Hedgehog pathway mediates early acceleration of liver regeneration induced by a novel two-staged hepatectomy in mice

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Abbreviations: ALPPS: Associating Liver Partition and Portal Vein Ligation Surgery, PVL: Portal Vein Ligation, Ts: Transection, LLLx: Left lateral lobe resection, Ihh: Indian hedgehog, pH3: phosphohistone 3, SFSS: small-for-size-syndrome, TSH: two-stage hepatectomy, FLR: functional liver remnant.

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Lay abstract

ALPPS, a novel two-staged hepatectomy, massively accelerates liver regeneration, thereby helping treat patients with otherwise unresectable liver tumors. The molecular mechanisms behind this accelerated regeneration are unknown. Here, we elucidate that Indian hedgehog, a secreted ligand important for fetal development, is a crucial mediator of the regenerative acceleration triggered by ALPPS surgery.

Abstract

Background & Aims: Occlusion of one liver section by portal vein ligation (PVL) induces compensatory liver regeneration in the remaining part. PVL-induced liver growth is being exploited for the surgical removal of large/multiple liver tumors. A novel approach coined ALPPS (*Associating Liver Partition and Portal vein ligation for Staged hepatectomy*) combines PVL with parenchymal transection and massively accelerates compensatory growth, enabling much faster and more extensive tumor removal. Regenerative acceleration following ALPPS seems to be mediated by plasma factors, which we sought to identify.

Methods: We compared a mouse model of ALPPS against PVL and additional control surgeries. Gene expression profiling was performed to identify candidate molecules unique to ALPPS liver. Recombinant protein and neutralizing antibody combined with appropriate surgeries were used to explore candidate function in ALPPS.

Results: ALPPS in mouse confirmed formidable acceleration of liver regeneration relative to PVL. Indian hedgehog (Ihh), a secreted ligand inducing hedgehog signaling, was uniquely upregulated in ALPPS liver. Ihh plasma levels rose 4h after surgery, along with hedgehog pathway activation and subsequent cyclin D1 induction in the liver. When combined with PVL, Ihh alone was sufficient to induce ALPPS-like acceleration of liver growth. Conversely, blocking Ihh markedly inhibited the accelerating effects of ALPPS. In human plasma, IHH was elevated early after ALPPS surgery.

Conclusions: Ihh and hedgehog pathway activation provide the first mechanistic insight into the acceleration of liver regeneration triggered by ALPPS surgery. The accelerating potency of recombinant Ihh and its elevations in human ALPPS suggest clinical potential for this morphogenic protein.

Introduction

The liver is unique with regards to its regenerative capacity. Its ability to regrow after tissue loss has permitted the surgical removal of large or multiple liver tumors with curative intent. Physiological limits to liver regeneration nevertheless exist. When the functional liver remnant is too small, the liver cannot recover vital function due to deficient hepatocyte proliferation [1, 2]. In the clinic, resection-induced liver failure is known as the Small-for-Size Syndrome (SFSS) and represents the most frequent cause of death due to liver surgery [3, 4].

To reduce the risk of SFSS and to expand the application of surgery, two-staged hepatectomies (TSHs) have been developed. In a first step, the portal veins of one (i.e. the diseased) liver section are occluded, causing compensatory growth of the non-occluded (i.e. healthy) part. The thereby increased liver volume enables extended liver resection in a second step. While the combination of portal vein ligation (PVL) with later resection have become standard strategies [4,5], liver growth after PVL is slow and sometimes insufficient to enable TSH completion or prevent SFSS [6]. Even if successful, several months can pass until resection can be performed, increasing the likelihood of disease progression and complicating surgery due to developing tissue adhesion [7].

In the past few years, a novel two-staged approach coined ALPPS (Associating Liver Partition and Portal vein ligation for Staged hepatectomy) has been designed which has the potential to avoid most of the drawbacks associated with conventional TSHs [8, 9]. ALPPS differs from conventional TSHs in that step one combines PVL with the additional transection (an *in situ* split) along the demarcation between the occluded and non-occluded liver. Intriguingly, the PVL-transection combination causes unprecedented acceleration of compensatory liver growth [8, 9]. Within 9 days after PVL-transection, an average 74% volume increase of the non-occluded lobe has initially been reported [9], greatly reducing the time interval to resection. Extrapolated liver growth rates after ALPPS may be up to 11 times higher than after PVL [7]. In other words, ALPPS enables surgery in patients otherwise deemed as unresectable due to marginal liver remnants with high SFSS risk [9, 7]. Whereas optimal conditions for the application of ALPPS are still being defined, a marginal size of the liver remnant appears to be of small concern for ALPPS [10].

The acceleration of liver regeneration observed after ALPPS inquires for the nature of underlying mechanisms. Consequently, we have developed the first animal model of ALPPS that is based on the adaptation of human surgery to mouse liver architecture [11]. Akin to the human operation, PVL plus transection in mice massively accelerates the regenerative speed compared to PVL alone. When PVL-transection as a first step is completed, the second step (i.e. extended resection) is performed 48h later, leading to completed regeneration within 4 days despite starting from a remnant with only 10% of the original liver volume [11]. In comparison, regeneration after standard 70%-hepatectomy (leaving a 30% remnant) needs about a week for completion in mice, while a 90%-hepatectomy is lethal [1, 2]. Intriguingly, plasma obtained from mice subjected to ALPPS step 1 comparably accelerates regeneration when injected into mice subjected to PVL only [11]. Therefore, ALPPS in mice seems to trigger unknown circulating factors that mediate regenerative acceleration and hence might be of therapeutic interest.

This study was set out to obtain a first insight into the molecular mechanisms underlying ALPPS-triggered acceleration of liver regeneration. In particular, we sought to uncover a plasma factor that is induced by ALPPS step 1 to accelerate PVL-mediated liver regeneration. By using an unbiased expression profiling approach in comparing ALPPS step 1 with PVL, transection, and control surgery, we identified indian hedgehog (Ihh) as a plausible candidate. We then explored the role of Ihh in the acceleration of PVL-induced regeneration and tested the dependency of ALPPS on circulating Ihh. Altogether, we describe circulating Ihh as a key component mediating early acceleration of liver regeneration following ALPPS surgery.

Materials and Methods

Animals

All animal experiments were performed in accordance with Swiss Federal Animal Regulations and approved by the Veterinary Office of Zurich. Male C57Bl/6 mice (Envigo, Horst, NL) aged 10-12 weeks kept on a 12-hour day/night cycle with free access to food and water were used.

Animal surgery and treatment

To mimic human ALPPS surgery in mice, 90% PVL was performed leaving a 10% functional remnant consisting of the left and a part of the right middle lobe. Then, a partial 80% transection was done through the middle lobe along the demarcation line of the occluded/non-occluded parenchyme. The left lateral lobe (LLL, 25% of liver volume) was also resected to simulate the cleaning of the liver from smaller tumors as often done in human ALPPS (Fig. 1A). Left lateral lobe resection (LLLx) does not induce significant regeneration in mouse and is comparable to sham. Details on isoflurane anesthesia, buprenorphine analgesia, ALPPS procedures, and the control operations (90% PVL, 80% transection, LLLx, and sham laparotomy) have been described [11]. After surgery, animals were allowed to recover on a warming pad in a separate cage until completely conscious. Standard (68%) hepatectomy was performed as described [1]. Animals were weighed before surgery and at sacrifice, where weight of resected and remnant liver was recorded. Tissue was formalin fixed or snap frozen, while plasma was stored at -80°C for analysis.

Recombinant Ihh (200 ng/kg, 160µl final volume; R&D, 1705-HH-025/CF Minneapolis, MN) or PBS was injected into the vena cava right after PVL. Ihh antibody (4ug/kg, 160µl final volume; R&D MAB8048) or PBS was injected via tail vein one hour before operation.

Liver regeneration

Liver regeneration was assessed by the percentage of functional-liver-remnant-to-the-body weight ratio (FLR/BW) derived from six animals per group and time point.

Quantitative real-time PCR

Total RNA was extracted from 50 mg of tissue using Trizol reagent following provided instructions (Invitrogen, Basel, Switzerland). RNA quality/quantity was spectrometrically assessed. qPCR was performed with two replicates/sample on cDNA (Thermo Script reverse transcription PCR System, Invitrogen) using an ABI Prism 7500 Sequence Detector system (PE Applied Biosystems Rotkreuz, Switzerland). Taqman gene expression assays are listed in Supplementary Table 1 and were from Applied Biosystems. Results represent mean fold induction ($2^{-\Delta Ct}$) $\pm$ SD relative to the normalization control *18S rRNA* [1].

Immunohistochemistry

Archived liver sections (3 μ m) were stained (antibodies in Supplementary Table 2) using the Dako Autostainer Link48 Instrument and the iView DAB kit (Dako Denmark A/S). Quantification of Ki67- and pH3-positive hepatocytes was done by blinded manual counting in 10 random visual fields (20x).

RNA deep sequencing

Total liver RNA prepared by DNA Column Clean-up (Qiagen, Basel, CH) was deep sequenced by the Functional Genomics Center Zurich using Poly-A selection on the Illumina Hiseq 2500. A QC report was generated and two-group analyses (comparing all procedures normalized to sham) were

done to retrieve differential gene expression data. Data were analyzed for pathways and genes unique to ALPPS through Metacore (filter criteria, $p < 0.05$ and $\log_2 \text{ratio} > 4$). All runs have been deposited to the European Nucleotide Archive (ENA) under the accession number PRJEB15593.

Western blotting

Blotting was performed as described [1]. Antibodies are listed in Supplementary Table 2. NE-PER Nuclear and Cytoplasmatic Extraction reagents (Thermo Fisher Scientific, Waltham, MA, PK207589) were used to isolate subcellular fractions, with their separation ascertained through β -tubulin (cytoplasmic) and lamin B1 (nuclear) blotting.

Hedgehog ligand levels

Ihh/IHH and Shh protein levels were measured by ELISA (mouse Ihh: Cusabio, CSB-E16517m; human IHH: CSB-E12007h; mouse Shh: R&D, MSH00).

Human samples

EDTA-blood was obtained from ALPPS patients (n=6) directly (t=0, baseline) or 2h, 24h, and 48h after transection (step 1). Plasma was stored at -80°C . The use of human samples was approved by the cantonal ethics committee Zürich (KEK-ZH-Nr. 2015-057). Informed consent was obtained from all patients. See Supplementary Table 3 for patient description.

Statistical Analysis.

Data are expressed as mean $\pm$ SD. Differences between groups (n=6/group unless for expression profiling where n=3/group) were determined using unpaired, two-tailed t tests assuming unequal variance via GraphPad Prism v4.0 (GraphPad, San Diego CA), with significance set at $P < 0.05$. Analyses were performed in a blinded way.

Results

The ALPPS model of accelerated liver regeneration

To study mechanisms underlying the ALPPS-associated acceleration of liver regeneration, we used our recently developed mouse model of ALPPS [11]. Liver weight gain induced by ALPPS (here specifically referred to PVL-transection-LLLx, see Fig. 1A and Methods for explanation) was compared to appropriate control surgeries (PVL, transection, LLLx, sham laparotomy) in order to ascertain acceleration of regeneration (see Supplementary Fig 1. for a comparison with hepatectomy). All procedures similarly affected body weight (Fig. 1A). LLLx alone did not induce significant liver weight regain, akin to transection only. As expected, PVL led to a steady weight gain, while ALPPS markedly accelerated regeneration relative to PVL (Fig. 1B). Acceleration was particularly evident during the first 12-24h, and ALPPS elevated the number of hepatocytes positive for Ki67 (cycling cells) and bold pH3 (marking mitotic cells [1]) at 4h and particularly 8h compared to PVL (Fig 1B). Next, we assessed the expression of key cell cycle molecules, the cyclins, most of which were elevated at 8h after ALPPS. *Ccnd1* (encoding cyclin D1, a crucial promoter of the hepatocyte cell cycle [12]) displayed an ALPPS-specific peak at 8h (Fig. 1C) akin to pH3 counts, while the cell cycle inhibitor p21 [1] was downregulated (Fig. 1D, see Supplementary Fig. 2 for immunoblot bands) in ALPPS versus PVL. At 12h however, p21 was upregulated in ALPPS and associated with a drop in Ki67/pH3 counts, perhaps due to a small negative feedback to prevent exuberant proliferation in response to the massive regenerative activity elicited through ALPPS [13]. Together, these findings indicate that ALPPS accelerates liver weight regain and hepatocellular cell cycle at early times after surgery. Therefore, key pathways must operate early after ALPPS to accelerate regeneration.

Indian hedgehog as a candidate plasma factor associated with ALPPS

To obtain unbiased insight into potential ALPPS-associated molecular alterations in the liver, we deep-sequenced hepatic RNA during the first 12h after ALPPS, PVL, transection, LLLx, and sham operation. A distinct gene expression profile was evident for ALPPS as early as 4h post operation (Fig.

2A, Supplementary Fig. 3). At this time point, metacore analysis (Fig. 2B) revealed two pathways unique to ALPPS, the "TGF-beta-dependent induction of EMT via MAPK" ($P=5.9^{-14}$, $FDR=3.18^{-12}$) and the "Sirtuin6 regulation and function" ($P=1.17^{-13}$, $FDR=5.48^{-12}$) pathway (Supplementary Table 4). Metacore analysis further revealed 50 independent genes unique to ALPPS (Fig. 2C, Supplementary Table 5). The only gene common to both the ALPPS-specific pathways and independent genes was *Ihh* ($\log_2\text{ratio}=6.0$, $FDR=2.96^{-30}$), a secreted hedgehog ligand. PCR revealed an ALPPS-specific *Ihh* mRNA peak at 4h, while hepatic protein levels were reduced (Fig. 2D). Plasma protein levels however peaked at 4h after ALPPS (Fig. 2D), together suggesting newly produced *Ihh* is not being retained within cells but immediately secreted into plasma. Thus, *Ihh* may fit the role of a postulated plasma factor released through ALPPS. No ALPPS-specific changes were detected for other hedgehog ligands (*Dhh*, *Shh*, Supplementary Fig. 4). Non-parenchymal cells, particularly stellate cells, were positive for *Ihh* across samples (Fig. 2E, Supplementary Fig 5), suggesting the ligand is sourced from the stellate population.

Ihh pathway activity is elevated after ALPPS

To substantiate *Ihh* as a candidate behind ALPPS-accelerated liver regeneration, we assessed hedgehog pathway components in liver. *Ihh* binds to its receptor *Ptch1*, relieving inhibition of *Smo*, which then causes nuclear translocation of the *Gli* transcription factors, the downstream effectors of hedgehog signaling [14]. No differences between ALPPS and PVL were observed for *Ptch1* and *Smo* expression, while *Gli1* and *Gli2* alterations were inconsistent. However, *Hhip* (encoding the hedgehog ligand inhibitor) was downregulated at 4h and particularly 8h post ALPPS relative to PVL/transection (Fig. 3A). Immunoblots on cytosolic and nuclear fractions revealed that nuclear *Gli1*, but not *Gli2*, was upregulated at 4h in ALPPS versus PVL/transection. Immunofluorescence localized upregulation of *Gli1* to hepatocyte nuclei of ALPPS liver (Fig. 3B). At 8h, *Gli1* was no more elevated in ALPPS nuclear fractions. Unlike in PVL/Ts however, the main *Gli1* signal remained in hepatocyte nuclei of ALPPS liver (Fig. 3B). Accordingly, both the *Gli1* target cyclin D1 (Supplementary Fig. 6, Fig. 1C for mRNA) and mitotic counts (Fig. 1B) peaked at 8h, associating hedgehog activity in hepatocytes with

their proliferation. Together with *Hhip* downregulation at 4-8h, *Ihh* signaling in liver may hence be conducive to acceleration of liver regeneration following ALPPS.

Recombinant Ihh accelerates PVL-induced liver regeneration

If *Ihh* is mediating acceleration of regeneration after ALPPS, combining *Ihh* with PVL should mimic ALPPS effects. Mouse recombinant *Ihh* was administered after PVL instead of transection. When assessing body weight after injection, no differences were observed between PVL-*Ihh* and PVL-PBS animals. In contrast, liver weight was significantly elevated after PVL-*Ihh* compared to PVL-PBS - akin to levels after ALPPS surgery, albeit with different kinetics (Fig. 4A). The assessment of proliferation markers revealed increasing pH3 counts, with elevations particularly evident at 12h after PVL-*Ihh* (Fig. 4B). qPCR suggested relative *Gli1* and *Ccnd1* upregulation as well as *Hhip1* downregulation in PVL-*Ihh* liver over time (Fig. 4C). *Gli2* (Fig. 4C), *Ptch* and *Smo* were largely unaffected by *Ihh* (Fig. 4C). Nuclear protein levels of Gli1, but not Gli2, were upregulated through *Ihh* relative to PBS treatment, however at times later than 4h (Fig. 4D). Accordingly, cyclin D protein levels (Fig. 4E) and hepatocellular expression (Supplementary Fig. 7) were elevated after PVL-*Ihh* particularly at 12h. Altogether, the combination of PVL with *Ihh* has effects on regeneration similar to ALPPS, albeit in a delayed manner. These findings identify *Ihh* as an accelerator of PVL-induced liver growth.

Ihh neutralization deaccelerates ALPPS-induced liver regeneration.

If *Ihh* is mediating acceleration of regeneration after ALPPS, blocking *Ihh* should inhibit the ALPPS effects. Neutralizing antibody against *Ihh* was injected 1h prior to ALPPS surgery. Compared to PBS injection, body weight remained unaffected. Instead, *Ihh* neutralization reduced liver weight gain after ALPPS, although not to levels seen after PVL only (Fig. 5A). Proliferation parameters confirmed the deaccelerating effect of *Ihh* inhibition, illustrated through the suppression of the 8h-pH3

peak seen after ALPPS (Fig. 5B). Relative to PBS, α Ihh upregulated *Hhip* but lowered *Gli1/2* and *Ccnd1* expression in liver of ALPPS mice (Fig. 5C). Moreover, nuclear levels of Gli1, but not Gli2, were reduced over time through α Ihh-treatment (Fig. 5D). Finally, both cyclin D levels (Fig. 5E) and hepatocyte expression (Supplementary Fig. 7) were lowered in the ALPPS- α Ihh relative to the ALPPS-PBS group. We conclude Ihh is an important mediator of the ALPPS effects.

Combined with our other data, these findings indicate the elevations in plasma Ihh observed after ALPPS are central to the acceleration of liver regeneration achieved through this surgical technique.

IHH is induced in human ALPPS

Hedgehog signaling is conserved among animals [15]. To estimate whether ALPPS-induced Ihh elevations in mice are of human relevance, we measured plasma IHH levels from six consecutive patients having undergone ALPPS surgery at our clinic. During the first 24h, IHH levels rose to about five times over baseline levels at the time of surgery (Fig. 6). Given the slower regenerative pace in man relative to mouse, these observations are consistent with an early upregulation of IHH plasma levels in patients subjected to ALPPS surgery. No correlation was detected between IHH levels and postoperative liver volume or complication scores (Ihh plasma levels correlated to % increase FLR, $R^2 = 0.1047$; Ihh plasma levels correlated to complication CCI scores, $R^2 = 0.4973$), however analysis was hampered through the small and heterogeneous patient group (Supplementary Table 3).

Discussion

The development of ALPPS surgery has pushed the limits of resectability in liver surgery. The need to keep a certain remnant volume for liver to recover after tissue resection is a serious constraint that hinders the management of liver malignancy. The ALPPS approach illustrates that a conceptually simple combination of existing surgical techniques with a novel maneuver can unleash biological processes which are surprising in their extent. In other words, ALPPS demonstrates that the intrinsic capacity of liver to regenerate is larger than we think, but usually is cabined, likely to gain in stability and reliability needed for long-term function.

Mechanistically, liver regeneration after simple resection is highly complex [16]. How PVL leads to contralateral liver growth is ill-understood [17], and how regeneration is then accelerated by a transection step is unknown. The observation however that plasma obtained from ALPPS mice can mediate acceleration [11] pointed to circulating molecules responsible for the ALPPS effects. Here, we identify *indian hedgehog* as one key plasma factor that is triggered by ALPPS step 1 to mediate early acceleration of liver regeneration.

Following an explorative transcriptome profiling approach, we found *Ihh* as the most likely gene to be specifically induced by ALPPS in liver. Its hepatic mRNA levels peak at 4h after surgery, paralleled by increases in *Ihh* plasma levels, consistent with *Ihh* acting as an auto/paracrine ligand following its secretion from stellate cells [14]. The time after the *Ihh* peak (4-8h) is associated with a marked increase in proliferation markers in ALPPS relative to PVL, along with an ALPPS-specific peak in the expression of *Ccnd1*, crucial for the hepatocellular cell cycle progression [12]. Cyclin D1 is a target of hedgehog signaling and required for successful liver regeneration after tissue loss [18, 19, 12], consistent with a key role for *Ihh* in the ALPPS effects. Although the levels of plasma *Ihh* and of nuclear Gli1 (the hedgehog effector inducing *Ccnd1*) in hepatocytes were highest at 4h after ALPPS, cyclin D and mitoses (pH3) peaked not before 8h. At 8h however, the nuclear Gli1 signal remained localized to hepatocytes particularly in ALPPS liver, despite overall expression levels similar to PVL and Ts. On the other hand, the hedgehog ligand inhibitor *Hhip* was most downregulated in ALPPS at 8h relative to PVL/Ts, providing an explanation for the persistence of hepatocellular Gli1 activity at

this time [14]. These findings suggest the ALPPS-specific *Ihh* wave induces sustained hedgehog activity in hepatocytes, eventually causing cyclin D upregulation to promote cell cycle progression. Therefore, ALPPS-triggered hedgehog pathway activity in hepatocytes appears to rely not only on the level of *Gli1*, but also on the duration and location of *Gli1* activity. The associated proliferative peak at 8h then likely leads to the rapid gain in liver weight early post surgery. Cellular hypertrophy or lipid accumulation might contribute to weight gain, however ALPPS causes neither hypertrophy (Supplementary Fig. 8) nor significant steatosis (Fig. 1B, H&E) at early times.

To establish a role for *Ihh* in ALPPS, we injected recombinant protein into PVL mice. Indeed, regeneration was accelerated and accompanied by increases in proliferation, *Gli1* and cyclin D1 along with *Hhip* downregulation. Of note, the response to recombinant *Ihh* was somewhat delayed compared to ALPPS. A likely explanation for this delay is that injection of recombinant *Ihh* cannot fully mimic the ALPPS-triggered *Ihh* wave, as both local *Ihh* concentrations and protein modifications will differ between ALPPS and *Ihh* injection [20]. Nonetheless, our findings demonstrate that plasma *Ihh* alone is sufficient to push regeneration induced by PVL. Conversely, ALPPS markedly lost its accelerating effects - again paralleled by according changes in proliferation and hedgehog components - when circulating *Ihh* was blocked, confirming that the regenerative potential unleashed by ALPPS strongly depends on the elevations in *Ihh*. However, some acceleration was still evident after *Ihh* neutralization, suggesting ALPPS induces additional factors with a pro-regenerative effect. In our screen, we did not detect ALPPS-specific changes in molecules classically involved in liver regeneration, such as *Hgf*, *Egf* or *Tnf* (Supplementary Fig. 9). However, we have already reported very early increases in plasma *Il-6* after ALPPS surgery, suggesting this molecule may contribute to the ALPPS effects [11]. Altogether, our findings reveal a key role for *Ihh* in the acceleration of PVL-induced liver growth. Future research will need to explore the nature and kind of additional factors that participate in the accelerating effects of ALPPS.

Hedgehog signaling is known to be required for liver regeneration after both chemical insults and resection. Interestingly, the pathway is thought to operate in activated stellate cells that then promote parenchymal proliferation [21, 22]. At the *Ihh* mRNA/plasma peak after ALPPS, we observed *Ihh*

staining in non-parenchymal cells often displaying typical stellate morphology (Supplementary Fig. 5). ALPPS hence appears to trigger *Ihh* production in stellate cells but not hepatocytes [14]. Additional contributions to the elevations in circulating *Ihh* perhaps might come from other non-parenchymal cells or from the extracellular matrix, which can release the ligand during regenerative remodeling [23]. Early after hepatectomy, hedgehog signaling first acts in an autocrine way to promote the conversion of stellate cells into epithelial progenitors able to repopulate a part of the lost parenchymal mass [21, 22]. At 48h after hepatectomy, signaling likewise becomes paracrine, resulting in *Gli* nuclear accumulation in hepatocytes to promote parenchymal reconstitution directly from epithelium [24]. After ALPPS, *Gli1* is observed in nuclei of hepatocytes and stellate cells already at 4h, suggesting that the *Ihh* wave either simultaneously induces autocrine and paracrine signaling, or greatly accelerates the events observed after hepatectomy [21, 22, 24]. Therefore, while hedgehog signaling may not be specific to ALPPS, the timing of pathway activation through early *Ihh* release is unique to this procedure. Whether hedgehog signaling after ALPPS otherwise operates in analogy to hepatectomy or induces distinct processes will require further study. Moreover, the regenerative mechanisms associated with PVL alone will need clarification to appreciate how *Ihh* signaling accelerates ALPPS-associated liver growth. However, our findings are consistent with the view that stellate cells are the main conveners of hedgehog signaling associated with ALPPS-triggered regeneration, akin to early regenerative responses after other liver insults.

The general involvement of hedgehog activity in various processes related to tissue remodeling, such as in response to growth or injury [14], portray *Ihh* signaling a plausible mechanism to contribute to ALPPS-enforced regeneration. Currently it is unclear how ALPPS may induce the *Ihh*-dependent cascade. Combining PVL with LLLx has no accelerating effect, because for ALPPS the left lateral lobe is being occluded anyway before its resection. On the other hand, transection is the key factor differentiating ALPPS from PVL and would be an obvious candidate. Liver injury induced by transection conceivably might induce a repair response including *Ihh* secretion. Plasma *Ihh* levels did raise over time after transection only, however to much lower levels than following ALPPS and without upregulating *Ihh* mRNA (Fig. 2). Transection is hence not the cause of active *Ihh* production seen after ALPPS (but may contribute perhaps by causing release of matrix-bound *Ihh*). Rather, the

combination of PVL with transection appears to elicit a synergistic effect, with ALPPS triggering much higher Ihh plasma levels than the sum of Ihh seen after each procedure alone (Fig. 2). How the combination of occlusion and injury is leading to Ihh secretion will require further study. Injury however seems to be an important component, and even injection alone had some accelerating effect on PVL-induced regeneration (see PVL+PBS/Ihh versus PVL/ALPPS, Fig. 4A). Ihh signaling indeed is the first molecular insight into ALPPS-related mechanisms, and much more work will be needed to clarify triggers, contributing sources, and the various cellular crosstalks that underlie Ihh-dependent acceleration of liver regeneration in response to ALPPS.

The powerful promotion of regenerative speed seen in PVL mice after Ihh injection suggests therapeutic potential for this protein. In support of such potential, we observed early IHH elevations in ALPPS patients. Future studies should address specific clinical situations where hedgehog activation might be of benefit. At present, IHH may be of value for liver patients in need of but unsuitable - e.g. due to a critical health state - for major surgery such as ALPPS step 1. Given that the usual indication for resections is malignancy, the well-known cancer-promoting activities of hedgehog signaling [25] will have to be carefully balanced against the benefits for individual patients. In this regard, a single therapeutic IHH injection might have a small enough impact on neoplasia to be applied to patients at high risk of death due to unresectable disease. However, the tumorigenic risks associated with an IHH bolus are currently unknown and will need meticulous assessment in the settings of PVL. Of note, recent (but still limited) numbers suggests that ALPPS - as we show inducing IHH in patients - is not associated with higher recurrence rates than PVL [7] despite existing concerns [26]. Clearly, the tumorigenic potential of an IHH bolus in the settings of PVL will need to be experimentally addressed.

In summary, we report the first insight into the molecular principles underlying the acceleration of liver regeneration through ALPPS. The elevations in circulating Ihh early after ALPPS are both sufficient to accelerate compensatory liver growth following occlusion and required for ALPPS to develop full effect. Our findings highlight the dependency of ALPPS on humoral factors and provide a basis for a pharmaceutical promotion of compensatory liver growth in the clinic. The identification of

Ihh as an accelerating factor is a first step in deciphering the mechanistic processes underlying ALPPS and in exploring according therapeutics for the expanded application of liver surgery.

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Figure Legends

Figure 1. Acceleration of compensatory liver growth through ALPPS. (A) ALPPS surgery in mouse and its effects on liver re-growth. Gray shading indicates occluded liver parts following PVL. The yellow line indicates the transection step, and the green line the LLLx step. Percent body weight and liver weight regain (expressed as percent functional-liver-remnant-to-body-weight-ratio) after surgery are shown in the left and right graph, respectively. (B) H&E, Ki67, and pH3 staining of liver after surgery. Graphs show counts of hepatocytes positive for Ki67 or bold pH3. Histology (H&E) for sham was consistent over time. (C) Hepatic gene expression of *Ccnd1*, *Ccne1*, *Ccna2*, *Ccnb1*, and *Ccnb2* after surgery. (D) Hepatic protein expression (normalized to Gapdh) of p21 after surgery. N=6/group. t-test, *P<0.05, ** P<0.01. ***P<0.001. Significances refer to ALPPS vs. PVL comparison unless otherwise stated.

Figure 2. *Ihh* induction after ALPPS. (A) Hepatic gene expression profiles derived from RNA sequencing at 4h after surgery. Note the unique ALPPS profile. (B) Visual presentation (Venny 2.0) of overlaps between top dysregulated pathways (fold change ≥ 2 , $p > 0.05$) derived from Metacore pathway analysis of 4h-expression profiles. (C) Venny visualization of dysregulated genes (fold change ≥ 4 , $p > 0.05$) in two-pair comparisons. N=3/group for expression profiling. (D) Hepatic *Ihh* gene expression, hepatic *Ihh* protein expression (normalized to Gapdh), and plasma *Ihh* protein levels (ELISA) after surgery. (E) *Ihh* immunohistochemistry. Note the similar staining patterns across all groups. N=6/group. t-test, *P<0.05, **P<0.01, ***P<0.001.

Figure 3. Hedgehog pathway components after surgery. (A) Gene expression for *Hhip1*, *Ptch1*, *Smo*, *Gli1*, and *Gli2*. N=6/group. (B) Cytosolic and nuclear protein levels (normalized to β -tubulin and lamin B1, respectively; see immunoblots in Supplementary Fig. 2) of *Gli1* and *Gli2*. N=6/group. Right panels: immunofluorescence for hepatic *Gli1*. Note for ALPPS liver the nuclear staining predominantly in hepatocytes. At 8h, *Gli1* expression was more prominent in parenchymal than non-parenchymal cells for ALPPS relative to Ts, PVL and sham. In sham liver, cytoplasmic staining was

increased in non-parenchymal cells at 8h relative to 4h. t-test, *P<0.05, **P<0.01, ***P<0.001 for PVL vs ALPPS unless indicated otherwise.

Figure 4. Effects of recombinant Ihh on PVL-induced liver regeneration. (A) Percent body weight and functional-liver-remnant-to-body-weight-ratio after PVL-Ihh, PVL-PBS, ALPPS, PVL and transection are shown in the left and right graph, respectively. (B) Corresponding H&E, Ki67, and pH3 staining of liver. Graphs show counts of hepatocytes positive for Ki67 or pH3. Note the increases in bold pH3-positive nuclei 12h after PVL-Ihh. (C) Hepatic gene expression of *Hhip*, *Gli1*, *Gli2*, and *Ccnd1* after PVL plus PBS or Ihh. (D) Cytosolic and nuclear protein levels (normalized to β -tubulin and lamin B1, respectively) of Gli1 and Gli2. (E) Quantified cyclin D1 immunoblots. N=6/group. t-test, *P<0.05, **P<0.01, ***P<0.001 for PVL-PBS vs PVL-Ihh.

Figure 5. Effects of neutralizing Ihh antibody on ALPPS-induced liver regeneration. (A) Percent body weight and functional-liver-remnant-to-body-weight-ratio after ALPPS- α Ihh antibody, ALPPS-PBS, ALPPS, PVL, and transection. (B) Corresponding H&E, Ki67, and pH3 staining of liver, with graphs showing Ki67 or pH3 counts. (C) Hepatic gene expression of *Hhip*, *Gli1*, *Gli2*, and *Ccnd1* after ALPPS plus PBS or α Ihh antibody. (D) Cytosolic and nuclear protein levels of Gli1 and Gli2. (E) Quantified cyclin D1 immunoblots. t-test, *P<0.05, **P<0.01, ***P<0.001 for ALPPS-PBS vs ALPPS- α Ihh.

Figure 6. Human IHH levels in clinical material. Plasma from ALPPS patients was available at 0h (baseline), 2h, 24h, and 48h after PVL and transection. IHH levels were assessed by ELISA.

Figure 1

A

B

C

D

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

PORTAL VEIN LIGATION

PORTAL VEIN
LIGATION
+
TRANSECTION

ALPPS step 1