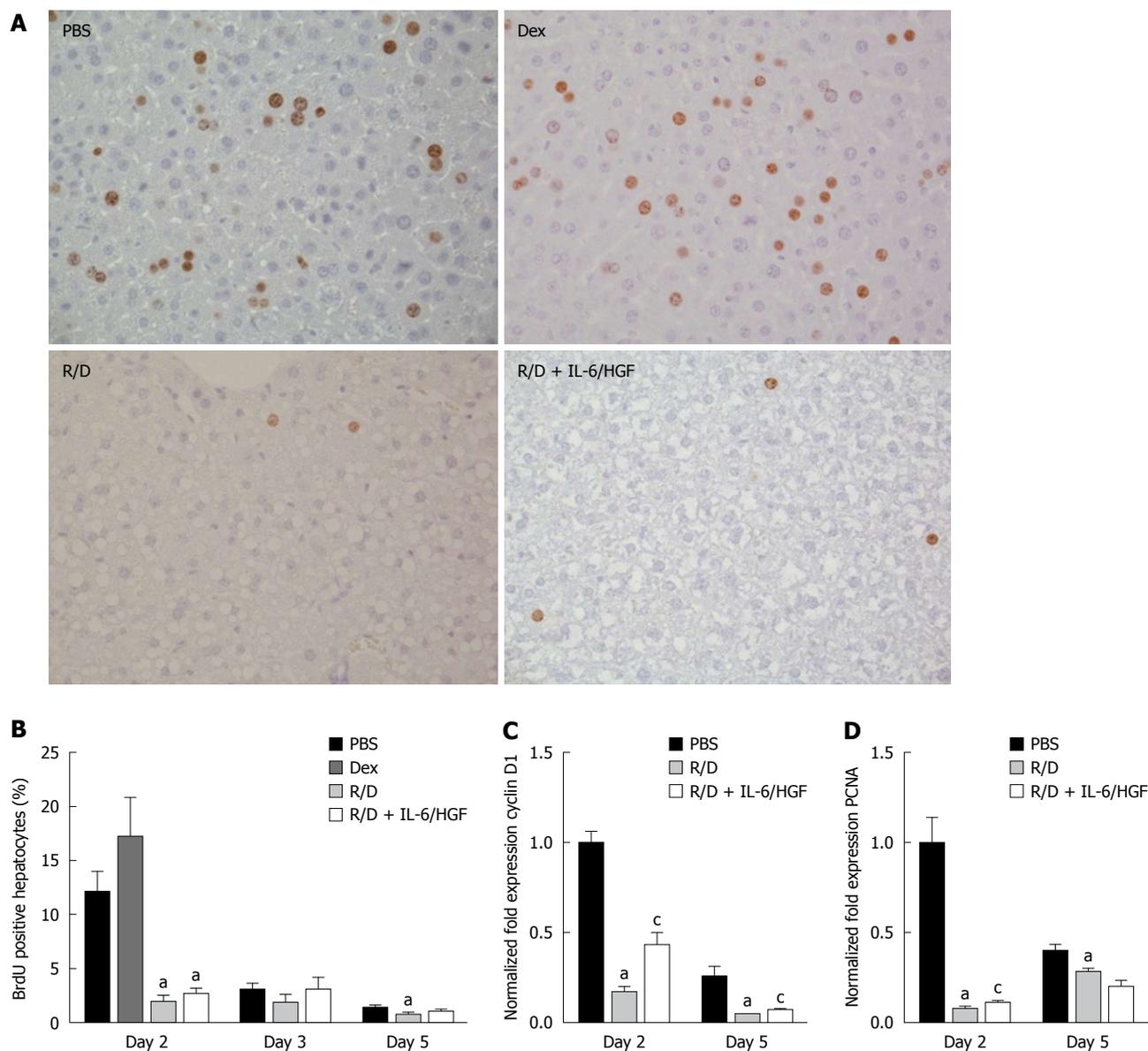
### Reduced liver mass reconstitution by mTOR inhibition can be overcome with exogenous IL-6 and HGF

After 70% PH in the control group, liver mass recovered to 54% of the initial total liver weight by day 2 and to 76% by day 3 (Figure 1B). Treatment with Rapa-Dex caused a significant inhibition in the reconstitution of liver mass



**Figure 1** Effects of mammalian target of rapamycin inhibition on body and liver weight. A: Harvest body weight at days 2, 3 and 5 after partial hepatectomy (PH) vs initial body weight; B: Harvest liver weight at days 2, 3 and 5 after PH vs total liver weight prior to PH. Data are shown as mean  $\pm$  SE. BW: Body weight; R/D: Rapa-Dex; IL-6: Interleukin 6; HGF: Hepatocyte growth factor; PBS: Phosphate buffered saline.

at day 3 *vs* control treatment (63% of initial total liver weight, *P* = 0.04). A similar trend was seen at day 5, but differences did not reach statistical significance. Treatment



**Figure 2** Effects of mammalian target of rapamycin inhibition on hepatocyte proliferation. A, B: Livers were processed for immunohistochemistry on 5-Bromo-2'-deoxyuridine (BrdU) to quantify hepatocyte proliferation. A: Representative pictures ( $\times 400$ ) of hepatocyte proliferation at day 2 after partial hepatectomy (PH); B: Quantification of hepatocyte proliferation at day 2, 3 and 5 after PH; C, D: Hepatic gene expression levels of cyclin D1 and proliferating cell nuclear antigen (PCNA) were determined by quantitative reverse transcription-polymerase chain reaction and normalized against TATA binding protein. C: Expression levels of cyclin D1 at day 2 and 5 after PH; D: Expression levels of PCNA at day 2 and 5 after PH. Data are shown as mean  $\pm$  SE. <sup>a</sup> $P \leq 0.05$  vs phosphate buffered saline (PBS); <sup>c</sup> $P \leq 0.05$  vs Rapa-Dex (R/D). HGF: Hepatocyte growth factor; IL-6: Interleukin 6.

with Dex alone did not show significant differences compared to controls (57% of initial total liver weight at day 2,  $P = 0.30$ ; data not shown). Combination of IL-6/HGF with Rapa-Dex completely restored liver reconstitution to control levels (75% of initial total liver weight at day 3,  $P = 0.02$  vs Rapa-Dex and  $P = 0.90$  vs controls).

**IL-6 and HGF treatment upregulates cell cycle progression-related gene expression of cyclin D1 and proliferating cell nuclear antigen, but does not restore mTOR-induced inhibition of hepatocyte proliferation**

Hepatocyte proliferation, quantified by the percentage of BrdU positive hepatocyte nuclei, was significantly reduced at day 2 after PH in animals treated with Rapa-Dex

compared to control treated animals (2% vs 12%,  $P < 0.01$ ; Figure 2A and B). mTOR inhibition delayed hepatocyte proliferation at least until day 5 (0.8% vs 1.4%,  $P = 0.02$ ). In contrast, treatment with Dex alone had no significant effect on proliferation at day 2. Addition of exogenous IL-6/HGF to Rapa-Dex treatment did not significantly stimulate hepatocyte proliferation at any time point after PH, although no significant difference compared to control treatment was seen at days 3 and 5. Combined treatment of Rapa-Dex with IL-6/HGF did, however, cause a decrease in the number of hepatocytes per HPF compared to treatment with Rapa-Dex alone (170 cells/HPF vs 206 cells/HPF,  $P = 0.05$ ; data not shown), suggesting an increase in cell size.

The inhibitory effect of mTOR inhibition on cell proliferation was also reflected in the hepatic gene expression levels of cyclin D1 and proliferating cell nuclear antigen (PCNA), known to be relevant for cell cycle progression and DNA synthesis. Compared to control treatment, Rapa-Dex treatment significantly downregulated expression of cyclin D1 (80% reduction,  $P < 0.01$ ; Figure 2C) and PCNA (90% reduction,  $P < 0.01$ ; Figure 2D) at day 2 after PH. Downregulation of cyclin D1 and PCNA gene expression after Rapa-Dex treatment continued at least until day 5 (80% and 30% reduction respectively,  $P < 0.01$ ). Addition of IL-6/HGF to Rapa-Dex treatment significantly upregulated both cyclin D1 (2.6-fold,  $P = 0.04$  at day 2 and 1.4-fold,  $P = 0.03$  at day 5) and PCNA (1.3-fold,  $P = 0.03$  at day 2) gene expression after PH compared to treatment with Rapa-Dex alone, but did not restore expression to control levels.

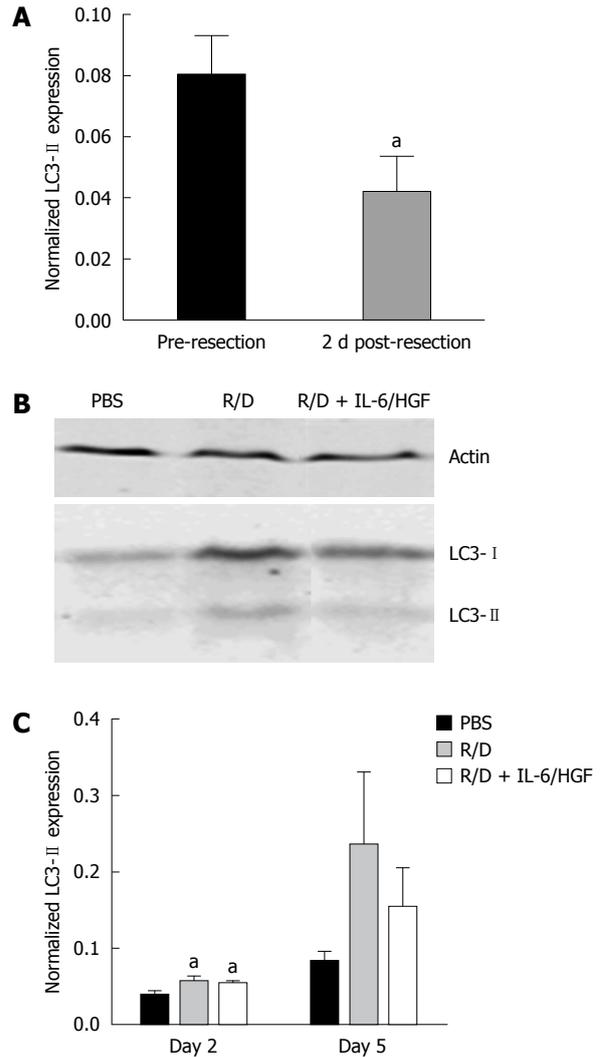
### Inhibition of mTOR increases autophagy and hepatocyte injury during liver regeneration

During autophagy, the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes and therefore a quantitative marker for autophagy. As shown in Figure 3A, LC3-II protein levels in control animals were significantly reduced at day 2 after PH compared to levels before resection (48% reduction,  $P = 0.05$ ). This finding suggests that baseline autophagy levels are reduced during liver regeneration. Compared to control treated animals, animals treated with Rapa-Dex showed a significantly higher LC3-II protein expression at day 2 (46% increase,  $P = 0.04$ ; Figure 3B and C). At day 5, LC3-II levels were back at pre-resection levels in control treated animals, but appeared further increased in Rapa-Dex treated animals. Treatment with Dex alone did not cause significant differences in hepatic LC3-II levels at day 2 (data not shown). Addition of exogenous IL-6/HGF to Rapa-Dex treatment had no significant effect on autophagy compared to Rapa-Dex alone, as LC3-II protein levels remained significantly elevated.

As shown in Figure 4A-C, treatment with Rapa-Dex furthermore significantly increased serum AST levels at day 2 (641 U/L *vs* 185 U/L,  $P = 0.02$ ) and caused a non-significant increase in ALT and bilirubin levels, compared to control treatment. Treatment with Dex alone did not cause changes in serum levels of these liver injury markers. Combined treatment with Rapa-Dex and IL-6/HGF significantly elevated levels of AST (1387 U/L,  $P < 0.01$ ), ALT (823 U/L *vs* 67 U/L,  $P < 0.01$ ) as well as bilirubin (39  $\mu\text{mol/L}$  *vs* 18  $\mu\text{mol/L}$ ,  $P = 0.04$ ). In accordance with serum levels of these injury markers, treatment with Rapa-Dex, either with or without IL-6/HGF, caused progressive changes in liver histology with formation of necrotic areas (Figure 4D).

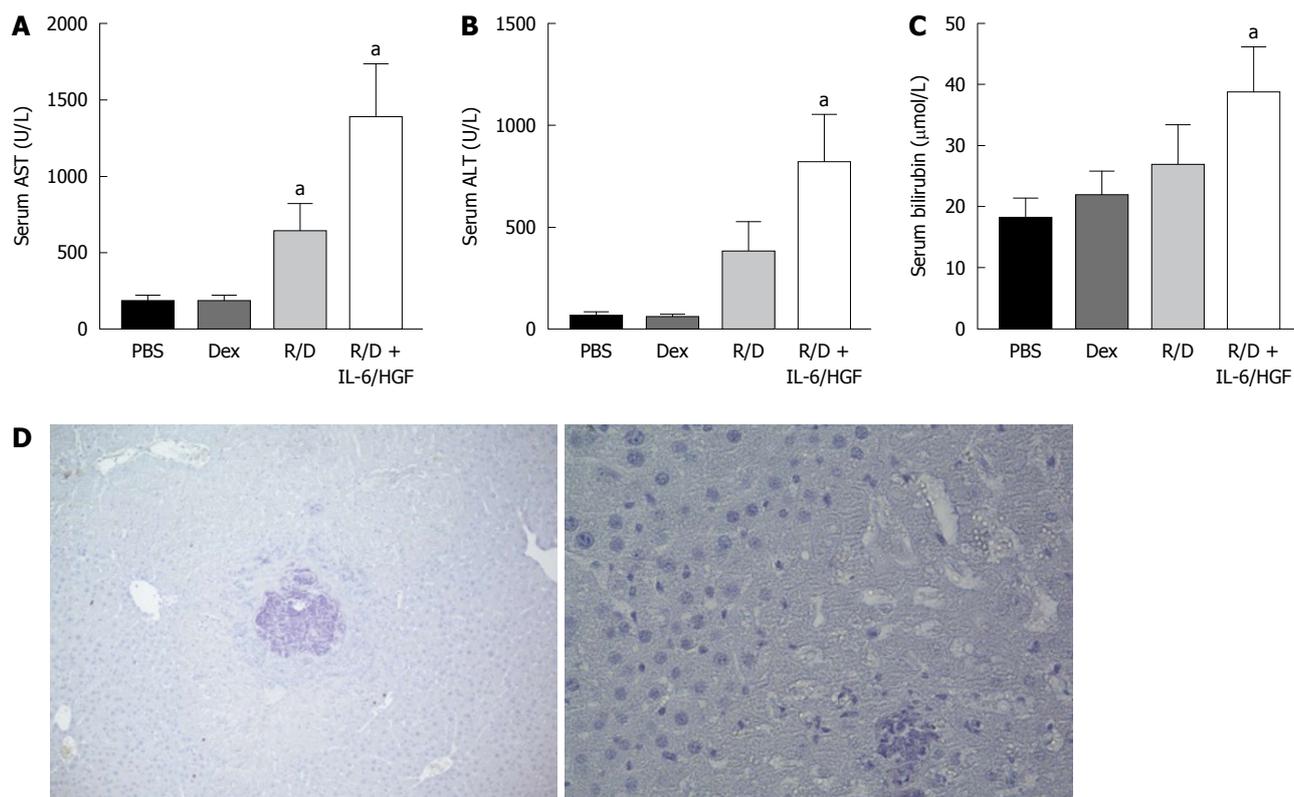
### mTOR inhibition alters expression of genes relevant for cell proliferation and inflammation

At day 2 after PH, treatment with Rapa-Dex significantly

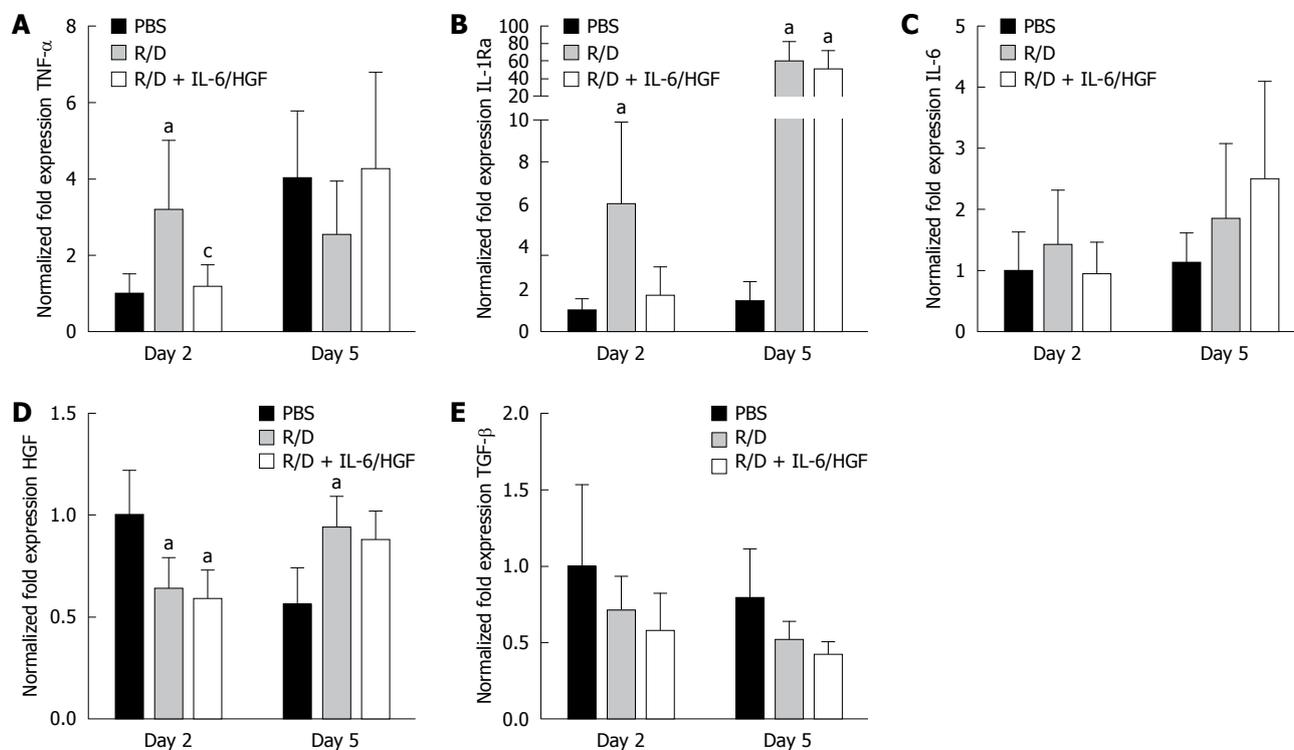


**Figure 3** Effects of partial hepatectomy and mammalian target of rapamycin inhibition on hepatic autophagy. Hepatic protein levels of the autophagy marker microtubule-associated protein 1 light chain 3 (LC3)-II were determined by Western blotting analysis and normalized against actin. A: Effects of liver resection on autophagy at day 2 after partial hepatectomy (PH); B: Western blotting showing effects of mammalian target of rapamycin inhibition on autophagy at day 2 after PH; C: Quantification of autophagy at day 2 and 5 after PH. Data are shown as mean  $\pm$  SE. <sup>a</sup> $P \leq 0.05$  vs phosphate buffered saline (PBS). R/D: Rapa-Dex; HGF: Hepatocyte growth factor; IL-6: Interleukin 6.

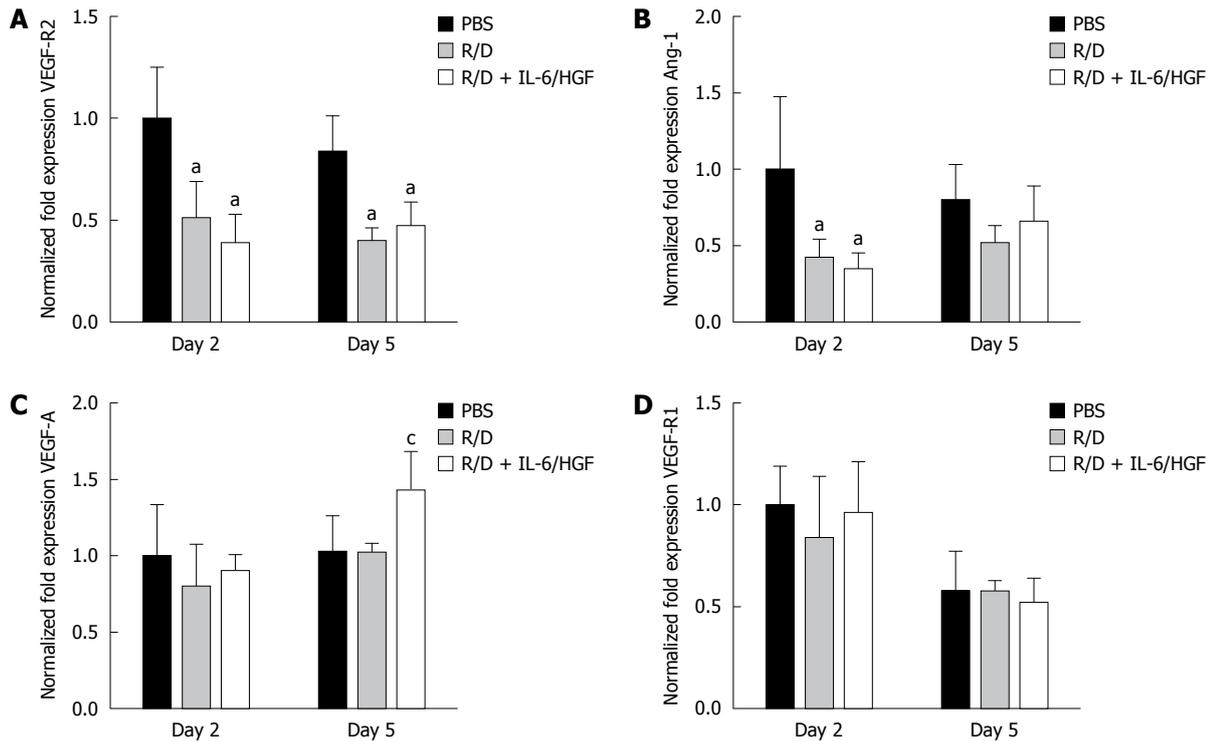
upregulated hepatic gene expression of the pro-inflammatory cytokine TNF- $\alpha$  (3.2-fold,  $P = 0.03$ ; Figure 5A) and the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra; 6.0-fold,  $P < 0.01$ ; Figure 5B) compared to control treatment. No significant effects were seen for IL-6 gene expression (Figure 5C). In contrast, gene expression of HGF was significantly downregulated (40% reduction,  $P < 0.01$ ; Figure 5D), whereas the observed reduced expression of transforming growth factor  $\beta$  (TGF- $\beta$ ) was not statistically significant (Figure 5E). Addition of IL-6/HGF to Rapa-Dex treatment restored the upregulated expression of TNF- $\alpha$  and IL-1Ra to control levels. Combined treatment did however not reverse the downregulated expression of HGF or TGF- $\beta$ . At day 5, treatment with Rapa-Dex led to progressive upregulation



**Figure 4** Effects of mammalian target of rapamycin inhibition on hepatocyte injury. Serum levels at day 2 after partial hepatectomy (PH) for aspartate aminotransferase (AST) (A), alanine aminotransferase (ALT) (B) and bilirubin (C); D: Histologic changes (× 400) at day 5 after PH in liver tissue from Rapa-Dex (R/D) treated animals. Data are shown as mean ± SE. <sup>a</sup>*P* ≤ 0.05 vs phosphate buffered saline (PBS). HGF: Hepatocyte growth factor; IL-6: Interleukin 6.



**Figure 5** Effects of mammalian target of rapamycin inhibition on inflammation and cell cycle related gene expression. Hepatic gene expression levels were determined by quantitative reverse transcription-polymerase chain reaction and normalized against TATA binding protein. A: Expression levels of tumor necrosis factor α (TNF-α) at day 2 and 5 after partial hepatectomy (PH); B: Expression levels of interleukin 1 receptor antagonist (IL-1Ra) at day 2 and 5 after PH; C: Expression levels of interleukin 6 (IL-6) at day 2 and 5 after PH; D: Expression levels of hepatocyte growth factor (HGF) at day 2 and 5 after PH; E: Expression levels of transforming growth factor β (TGF-β) at day 2 and 5 after PH. Data are shown as mean ± SE. <sup>a</sup>*P* ≤ 0.05 vs phosphate buffered saline (PBS); <sup>c</sup>*P* ≤ 0.05 vs Rapa-Dex (R/D).



**Figure 6** Effects of mammalian target of rapamycin inhibition on angiogenic gene expression. Hepatic gene expression levels were determined by quantitative reverse transcription-polymerase chain reaction and normalized against TATA binding protein. A: Expression levels of vascular endothelial growth factor receptor 2 (VEGF-R2) at day 2 and 5 after partial hepatectomy (PH); B: Expression levels of angiopoietin 1 (Ang-1) at day 2 and 5 after PH; C: Expression levels of VEGF-A at day 2 and 5 after PH; D: Expression levels of VEGF-R1 at day 2 and 5 after PH. Data are shown as mean  $\pm$  SE. <sup>a</sup> $P \leq 0.05$  vs phosphate buffered saline (PBS); <sup>c</sup> $P \leq 0.05$  vs Rapa-Dex (R/D). HGF: Hepatocyte growth factor; IL-6: Interleukin 6.

of IL-1Ra gene expression (42.3-fold,  $P < 0.01$ ) as well as upregulation of HGF gene expression (1.7-fold,  $P = 0.03$ ) compared to control treatment. Addition of IL-6/HGF to Rapa-Dex could not restore IL-1Ra and HGF gene expression at this time point.

#### Treatment with Rapa-Dex impairs pro-angiogenic gene expression

As shown in Figure 6, treatment with Rapa-Dex significantly downregulated hepatic gene expression levels of vascular endothelial growth factor receptor 2 (VEGF-R2; 50% reduction,  $P = 0.01$ ) and angiopoietin 1 (Ang-1; 60% reduction,  $P < 0.01$ ) at day 2 after PH compared to control treatment. Downregulation of VEGF-R2 expression continued at least until day 5 (50% reduction,  $P < 0.01$ ). Addition of IL-6/HGF to Rapa-Dex treatment did not affect the downregulated expression levels of VEGF-R2 or Ang-1. Gene expression levels of VEGF-A and VEGF-R1 were not significantly reduced after Rapa-Dex treatment.

## DISCUSSION

Current immunosuppressive strategies in the first period after liver transplantation mostly involve treatment with steroids in combination with mycophenolic acid, IL-2 receptor antagonists or calcineurin inhibitors<sup>[31]</sup>. These regimes are however associated with chronic renal failure, with an incidence of up to 20% kidney dysfunction over

time<sup>[18]</sup>. The mTOR inhibitor and immunosuppressant rapamycin, in contrast to the calcineurin inhibitors tacrolimus and cyclosporin, does not cause nephrotoxicity and is suggested to be a good alternative in transplant patients with deteriorating renal function<sup>[32-34]</sup>.

Recently, mTOR inhibition has gained wide interest in the treatment of cancer<sup>[35,36]</sup>. Therefore, also in patients transplanted for hepatocellular carcinoma, mTOR inhibitors are an attractive alternative with reported inhibitory effects on tumor growth and recurrence<sup>[37-40]</sup>. However, mTOR is a key regulator of cell growth and proliferation and its inhibition is reported to have detrimental effects on liver regeneration<sup>[23-25]</sup>. There may however be a more intricate relation as mTOR also regulates metabolism and inhibition of mTOR may preserve energy supplies for the remaining hepatocytes after liver resection to keep up metabolic function. This is supported by a recent publication showing excellent results in patients treated *de novo* with rapamycin after living-donor liver transplantation as well as data from animal experiments showing no increase in mortality with rapamycin treatment, even after a 90% liver resection and despite inhibited hepatocyte proliferation<sup>[41,42]</sup>.

Additionally, mTOR has been implicated to be of paramount importance in the control of autophagy, a general term for pathways in which cytoplasmic material, including soluble macromolecules and organelles, are delivered to lysosomes for degradation<sup>[13,43-45]</sup>. Autophagy is thought to have evolved as a stress response mechanism

that allows organisms to survive during harsh conditions, probably by regulating energy homeostasis<sup>[16]</sup>. Early histomorphologic studies showed a decrease in autophagic bodies of up to 98% at day 1 after PH<sup>[46-48]</sup>. This can support the hypothesis that the inhibition of intracellular autophagic degradation in regenerating liver has its biochemical equivalent, *i.e.*, inhibited protein catabolism, and is interpreted as an important and adequate mechanism to shift from the physiological steady state to compensatory growth of the liver after PH. Degli Esposti *et al*<sup>[49]</sup> showed the presence of autophagy in 21% of good functioning human liver grafts 2 h after reperfusion, without differences between normal and steatotic livers. Ischemic preconditioning in this study increased autophagy only in steatotic livers, which appeared to have a protective effect on post-operative function. Wang *et al*<sup>[50]</sup> showed that autophagy is essential for hepatocyte resistance to oxidant stress and that loss of macroautophagy led to overactivation of the c-Jun N-terminal kinase signaling pathway that induced cell death. Therefore we studied the interplay between liver regeneration, mTOR inhibition and autophagy in a transplant-related 70% PH model. In accordance with the findings of others, we found a significant decrease in proliferating hepatocytes from 12% to 2% after mTOR inhibition, with concomitant decreases in hepatic gene expression of the cell cycle genes cyclin D1 and PCNA<sup>[25,42,51]</sup>. This was furthermore accompanied by increased serum transaminases, suggesting increased liver injury.

Rupertus *et al*<sup>[40]</sup> recently described that rapamycin had no detrimental effects on liver regeneration, yet in their study hepatocyte proliferation was not actually measured, but only estimated from wet liver weight at 12 d after hepatectomy. In our experiment, wet liver weight after mTOR inhibition was still lower at day 5 after liver resection. In the study of Dahmen *et al*<sup>[42]</sup> BrdU incorporation decreased from 17% to less than 1% at 2 d after 90% hepatectomy, without effects on survival. In the study of Palmes *et al*<sup>[25]</sup> the same effects were found, with decreased gene expression levels of TNF- $\alpha$ , HGF and TGF- $\beta$  at day 2 after a 70% liver resection. Interestingly, in our series, we found a significant upregulation of TNF- $\alpha$ , downregulation of HGF, but no significant changes in IL-6 and TGF- $\beta$  gene expression.

Similar to the Palmes study, gene expression of the angiogenic factors VEGF-R2 and Ang-1 was downregulated in our experiments. Inhibition of angiogenesis is suggested to be one of the most relevant mechanisms by which tumor growth and recurrence is inhibited<sup>[39,40]</sup>.

In our study, mTOR inhibition furthermore resulted in a profound upregulation of IL-1Ra gene expression, which was not reported before. IL-1Ra is an anti-inflammatory cytokine, reported to be released in response to both surgical as well as toxic liver injury and to have a protective effect after CCl<sub>4</sub>-induced toxic liver injury<sup>[52-54]</sup>.

We investigated whether the inhibition in hepatocyte proliferation could be overcome by kick-starting the priming phase of liver regeneration by pre-resection

administration of IL-6 and HGF, both described to stimulate liver regeneration, especially in combined treatment<sup>[55-57]</sup>. It appeared that treatment with exogenous IL-6 and HGF partly reversed the negative effects of rapamycin by restoring TNF- $\alpha$  and IL-1Ra gene expression to control levels, significantly increasing gene expression of Cyclin D1 and PCNA and normalizing liver weight reconstitution. However, no significant increase in hepatocyte proliferation was found and serum transaminases were even further elevated, suggesting increased hepatocyte damage. This is in line with the findings of Haga *et al*<sup>[9]</sup>, who found in their model of LPdk1KO mice that the PI3K/PDK1/Akt/mTOR pathway was regulated independent of the IL-6/JAK/STAT3 pathway. An alternative explanation for the increase in liver weight could be cellular hypertrophy *q.* edema, which is supported by the decreased number of hepatocytes per HPF in this treatment group.

For the first time, we describe that mTOR inhibition also significantly increased hepatic autophagy during liver regeneration after PH. Earlier, Kondomerkos *et al*<sup>[58]</sup> showed that mTOR inhibition by rapamycin increased autophagy in the liver and heart of newborn animals. This effect may compensate for the decreased hepatocyte proliferation, as increased autophagy ameliorates oxidative stress and saves cellular energy.

Finally, the ongoing loss of body weight in mice treated with rapamycin is noteworthy. Similar effects of rapamycin on body weight have previously been reported by DiJoseph *et al*<sup>[59]</sup> and Zafar *et al*<sup>[60]</sup>. The role of mTOR in metabolism is complicated; it has been described that chemical inhibitors of glycolysis and mitochondrial function suppress mTORC1 activity, indicating that mTORC1 senses cellular energy<sup>[35]</sup>. This is crucial, because mTORC1-driven growth processes consume a large fraction of cellular energy and thus could be deleterious to starving cells. The mTORC1 pathway indirectly senses low ATP by a mechanism that is centred on the AMP-activated protein kinase<sup>[61]</sup>. During starvation, mTOR must be downregulated to avoid energy expenditure in absence of nutrients. Therefore pharmacological inhibition of mTORC1 could lead to a defective energy sensing system, mimicking starvation. On the other hand, rapamycin, as mTORC1 inhibitor, may protect the regenerating liver through this mechanism by slowing down the anabolic processes and saving energy and this may account for the fact that animals survive, despite seriously hampered liver regeneration.

In summary, this study investigated the role of mTOR in liver regeneration *in vivo* and more specific in IL-6 and HGF stimulated signaling pathways. mTOR inhibition resulted in inhibited liver regeneration and increased hepatic autophagy. Although exogenously administered IL-6 and HGF could overcome the rapamycin-induced inhibited reconstitution of liver mass and furthermore upregulated gene expression of factors known to be downstream of mTOR, no significant beneficial effects on body weight, hepatocyte proliferation, autophagy or

markers of liver injury were seen. To interpret these data on mTOR inhibition in relation to the clinical setting of living-donor liver transplantation, it is important to realize that the model used is limiting in that it is purely a liver regeneration model without ischemia and reperfusion injury or alloreactivity. However, from these results, the use of mTOR inhibitors in the early post-transplant setting can currently not be recommended, despite their recently reported beneficial effects on cancer development and kidney function.

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## COMMENTS

### Background

The liver has a remarkable regenerative capacity to compensate for lost or damaged liver tissue after injury. This process enables living-donor liver transplantation, a setting in which 40%-60% of the liver of a healthy donor is transplanted into a recipient with end-stage liver disease. Treatment of the recipient with immunosuppressive medication is necessary to prevent rejection of the liver graft. Inhibition of the protein mammalian target of rapamycin (mTOR) represents an important immunosuppressive strategy. In the initial phase after living-donor liver transplantation, the mTOR inhibitor rapamycin is rarely used, as mTOR is a key regulator of cell growth and proliferation and concerns have been raised regarding adverse effects on liver regeneration. However, the exact mechanisms by which mTOR inhibition attenuates liver regeneration are largely unknown.

### Research frontiers

The mTOR inhibitor rapamycin, in contrast to most immunosuppressive agents, does not cause nephrotoxicity and has recently gained wide interest in the treatment of cancer. mTOR inhibitors are therefore an attractive alternative in patients with deteriorating kidney function and also in patients transplanted for hepatocellular carcinoma. Furthermore, besides being a key regulator of cell growth and proliferation, mTOR was recently identified to play an important role in the control of autophagy. Autophagy is a degradation pathway that plays a protective role in case of cellular injury. It has been implicated as a survival response as well as a mediator of cell death during stress conditions, and might therefore play a role in liver regeneration.

### Innovations and breakthroughs

Previous studies have reported detrimental effects of mTOR inhibition on liver regeneration. In contrast, a recent publication shows excellent results in patients treated *de novo* with rapamycin after living-donor liver transplantation. Here we report that mTOR inhibition severely impairs liver regeneration and increases autophagy after liver resection in mice. The most novel finding of this study is that this impaired regeneration can be partly reversed by treatment with the cytokine interleukin 6 (IL-6) and growth factor hepatocyte growth factor (HGF), both described to stimulate liver regeneration, especially if combined.

### Applications

From the authors' results, the use of mTOR inhibitors in the early post-transplant setting can currently not be recommended, despite their recently reported beneficial effects on cancer development and kidney function. However, this study contributes to a better understanding of the role of mTOR and autophagy in liver regeneration and more specific in IL-6 and HGF stimulated signaling pathways.

### Terminology

Regeneration is the process of restoration, growth and renewal that makes cells, tissues or organisms resilient to natural fluctuations or events that cause injury or loss. mTOR is a protein kinase that regulates cell growth, proliferation and survival, as well as protein synthesis and transcription. Autophagy is the basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the lysosomal machinery, thereby enabling recycling of cellular components and ensuring cellular survival during starvation.

## Peer review

The summary is complete and serves to provide the relevant information of the paper. The introduction is adequate. The methodology is descriptive and logical. The results are very well described. The discussion fully satisfies the requirements to compare the results with existing data from the literature. The current literature is related to the topic. The figures are well prepared and properly described.

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