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## Molecular mechanisms of platelet-mediated liver regeneration after partial hepatectomy

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**TRANSIENT VON  
WILLEBRAND  
FACTOR-MEDIATED  
PLATELET INFLUX  
STIMULATES LIVER  
REGENERATION  
AFTER PARTIAL  
HEPATECTOMY IN  
MICE**

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## Abstract

**Background & aims:** In addition to their function in thrombosis and hemostasis, platelets play an important role in the stimulation of liver regeneration. It has been suggested that platelets deliver mitogenic cargo to the regenerating liver, and accumulation of platelets in the regenerating liver has been demonstrated. We studied kinetics of platelet influx in the regenerating liver and investigated the signal that initiates platelet influx.

**Methods:** We visualized platelets in the liver remnant after partial hepatectomy in mice using intravital microscopy and assessed liver regeneration by examination of liver/body weight ratio and the number of proliferating hepatocytes examined by immunohistochemistry.

**Results:** We demonstrated rapid but transient platelet influx into the liver remnant after a partial liver resection. Liver regeneration in thrombocytopenic mice was substantially impaired as evidenced by a reduced liver-to-body weight ratio and decreased numbers of proliferating hepatocytes at day 3 compared to mice with normal platelet counts. In contrast, liver regeneration was only mildly impaired when thrombocytopenia was induced 2 hours after partial liver resection. Platelet influx into the liver remnant was virtually absent in the presence of an antibody to von Willebrand factor (VWF) suggesting that VWF release from liver sinusoidal endothelial cells mediates platelet influx. Additionally, liver regeneration in mice deficient in VWF was markedly impaired.

**Conclusions:** A rapid but transient VWF-dependent platelet influx into the liver remnant drives platelet-mediated liver regeneration.

## Introduction

The liver has a unique regenerative capacity following damage or surgical resection. Liver regeneration starts with a well-organized and complex series of signals which is generated by cytokines and growth factors (1). Accumulating evidence from *in vitro* and *in vivo* studies suggests that platelets have a pivotal role in liver regeneration (2-11). In animal models in which platelets were depleted or functionally impaired, liver regeneration was substantially delayed after a partial liver resection (2,3). Conversely, induction of thrombocytosis stimulated liver regeneration (3,4,12). In a clinical study, we showed that a decreased platelet count is an independent predictor of delayed postoperative liver function recovery after a partial liver resection (10). More recently, it was demonstrated that intraoperative platelet count and platelet transfusion were associated with faster liver regeneration in living donor transplant recipients (11). The molecular mechanisms of platelet-mediated stimulation of liver regeneration are, however, still largely unknown (13).

Platelets bind to liver sinusoidal endothelial cells *in vitro* and this interaction stimulates hepatocyte proliferation (14). In addition, platelets were found to accumulate in the liver parenchyma after a partial liver resection in experimental animal models (3,9). A direct interaction between platelets and hepatocytes is crucial for platelet-mediated stimulation of hepatocyte proliferation *in vitro* (5) but the significance of these findings for liver regeneration *in vivo* are unclear (15). Release of growth factors that are stored within platelets may be responsible for platelet-mediated liver regeneration (5,16,17), but direct evidence from *in vivo* experiments for this is lacking (15,18). For example, release of serotonin from platelet dense granules has been suggested by some to mediate platelet-mediated liver regeneration in mice and humans (2,16). However, although mice lacking serotonin in their platelets have reduced regenerative capacity, this may be explained by a reduced functional capacity of serotonin-deficient platelets rather than by a specific defect in mitogenic activity of the platelets (19). Furthermore, human studies have provided additional evidence against a role of serotonin in liver regeneration in humans (20).

An alternative scenario for platelet mediated liver regeneration involves transfer of RNA from platelets to hepatocytes as has been recently demonstrated in an *in vitro* model (9). Although platelet influx of the liver remnant shortly after partial hepatectomy has been well established in rodent models, it is yet unknown what triggers platelet accumulation and whether platelets persist in the liver remnant over time.

It has been shown that levels of the platelet adhesive protein von Willebrand factor (VWF) are increased in plasma following a partial liver resection in rats and in mice (21,22). VWF protein is also highly upregulated in liver sinusoidal endothelial cells following a partial hepatectomy (21). Although the latter finding was interpreted as a potential role for VWF in tissue remodeling during liver regeneration, it may also be that VWF release from liver sinusoidal endothelial cells leads to platelet influx into the liver remnant following partial hepatectomy.

In this study we tested the hypothesis that VWF is involved in platelet influx into the liver

remnant. By use of intravital microscopy we studied the dynamics of platelet influx into the liver sinusoids upon partial liver resection in mice. We demonstrated rapid and transient platelet influx into the liver parenchyma after partial liver resection, which was dependent on VWF.

## Material & Methods

### Partial liver resection in mice

Male C57Bl6 mice (Charles River, Leiden, The Netherlands) of 8-10 weeks of age or male mice deficient in VWF (VWF<sup>-/-</sup>, on a C57BL/6 background (23)) underwent a 70% partial liver resection according to published protocols (24). In sham surgeries, mice underwent an identical procedure with the exception of ligation and removal of the liver lobes. Surgical procedures were performed under isoflurane inhalation anesthesia (Abbott, Chicago, IL). Thrombocytopenia was induced by intravenous injection of a rat monoclonal antibody directed against mouse Gplb $\alpha$  (4 $\mu$ g/g body weight) (Emfret, Würzburg, Germany). In selected experiments, 50 $\mu$ g of the polyclonal rabbit anti-VWF antibody A0082 (DAKO, Glostrup, Denmark) was intravenously injected 30 minutes prior to resection. The Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands approved these studies.

Mice were terminated by exsanguination from the inferior vena cava after injection of 150 $\mu$ l 3.4% sodium citrate (Merck, Germany) diluted in NaCl (0.9%) in the spleen. Collected blood samples were centrifuged at 1400g for 10 minutes (without brake) to obtain plasma and were stored at -80°C. Livers were fixed in 4% formaldehyde or were snap frozen in liquid nitrogen for immunohistochemical analyses.

### Confocal intravital microscopy

Platelets were imaged in living mice shortly after the hepatectomy by intravital microscopy as described previously (25). Platelets were labeled *in vivo* by intravenous injection of 1.6 $\mu$ g phycoerythrin (PE)-conjugated hamster anti-mouse CD49b (clone HMA2) (BD Pharmingen, San Diego, CA) just prior to an imaging session as described (25). After partial liver resection or sham operation the animal was placed in a right lateral position on an adjustable microscope stage. Mouse body temperature was maintained at 37 °C. After the liver was exteriorized, it was placed on the inverted microscope, the liver surface was covered with a small piece of saline-soaked KimWipe (Kimberly-Clark, Roswell, GA) to keep the organ moist and hold the organ in position. Image acquisition was started as soon as possible and was performed for 1 hour. Alternatively, mice were examined after 4 hours, 1 day or 3 days after the hepatectomy by relaparotomy. We used two different confocal microscope set-ups in this study. The first set-up has been described previously (25). Another set of experiments was performed using an inverted Zeiss LSM 780 NLO microscope (Axio Observer.Z1; Carl Zeiss, Ulm, Germany) equipped with a temperature controlled incubator (XL S1 DARK; Pecon, Erbach, Germany). For these experiments a 20 $\times$ PlanApo, 0.8 NA

objective was used. Images were captured using 488nm Argon laser and a gallium arsenide phosphide (GaAsP) spectral detector (Carl Zeiss, Ulm, Germany) at 508 nm to 561 nm for autofluorescence detection, revealing the vasculature, and 569 nm to 655 nm for PE-detection. Hardware control was via the ZEN Black acquisition software (Carl Zeiss, Ulm, Germany).

### Intravital microscopy image processing and platelet aggregate analysis

For IVM data analysis, tif images were exported from the Volocity (Improvision Inc., Lexington, MA) acquisition software or from ZEN Black software. Images for platelet aggregate quantification were imported directly into ImageJ (version 1.45; US National Institutes of Health) and image contrast was set to maximum for sharp definition of the borders of each platelet aggregate. The same settings were applied to images from all treatment groups within a single experiment. Analysis of platelet aggregates was performed using the Analyze Particles function within ImageJ. Videos underwent contrast enhancement within the acquisition software package, adjusting the Black Point for each fluorescence channel. Again, the same settings were applied to the videos of all treatment groups within a given experiment. Videos were exported as .avi files and were converted to an appropriate size, resolution, and frame rate using Microsoft Movie Maker (Microsoft, San Jose, CA). A platelet aggregate was defined as a positive signal of 10 pixels (1  $\mu$ m) or more.

### Immunohistochemistry

Deparaffinized liver sections were subjected to antigen retrieval. Ki-67 sections were incubated for 20 minutes in boiling Tris/EDTA buffer, pH 9.0. Endogenous peroxidase was blocked by 3% H<sub>2</sub>O<sub>2</sub> for 30 minutes. Sections were incubated with monoclonal rabbit anti-Ki67 antibody (1:200 in TBS + 1%BSA) (Abcam, Cambridge, UK) at 4°C for at least 16h. Next, a secondary peroxidase-conjugated goat-anti-rabbit antibody (1:100, DAKO, Glostrup, Denmark) and a tertiary peroxidase-conjugated rabbit-anti-goat antibody (1:100, DAKO, Glostrup, Denmark) were used. Prior to the incubation with the secondary and tertiary antibody normal rabbit serum (1:100) was added for 30 minutes. The peroxidase activity was visualized by a 10 minute incubation in 3,3-diaminobenzidine tetrachloride (Sigma, St.Louis, MO). Subsequently the sections were counterstained for 1 minute with haematoxylin and mounted with Kaiser's glycerin gelatin. Ki-67-positive hepatocytes were manually counted in at least 5 high-power fields per mouse and expressed as percentage of all hepatocytes.

### Statistical analysis

Statistical analysis was performed with the GraphPad Prism 5 (San Diego, CA) software package. Continuous variables were expressed as mean  $\pm$  SD or median and range. Values are representative of at least 3 independent experiments performed in triplicate. Continuous data were tested for normality and analyzed by t-test, Mann-Whitney U-test or one-way ANOVA, as appropriate. A P value of less than 0.05 was considered statistically significant.

## Results

### Transient platelet influx into the liver remnant immediately after partial liver resection

We studied platelet influx after partial liver resection in mice using intravital microscopy. Following partial liver resection, an immediate platelet influx in the remnant liver was observed (Fig. 1A). Platelets appeared to attach to the endothelial cells and formed both small and larger aggregates in the sinusoid, but the aggregates never became occlusive (Supplementary video 1). The aggregates were unstable as platelets or groups of platelets frequently detached from these aggregates, and continuous reattachment of new platelets was observed. In sham-operated mice some platelets transiently attached to the endothelial cells but the quantity and size of platelet aggregates was substantially less compared to the mice that underwent partial hepatectomy (Fig. 1A, supplementary video 2). Intravital imaging at 4h, 1 day, and 3 days after liver resection showed minimal platelet deposition compared to the early phase (<1h) after liver resection (Fig. 1B, supplementary video 3).

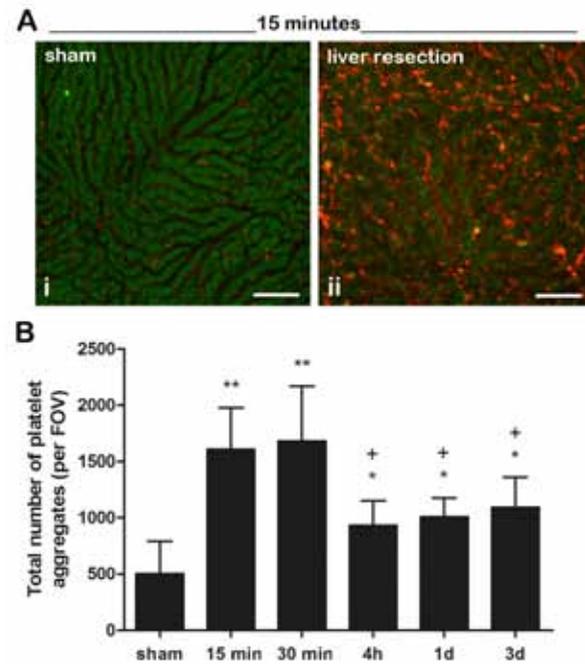


Figure 1: Platelet influx into the liver remnant immediately after partial liver resection

(A) Representative intravital microscopy images of livers of c57/bl6 mice at 15 minutes after a partial liver resection or sham operation. Platelets were labeled in vivo with PE-conjugated anti-CD49 (red). The autofluorescence signal of the liver is displayed to visualize liver anatomy (green). Original magnification 200x. Scale bar denotes 50 $\mu$ m.

(B) Quantification of the total number of platelet aggregates in the sinusoids of the liver remnant. Platelet aggregates were quantified in sham operated mice at 15 minutes after the sham surgery and at different time points after partial liver resection. \*P < 0.05, \*\*P < 0.01 versus sham. + P < 0.05 versus 30 min hepatectomy. Data represent the mean of three animals. Error bars indicate SD.

### Transient platelet influx stimulates liver regeneration

To study whether the transient influx of platelets following a partial hepatectomy is sufficient to stimulate liver regeneration, we compared liver regeneration in mice that were rendered thrombocytopenic 2 hours prior to or two hours after a partial hepatectomy. Intravenous injection of platelet-depleting antibodies resulted in a >90% reduction in platelet count within 15 minutes (data not shown). Compared to mice with a normal platelet count, mice that were rendered thrombocytopenic prior to partial hepatectomy showed markedly impaired regeneration as evidenced by a reduced liver/body weight ratio (Fig. 2A) and a reduction in ki67-positive cells at day 3 after partial hepatectomy (Fig. 2B-C). In contrast, liver regeneration was only marginally impaired in mice that were rendered thrombocytopenic 2 hours after partial hepatectomy. Importantly, no excessive perioperative bleeding was observed in thrombocytopenic animals, nor was there evidence of bleeding observed during termination.

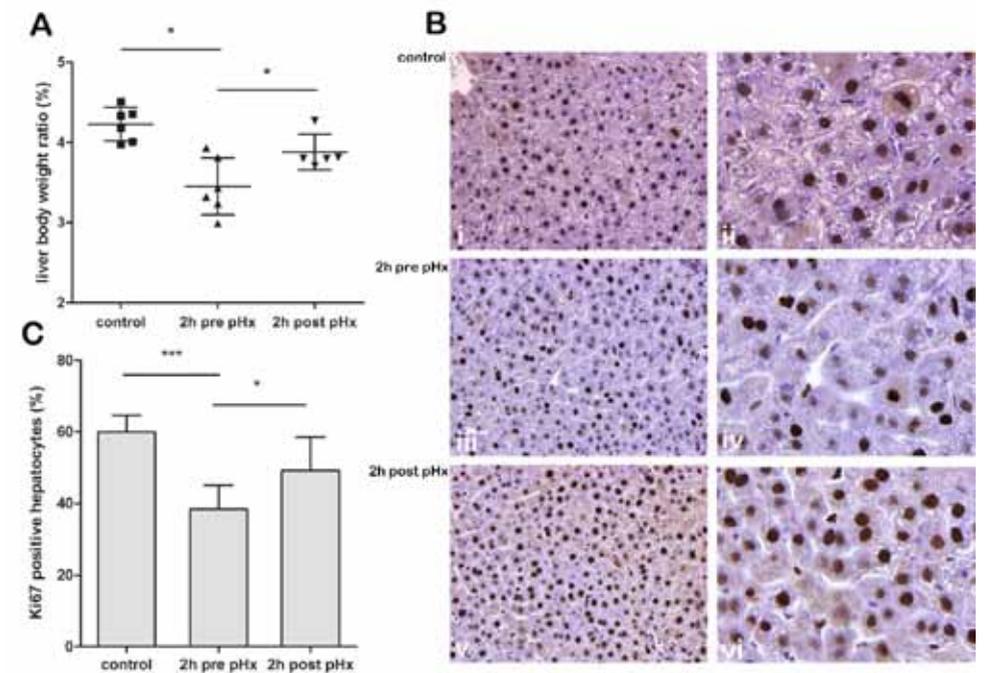


Figure 2: Transient platelet influx stimulates liver regeneration.

(A) Quantification of liver to body weight ratio in mice 3 days after partial liver resection. Thrombocytopenia was induced either 2h prior to or 2h after partial liver resection (pHx) and compared to untreated (control) mice. \*P < 0.05. Horizontal bars represent means. Error bars indicate SD.

(B) Immunohistochemical staining of Ki-67 on liver paraffin sections. Mice were sacrificed 3 days after partial liver resection. (i, ii) Ki-67 staining on mice with a normal platelet count (i) original magnification 200x, (ii) original magnification 400x, (iii, iv) mice that were rendered thrombocytopenic two hours prior (iii) 200x, (iv) 400x and (v, vi) mice that were rendered thrombocytopenic two hours after partial liver resection (v) 200x, (vi) 400x. Images are representative for six animals.

(C) Quantification of Ki-67-positive hepatocytes in groups represented in (B). \*\*\*P < 0.001. \*P < 0.05. Data represent the mean of six animals. Error bars indicate SD.

### Platelet influx into the liver is mediated by VWF

To assess the role of VWF in platelet influx into the liver remnant, we blocked VWF function by a polyclonal antibody to VWF and performed intravital imaging of platelets immediately following partial hepatectomy. As shown in figure 3, VWF blockade substantially reduced platelet influx following partial hepatectomy to levels similar to that observed in sham operated mice (Fig. 3A). We observed a significant reduction of the number of platelet aggregates after partial liver resection when VWF was inhibited (Fig. 3B).

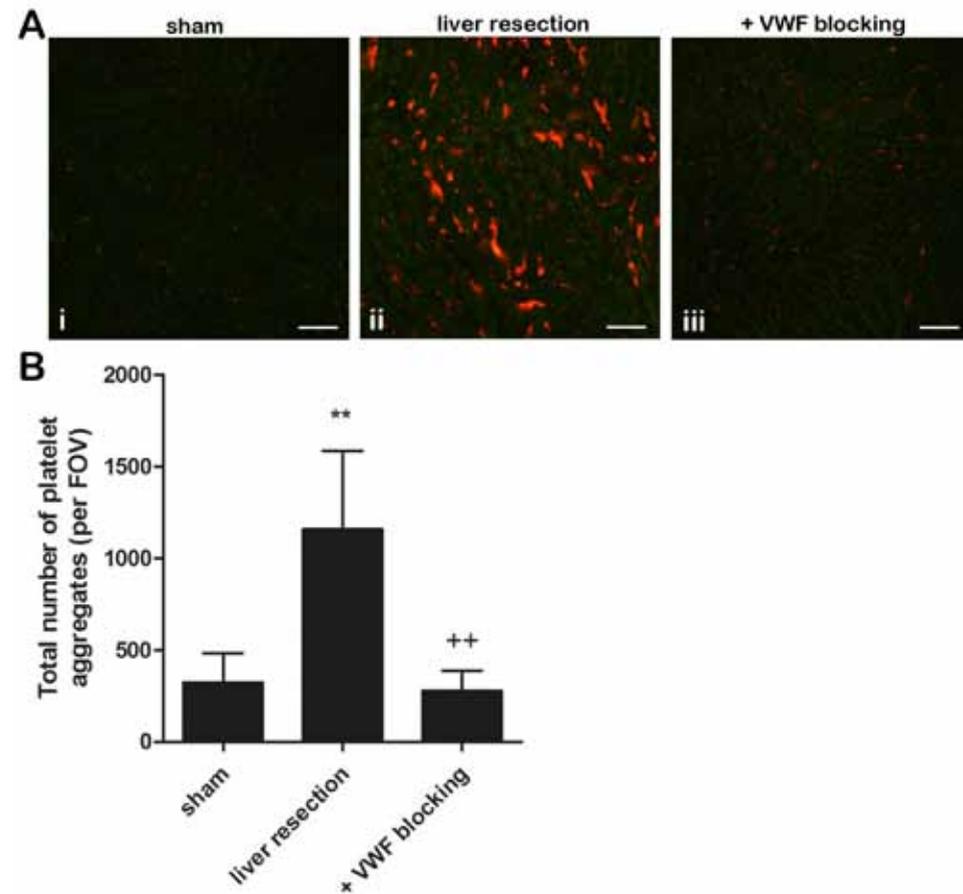


Figure 3: VWF mediates platelet influx into the liver

(A) Representative intravital microscopy images of c57/bl6 control mice or mice treated with an inhibitory antibody to VWF. Platelets were labeled in vivo with PE-conjugated anti-CD49 (red). The autofluorescence signal of the liver is displayed to visualize liver anatomy (green). (i) 15 minutes after sham operation (ii) 15 minutes after partial liver resection (iii) or 15 minutes after partial liver resection in the presence of an inhibitory antibody to VWF. Original magnification 200x. Scale bar denotes 50µm.

(B) Quantification of the total number of platelet aggregates in the sinusoids of the liver remnant. Platelet aggregates were analyzed in sham operated mice (n=3), mice that underwent partial liver resection (n=6) and mice that were treated with a blocking VWF antibody prior to partial liver resection (n=6). \*\*P < 0.01 vs sham. ++ P < 0.01 vs liver resection. Error bars indicate SD.

### VWF deficiency impairs liver regeneration

As VWF mediates transient platelet influx following partial hepatectomy and transient platelet influx stimulates liver regeneration, we next studied liver regeneration in VWF deficient mice. The liver/body weight ratio was similar in WT and VWF<sup>-/-</sup> mice at day 1 after partial hepatectomy, but was significantly higher in WT compared to VWF<sup>-/-</sup> mice at day 3 (Fig. 4A). In line with these results, the proportion of ki-67-positive hepatocytes was substantially higher in WT compared to VWF<sup>-/-</sup> at day 3 (Fig 4B-C). Importantly, no excessive perioperative bleeding was observed in VWF<sup>-/-</sup> mice, nor was there evidence of bleeding observed during termination.

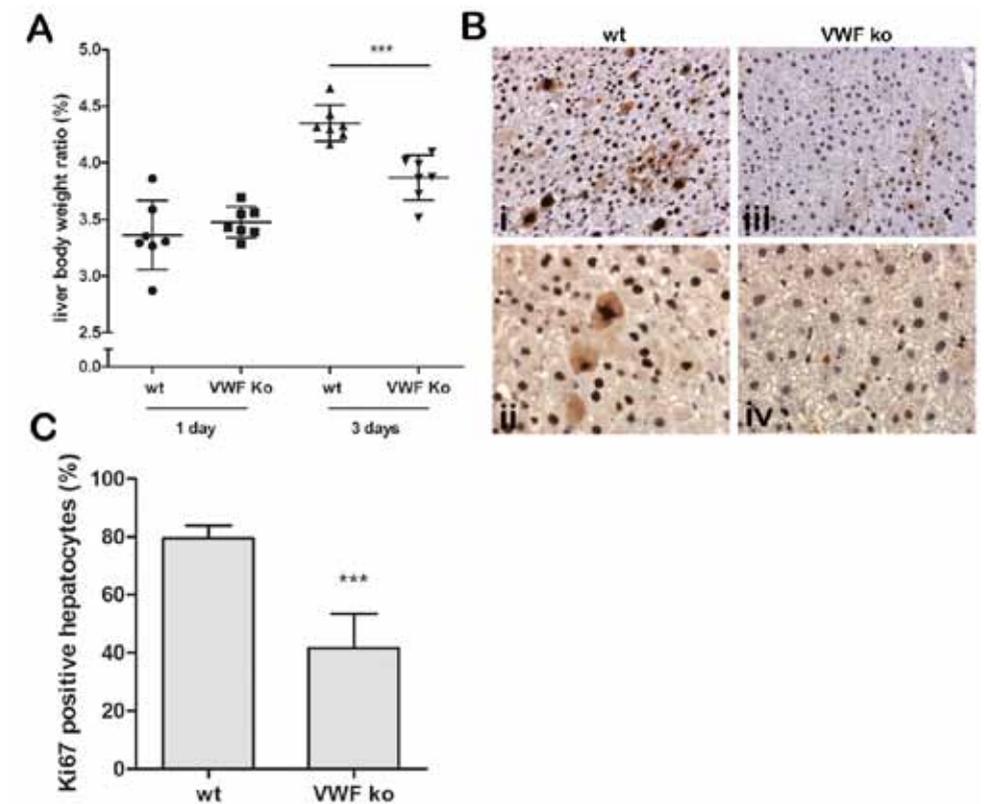


Figure 4: Mice deficient in VWF show impaired liver regeneration

(A) Liver to body weight ratio of WT mice compared to VWF knock out mice at day 1 and day 3 after liver resection. \*\*\*P < 0.001. Horizontal bars indicate means. Error bars indicate SD.

(B) Immunohistochemical staining of Ki-67 on liver sections from mice 3 days after partial liver resection. Representative images of Ki-67 staining of WT mice (i) original magnification 200x, (ii) original magnification 400x and of VWF knock out mice (iii) 200x, (iv) 400x.

(C) Quantification of the percentage Ki-67-positive hepatocytes in WT mice and VWF knock out mice at day 3 after partial liver resection. \*\*\*P < 0.001. Data represent the mean of seven animals. Error bars indicate SD.

## Discussion

Using intravital imaging, we have demonstrated transient influx of platelets in the liver within 15 minutes after a partial hepatectomy in mice. Platelet accumulation within the liver was critically dependent on VWF. Early platelet accumulation within the liver sinusoids appears sufficient to support liver regeneration as removal of >95% of the circulating platelets 2 hours after partial hepatectomy had little effect on liver regeneration, whereas regeneration was substantially inhibited when platelets were depleted just prior to resection. In addition, decreased liver regeneration in VWF-deficient mice supports the notion that platelet recruitment to the liver remnant, mediated by VWF, is critical in platelet-mediated liver regeneration.

Our study confirms that platelets accumulate in the sinusoids immediately after partial hepatectomy (3) and importantly, we show that within one hour after partial hepatectomy platelet aggregates have largely disappeared again. Using post-hepatectomy platelet depletion we show that platelets are only required in the early period after partial hepatectomy for stimulation of liver regeneration. These combined results suggest that platelets deliver mitogenic signals within the liver early after partial hepatectomy. Whether such signals involve growth factors secreted from platelet granules (5), RNA transfer (9), or a yet-to-be identified mitogenic stimulus remains to be determined.

Our study also extends previous data on the role of VWF in liver regeneration (21). As it had been previously established that VWF levels in plasma and within the liver rapidly rise following a partial hepatectomy (21,22), we surmised that VWF may be involved in platelet accumulation in the regenerating liver. Indeed, both platelet accumulation and liver regeneration were strongly dependent on VWF, although we have not conclusively shown that LSEC (and not plasma or platelet VWF) mediates these processes. We have recently demonstrated that plasma VWF levels increase and levels of the VWF-cleaving protease ADAMTS13 decrease following partial hepatectomy in humans (26). Acute VWF release within the liver accompanied by inadequate or delayed removal of reactive VWF molecules by decreased ADAMTS13 levels may explain the transient VWF-dependent platelet accumulation after partial hepatectomy.

The mechanisms leading to VWF release from liver sinusoidal endothelial cells are incompletely understood but may include hemodynamic effects as the blood flow through the liver is substantially increased by partial hepatectomy. Regardless of the mechanism involved, stimulation of VWF release, for example by administration of 1-desamino-8-D-arginine vasopressin (DDAVP) might be an interesting clinical approach to stimulate liver regeneration. In conclusion, we find rapid and transient platelet influx into the liver remnant following a partial hepatectomy in mice in a process dependent on VWF. These results suggest that platelets deliver mitogenic cargo rapidly after liver resection, and knowledge of the mechanism behind this rapid and transient platelet influx may assist development of novel strategies to support liver regeneration.

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## Conflict of interest statement

None of the authors have a conflict of interest to report.

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