Car-driven regeneration protects liver from failure following tissue loss and bears therapeutic potential

Authors: Christoph Tschuor¹, Ekaterina Kachaylo¹, Limani Perparim¹, Dimitri A Raptis¹, Michael Linecker¹, Yinghua Tian¹, Uli Herrmann², Kamile Grabliauskaite¹, Achim Weber³, Amedeo Columbano⁴, Rolf Graf¹, Bostjan Humar^{1*} & Pierre-Alain Clavien^{1*}

*shared senior authorship

¹Laboratory of the Swiss HPB and Transplantation Center, Department of Surgery, University Hospital Zürich, Switzerland.

²Department of Neuropathology, University Hospital Zürich, Switzerland.

³Institute of Surgical Pathology, University Hospital Zürich, Switzerland.

⁴Department of Biomedical Sciences, University of Cagliari, Italy.

Correspondence: Pierre-Alain Clavien or Bostjan Humar, University Hospital Zurich, Department of Surgery, Raemistrasse 100, CH-8091 Zurich, Switzerland.
Phone: +41 (0)44 255 33 00.

Email: clavien@access.uzh.ch or bostjan.humar@usz.ch

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Abbreviations: Car, constitutive androstane receptor; CITCO, (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl)oxime); eHx, extended hepatectomy; huCAR, humanized CAR; SFSS, Small-For-Size Syndrome; sHx, standard hepatectomy; TCP, 1,4-bis(2-(3,5-dichloropyridyloxy))benzene

Key words: Small-for-Size Syndrome, TCPOBOP, CITCO, hepatomegaly

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Author's contributions: CT, BH and RG designed the experiments. CT, EK, LP, DAR, and ML performed molecular analyses. KG performed immunohistochemistry. YT performed mouse liver transplantations. UH provided expertise for the *ex vivo* experiments. AW evaluated human liver samples and contributed to tissue array studies. AC provided intellectual input and critically revised the manuscript. BH designed the study. BH, CT, RG and PAC wrote the manuscript. BH, RG and PAC supervised the study.

Abstract

BACKGROUND & AIMS: Liver can recover following resection. If tissue loss is too excessive, however, liver failure will develop as is known from the Small-for-Size-Syndrome (SFSS). The molecular processes underlying liver failure are ill-understood. Here, we explored the role and the clinical potential of Nr1i3 (constitutive androstane receptor, Car) in liver failure following hepatectomy.

METHODS: Activators of Car, various hepatectomies, Car^{-/-} mice, humanized CAR mice, human tissue and *ex vivo* liver slice cultures were used to study Car in the SFSS. Pathways downstream of Car were investigated by *in vivo* siRNA knockdown.

RESULTS: Excessive tissue loss causing liver failure is associated with deficient induction of Car. Re-activation of Car by an agonist normalizes all features associated with experimental SFSS. The beneficial effects of Car activation are relayed through Foxm1, an essential promoter of the hepatocyte cell cycle. Deficiency in the CAR-FOXM1 axis likewise is evident in human SFSS. Activation of human CAR mitigates SFSS in humanized CAR mice and improves the culture of human liver slices.

CONCLUSIONS: Impaired hepatic Car-Foxm1 signaling provides a first molecular characterization of liver that fails to recover after tissue loss. Our findings place deficient regeneration as a principal cause behind the SFSS and suggest CAR agonists may bear clinical potential against liver failure.

Key words: Small-for-Size Syndrome, TCPOBOP, CITCO, hepatomegaly

Lay abstract

The unique regenerative capacity of liver has its natural limits. Following too excessive tissue loss, such as through extended resection in the clinic, liver failure may develop. This entity is known as Small-for-Size Syndrome (SFSS) and represents the most frequent cause of death due to liver surgery. Here we show that deficient induction of the protein Car, a central regulator of liver function and growth, is a cause of liver failure following extended resection; re-activation of Car through pharmacological means is sufficient to prevent or rescue from the SFSS.

Introduction

The unique ability of liver to regenerate after tissue loss has permitted the surgical removal of large liver parts and the transplantation of partial liver grafts. The capacity of liver to regain function following tissue loss however is limited. In mice, standard hepatectomy (sHx, removal of 70% volume) leads to complete recovery within a week[1], whereas extreme resection (91% removed) induces liver failure and death within 48h[2]. Therefore, remnant volume is a key determinant for successful recovery after tissue loss.

The requirement for a sufficient liver volume is a factor significantly limiting the application of liver surgery. The transplantation of marginal liver grafts puts recipients at risk of developing liver failure, a clinical entity known as the Small-for-Size syndrome (SFSS)[3, 4]. Likewise, a congruent entity can be observed following extended hepatectomy, the most frequent intervention against highly prevalent liver tumors. In both cases, patients present with metabolic liver dysfunction (e.g. hypoalbuminemia, hyperbilirubinemia), persistent hepatostasis, and an elevated mortality. Indeed, SFSS following liver resection or transplantation represents the most frequent cause of death due to liver surgery[3, 4].

Why small liver remnants/grafts fail to recover is incompletely understood. Following tissue loss, portal blood flow into remnants/grafts increases; an excessive elevation in portal pressure may damage the sinusoidal endothelium, eventually causing parenchymal injury, but its role in the SFSS remains controversial[5, 6]. Liver surgery often is performed with clamping of hepatic blood supply; the resulting ischemic insult (which is unavoidable in transplantation) is known to counteract liver recovery and certainly will impact marginal remnants[7]. Likewise, the accrual of injury has repeatedly been proposed to account for resection-induced liver failure in

the absence of ischemia[8-10]. However, hepatectomies in mice are technically challenging and *per se* may augment liver injury[11]. To avoid confounding by surgical damage, we have introduced in mice a modified version of extended hepatectomy (eHx, 86% removed) that induces little injury as assessed by diverse parameters[1]. Despite the absence of significant injury, mice following eHx display metabolic liver dysfunction, hepatosteatosis and an elevated mortality akin to human SFSS[1]. Therefore, injury is not required for liver failure to develop after extended tissue loss in mice.

Our experimental SFSS model further was associated with delayed regeneration due to arrest at the S and particularly M phase of the hepatocyte cell cycle. When repeating eHx in mice lacking the generic cell cycle inhibitor p21, liver regeneration was restored and most metabolic SFSS features were ameliorated, as was survival[1]. These improvements suggest an impaired regenerative capacity may suffice to cause SFSS.

Regenerative deficits indeed are a consistent finding in models of resection- or transplantation-induced SFSS[8-10, 12]. The road to impaired hepatocyte proliferation however remains ill-understood, and no clear-cut molecular defects are known for human SFSS. The notion that impaired regeneration and metabolic dysfunction go hand in hand with a marginal liver volume may hint to a pathway that coordinates hepatocyte proliferation with the liver's metabolic tasks. Nr1i3 (constitutive androstane receptor, Car) is a nuclear receptor that regulates P450 cytochromes and has diverse metabolic functions[13], including the clearance of xeno/endobiotics such as toxic bilirubin[14]. Notably, Car activation through phenobarbital-like agents induces spontaneous hepatomegaly[15]. Likewise, Car appears to be required for liver regeneration after hepatectomy[16].

To this end, we investigated (i) whether disturbed Car-dependent signaling is associated with the development of liver failure after tissue loss in mice, (ii) whether putative deficits are relevant for human SFSS, and (iii) whether Car modulation may be exploited for the clinical management of SFSS.

Materials & Methods

Animals

Animals aged 8-10 weeks were kept on a 12-hour day/night cycle with free access to food and water. Male wild type mice (C57BL6, Harlan) were used unless otherwise stated. CAR knockout animals (9103-M, C57BL/6-*Nr1i3tm1.1Arte*) and corresponding wild type controls were obtained from Taconic Laboratories, as were humanized CAR mice (9101-M, C57BL/6-*Nr1i3tm1(NR1I3)Arte*). Due to local requirements, breeding was started with offspring from in-house C57BL6 following embryonic transfer.

Animal Surgery

Standard hepatectomies (sHx, 70%, fully regenerating, 100% survival) and extended hepatectomies (eHx, 86%, regenerative delay, >75% survival, SFSS model) were performed as reported[1]. The same surgical technique was applied for extreme hepatectomy (91%, 0% survival within 48h), except that all segmental portal vessels of the right, left, and middle lobes were ligated. Sham operation consisted of cholecystectomy. SFSS orthotopic partial liver transplantations (using 30% (v/v) grafts) were performed according to Tian *et al.*[17]. The gain in liver weight, a physical measure of liver regeneration, was expressed through the ratio of liver weight to body weight (LW/BW).

Activation of mouse Car and human CAR

BL6 and *Car* knockout mice were treated with the murine Car agonist TCP (1,4-bis(2-(3,5-dichloropyridyloxy))benzene, Sigma Aldrich) directly prior to surgery or as indicated (see Supplementary Figure 1A for TCP effects in the absence of surgery).

TCP was dissolved in DMSO (5mg/ml), mixed with prewarmed PBS (final vol. 100µl) and given by oral gavage (1-3mg/kg). The human CAR agonist CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime, Sigma Aldrich, C6240, dissolved in DMSO at 5mg/ml) was i.p. injected with prewarmed PBS (final vol. 100µl) into humanized *CAR* (huCAR) mice at 50mg/kg directly before hepatectomy and then daily until harvest. For *ex vivo* liver slice cultures, 250nM TCP and 1 or 100µM CITCO were added to mouse and human media, respectively.

Foxm1 knockdown

siRNAs targeting *Foxm1* and the controls *Aha1* and *Luciferase* were designed by Axolabs GmbH (Kulmbach, D) and packed into company-owned formulations designed to preferentially target murine hepatocytes. Formulations were injected into the tail vein 48 hours before hepatectomy. The lack of significant toxicity was ascertained through the assessment of liver injury markers.

Immunohistochemistry and tissue microarray

These techniques were performed according to standard protocols and are described in the Supplements, including a description of human biopsy material.

Western Blotting

The procedure was performed as reported[1]. Antibodies are described in the Supplements.

Quantitative Real-Time Polymerase Chain Reaction

Sequence amplification and data analysis were performed on the ABI Prism 7000 Sequence Detector System (PE Applied Biosystems) as detailed in the Supplements. If not otherwise stated, expression values were normalized to time-matched samples from sham-operated mice.

Ex vivo culturing of liver slices

Ex vivo cultures of liver slices were prepared as described by de Graaf *et al.*[18] with slight modifications. Liver biopsies were obtained from three mice (C57BL6) and one human subject (diagnosed with colorectal liver metastasis, tissue from an unaffected lobe). Biopsies were embedded in 10ml liquid (2%wt/vol) ultralow-melting-point agarose dissolved in Krebs-Henseleit buffer (KHB, 5mM NaCl, 118mM KCl, 1.1mM MgSO₄·7H₂O, 1.2mM KH₂PO₄, 25mM NaHCO₃, 2.5mM CaCl₂·2H₂O, 25mM D-Glucose, 9mM HEPES in ultrapure water), cut with a vibratome into 200µm thick slices and kept in KHB. Five to six slices were then plated on 0.4µm inserts (30mm diameter, Millicell) and the residual buffer was removed before placing the inserts into a cell culture plate containing culturing medium (Williams E medium (+L-glutamine) supplemented with antibiotics and 14mM D-glucose (Sigma), 30nM insulin (Gibco Life Technologies), 100nM glucagon (Sigma), 1nM corticosterone (Sigma), 1nM Egf (Sigma) plus 5% FCS. The tissue cultures were kept in a standard cell incubator (37°C, 5% CO₂ and 95% humidity) and the medium was changed daily. Integrity of explants was assessed by HE staining (i.e. the presence of a nucleus and a normal cell structure on histology). Experiments were limited to 24/48h cultures due to inconsistent tissue integrity at later times.

Experiments with liver biopsies from three mice and one human subject were run in triplicates. Ethical approval for the use of human biopsy tissue was granted by the local ethics committee from Zürich (KEK-ZH-Nr. 2012-01 08).

Statistical analysis

Data are presented as mean $\pm$ SD. Differences between the groups were assessed by a two-tailed *t-test* assuming unequal variance. In general, at least 5 mice/group were analyzed. For survival after sHx/eHx in wt/ Car^{-/-} mice and *Foxm1* knockdown, 10 animals/group were included. For the molecular analyzes following siRNA knockdown, at least three mice/group were used. Differences were considered significant at $P < 0.05$ and indicated in figures by an asterisk (*). Statistical analyzes were performed using Prism 6.0 (GraphPad).

Study approval

All animal experiments were in accordance with Swiss Federal Animal Regulations and approved by the Veterinary Office of Zurich. Ethical approval for the human sections was granted by the regional ethics committee (KEK-ZH -Nr. 2012-01 08). Written consent to study tissue for research purposes was received from the patient prior to inclusion in the study.

Results

Car activation after eHx is defective. To assess Car activity following resection, we measured its mRNA levels, protein levels/localization and downstream targets following resection. Compared to sHx, Car mRNA induction and nuclear localization were impaired after eHx (Figure 1A, Supplementary Figure 1B). Likewise, the prototypical Car target *Cyp2b10*[13] and the proliferation-related downstream molecule *Foxm1*[19] were hardly induced (Figure 1A, Supplementary Figure 1C), indicating defective Car activation following eHx.

Car deficiency causes an SFSS phenotype after sHx. Car is thought to be needed for efficient liver regeneration[16]. To detail its function in regeneration, we analyzed *Car*^{-/-} mice following resection and compared to wild type (wt) mice post sHx/eHx. Although usually at 100%, survival after sHx in *Car*^{-/-} mice was reduced to levels comparable to eHx in wt mice (Figure 1B). Liver weight gain, pH3 staining, mitoses, and *Cyp2b10* expression were decreased compared to wt sHx. Similarly, *Foxm1* and its cyclin targets *Ccna2/b2* were reduced, whereas *Cdkn1a* (p21, repressed by *Foxm1*)[20] was upregulated; the SFSS-associated hepatosteatosis (see also Supplementary Figure 1D) and metabolic liver dysfunction (hypoalbuminemia, hyperbilirubinemia) were present (Figure 1B). Therefore, sHx in *Car*^{-/-} mice induces a phenotype akin to eHx in wt mice. Together with impaired Car activity post eHx, these results identify Car deficiency as a cause of experimental SFSS.

Car re-activation rescues from SFSS. Car ligands such as phenobarbital-like compounds induce Car activity[15]; TCP (1,4-bis(2-(3,5-dichloropyridyloxy))benzene) is the most potent agent of this class, with one TCP gavage nearly doubling mouse liver weight within nine days[21]. We found TCP-induced hepatomegaly was accompanied by Car nuclear accumulation/downstream

gene induction (Supplementary Figure 1A). To determine whether TCP is able to re-activate Car after eHx, TCP was given to mice concomitant with eHx. One gavage was sufficient to restore Car nuclear translocation (see also Supplementary Figure 1B) and downstream gene induction. Moreover, TCP suppressed p21, re-elevated pH3 and mitotic counts, accelerated liver weight gain, and normalized metabolic SFSS features after eHx (Figure 1C). TCP lost these effects in Car^{-/-} mice, confirming dependency on Car (Supplementary Figure 2). Next, we assessed the impact of TCP on survival. Given that most mice (>75%) survive eHx-induced SFSS, TCP was tested in an alternative SFSS model featuring 0% survival (91% hepatectomy). One-week-survival was assessed, as this is the critical period after hepatectomy. TCP rescued 40% of mice after 91% hepatectomy. When TCP was tested in another lethal SFSS model (transplantation of 30% (v/v) SFSS grafts)[17], it again raised survival to 40% (Figure 1D). These findings demonstrate that Car reactivation through TCP leads to a functional recovery of marginal liver remnants and grafts.

Foxm1 mediates Car effects in experimental SFSS. TCP-induced hepatomegaly (Supplementary Figure 1) is paralleled by *Foxm1* induction[19], however the dependency of Car effects on *Foxm1* remains unexplored. On the other hand, sHx in *Foxm1*^{HEP^{-/-}} mice induces delayed progression through the S- and M-cell cycle phases, akin to eHx in wt mice[1, 20]. To determine whether *Foxm1* may be associated with SFSS, we mimicked its deficiency by *αFoxm1*-siRNA-mediated knockdown before sHx. Knockdown was observed during the S and M phase peaks (32h and 48h, respectively, Figure 2A, Supplementary Figure 1E) when *Foxm1* is maximally induced after sHx (Figure 1A, Supplementary Figure 1C). *Foxm1* knockdown reduced proliferative parameters, diminished liver weight gain, and caused hypoalbuminemia, hyperbilirubinemia and hepatosteatosis (see also Supplementary Figure 1D). Together

with the compromised survival following knockdown and sHx (Figure 2A), these findings indicate a crucial contribution of Foxm1 deficiency to the development of SFSS. When knocking down *Foxm1* before eHx, TCP lost its effects, with liver remnants remaining small and steatotic (Figure 2B, Supplementary Figure 1D). We conclude that Car activation via TCP requires signaling through Foxm1 to prevent the development of experimental SFSS.

Human SFSS displays pathobiological changes akin to mouse SFSS. Human SFSS has not yet been investigated at a molecular level. We analyzed liver tissue from SFSS patients and those without complications after resection. Regenerating human livers, but not SFSS livers, were positive for nuclear CAR and FOXM1 (Supplementary Figure 3). Both regenerating and SFSS livers expressed KI67, indicating hepatocytes have entered the cell cycle[1]. In contrast, p21 was induced whilst pH3 was hardly detectable in SFSS livers, consistent with deficient cell cycle progression. Human and mouse SFSS thus seem to share basic pathophysiological mechanisms, implying the activation of human CAR might prevent SFSS in the clinic.

CAR activation for human SFSS. Because TCP has little activity towards human CAR[22], we examined the human CAR agonist CITCO (6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime)[23] for its ability to prevent experimental SFSS in transgenic mice bearing a human CAR (huCAR mice, where mouse Car has been replaced with human CAR)[14]. In response to CITCO alone, huCAR mice developed spontaneous hepatomegaly (Figure 3A), albeit less pronounced than wt mice on TCP. In fact, huCAR mice following sHx displayed impaired regeneration accompanied by metabolic SFSS features and increased mortality, indicating that huCAR mice do not

retain the full regenerative capacity of wt mice (Figure 3B). Nonetheless, CITCO improved liver weight gain, pH3 counts, steatosis and hyperbilirubinemia in huCAR mice after eHx (Figure 3C). To provide further evidence for the clinical benefit of CAR activation, we treated *ex vivo* cultures of liver slices from a patient biopsy. Similar to mouse liver slices on TCP (Figure 4A), CITCO improved histology (see also Supplementary Figure 4) and increased pH3 counts in human slices (Figure 4B). Importantly, CITCO at high doses also improved viability (AST, Hmgb1, Casp3), but was less potent than TCP. Therefore, CITCO mitigates liver failure in huCAR mice and exerts proregenerative and protective effects on human liver slices, suggesting CAR activation may be effective against human SFSS.

Discussion

In this study, we demonstrate a vital role for the nuclear receptor Car in the development of liver failure following tissue loss. Unlike in normally regenerating liver, Car is not activated following extended resection of the liver. Standard hepatectomy in mice lacking Car provokes a phenotype akin to that seen in our SFSS model after eHx. A key consequence of Car deficiency is the failed induction of the cell cycle promoter Foxm1, knockdown of which is sufficient to induce most SFSS features following sHx. Re-activation of Car through its species-specific ligand TCP normalizes all of these features, illustrating the importance of Car activity for the prevention of experimental SFSS.

The association between Foxm1-dependent cell cycle deficits and the SFSS phenotype suggests delayed liver regeneration is the underlying cause of resection-induced liver failure. Foxm1 is considered a proliferation-specific transcription factor also in hepatocytes[24][20]. Foxm1 knockdown not only induced an SFSS-like phenotype, but also blunted the rescuing effects of TCP. As a limitation, only one siRNA against Foxm1 was used, and off-target effects hence cannot be excluded. However, Foxm1 knockdown before sHx causes cell cycle defects similar to sHx in hepatocyte-specific Foxm1 knockouts[20], sHx in *Car*^{-/-} mice, or eHx in wt mice[1]. We therefore propose that delayed progression through the S and particularly M cell cycle phase is a basic cause of liver failure.

Besides regulating Foxm1, the functions of Car in hepatic metabolism will add to the full prevention of an SFSS phenotype. Simply due to volume loss, marginal remnants may be overwhelmed by the metabolic needs posed on liver. Acting as a xeno/endobiotic sensor[13], Car may react via Foxm1 to augment liver mass, but also by inducing a panel of metabolizing enzymes, including those needed for the

clearance of elevated bilirubin levels[14]. The unusual ability of Car to modulate organ size restraints while controlling hepatocyte proliferation and function[25] implies its main task is the coordination between liver volume, its metabolic capacity, and the current metabolic demands. These qualities, and the possibility to induce its activity by exogenous ligands, render Car an attractive candidate for the mitigation of SFSS in the clinic.

For Car to be a clinically viable target, (i) human SFSS should display functional deficits in the Car axis, (ii) activation of human CAR should lead to similar outcomes as with mouse Car, and (iii) clinical situations should be amenable to CAR-based strategies. The alterations we observed in human SFSS liver were consistent with a deficient CAR-FOXM1 axis along with defective cell cycle progression. As for the activation of human CAR, the human agonist CITCO was tested in two different systems, huCAR mice and *ex vivo* human liver slices. Although huCAR mice have been reported to efficiently induce Car-dependent metabolic enzymes[14], the CITCO responses we observed (i.e. the development of mild SFSS following sHx, volatile CAR downstream gene expression patterns) suggest a subpar communication between human CAR and its downstream mouse partners, perhaps rooting in the protein structure considerably differing between man and mouse (huCAR/mCar size ratio=1.66, with 70% identity only in the common sequence (<http://www.uniprot.org>)). These observations however also emphasize the need of full Car activity for the prevention of liver failure. Despite the above inadequacies, CITCO did mitigate SFSS in huCAR mice and efficiently improved liver weight gain, likely owing to the induction of Foxm1. Similar to TCP, CITCO also was able to improve the integrity of human liver slice cultures and promote their proliferation. Again, CITCO was less effective than TCP; unlike TCP, repeated injection of CITCO is required to induce a

response *in vivo*[26], indicating a relatively low efficacy of the ligand. Novel CAR ligands with improved potency/stability will likely be needed to achieve full activation of human CAR. The effects of CITCO on huCAR mice and human liver slices however provide a proof-of-concept for the potential clinical utility of CAR activation.

Apart from remnant/graft volume and the presence of pre-existing liver disease[3, 4], no clinical predictors of SFSS currently exist, placing treatment over prevention. To estimate the therapeutic potential of Car activation, we applied TCP to our lethal SFSS model in a delayed mode (i.e. *after* surgery). Indeed, TCP maintained its beneficial effects and again rescued 40% of mice (Supplementary Figure 5), implying a therapeutic window exists for the rescue from SFSS. Another obstacle to clinical translation is the malignant potential associated with CAR activation. The prime indication for extended hepatectomies is liver malignancy, and Car ligands are non-genotoxic promoters of rodent liver tumors[27]. CAR activation hence might potentially increase the risk of recurrence, however both TCP and Car activation are associated with malignancy only in chronic settings, suggesting a single application bears little risk [27, 28]. No according data is available for human CAR, however phenobarbital treatment seems not to increase liver cancer incidence in patients[29]. To minimize risks, putative trials might focus on hepatectomies for liver cancer displaying CAR downregulation (Supplementary Figure 6). Given that TCP was likewise efficient in a model of SFSS transplantation, live liver donors may safely benefit from CAR activation, i.e. for the treatment of SFSS developing in donors following partial graft retrieval, or for a pre-operative enlargement of donor liver size to enable the riskless removal of sufficient volume for transplantation. Finally, unlike human liver, mouse liver is composed of distinct lobes that can be resected for

hepatectomy. The more compacted architecture of human liver however usually requires parenchymal transection for hepatectomy, causing liver injury. Although injury is not necessary for SFSS to develop, it will increase the SFSS risk. Therefore, effective clinical strategies could be based on a combined approach to promote the regenerative capacity (i.e. CAR) and to prevent injury. CAR activation itself has some protective effects, however adding compounds specifically targeting injury may be more effective[10].

Taken together, our study identifies Car deficiency as a key mechanism underlying the development of liver failure following extended tissue loss, and provides the means to correct these deficits. The function of Car in hepatic regeneration points to the translational potential of CAR activation, creating demand for novel, powerful agonists of human CAR. Moreover, the dependency of the beneficial Car effects on cell cycle-associated molecules imply that liver failure, including many of its metabolic features, arise from an insufficient capacity of marginal remnants to regenerate, illustrating the intimate nexus between proliferative pathways and the metabolic function of liver.

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Author names in bold designate shared co-first authorship

Figure Legends

Fig.1. Car deficiency underlies liver failure after eHx. (A) Car gene/protein expression and downstream gene expression after eHx. Despite similar protein levels, Car nuclear accumulation is impaired after eHx. (B) sHx in Car^{-/-} mice induces an SFSS phenotype, as evinced through the assessment of relevant SFSS parameters (at 48h post sHx/eHx for wt mice and post sHx for Car^{-/-} mice). Note that LW/BW (liver-to-body-weight ratio) cannot be compared between sHx and eHx due to a

different starting value. For survival, 10 mice/group were used. (C) TCP reactivates Car and normalizes SFSS features (shown for 48h post Hx) after eHx. TCP-induced Foxm1 re-elevation at 32/48h post eHx is illustrated in a magnified square. (D) TCP improves survival in lethal SFSS models. N=5/group unless otherwise stated; *P<.05.

Fig. 2. The beneficial effects of TCP in SFSS rely on signaling through Foxm1. (A) Foxm1 knockdown before sHx provokes SFSS-like features. Control siRNAs against *Luc* and *Aha1* (shown for 48h only) were used. Note the comparison to plain sHx/eHx for LW/BW, albumin and bilirubin. Survival following sHx was reduced by *Foxm1*-siRNA, reflecting compromised liver function. (B) Foxm1 knockdown prior to eHx abrogates the beneficial effects of TCP. In the siRNA-treated samples, (-) indicates a control without siRNA. Note the small size and the pale complexion (typifying steatosis) of liver remnants after eHx, or eHx plus TCP following *Foxm1*-siRNA-pretreatment. N≥3/group for molecular analyses, n=10/group for survival, otherwise n=5/group.

Fig. 3. CITCO induces spontaneous hepatomegaly and mitigates most SFSS features in huCAR mice. (A) Spontaneous hepatomegaly in huCAR mice through CAR activation via CITCO as evinced through the assessment of SFSS parameters. Note upregulation of *Cdkn1a*, and the marginal elevation in *Ccna2/b2*. (B) sHx in huCAR mice induces a mild SFSS phenotype. Note the reduced LW/BW and survival, the high gene expression, and elevated steatosis/bilirubin in huCAR mice. (C) CITCO mitigates liver failure in huCAR mice after eHx. Note the improvements in LW/BW, *Foxm1*, *Cdkn1a*, *Ccna2/b2*, pH3 and bilirubin, and the lack of significant effects on steatosis and albumin through CITCO. N=5/group.

Fig. 4. CAR activation promotes the proliferative state and the integrity of *ex vivo* liver slice cultures. (A) TCP effects in mouse liver slices cultured for 24h or 48h. TCP added to media improves liver histology (HE: appearance of nuclei and regular cell structure, see also Supplementary Figure 4), promotes nuclear pH3 positivity, accompanied by reduced supernatant levels of injury markers AST/Hmgb1. Staurosporin served as positive injury control. (B) CITCO effects in human liver slices. At 1 μ M, CITCO induces modest improvements in histology and proliferative markers. At 100 μ M, these effects are stronger, along with reduced apoptotic counts (Casp3) and Hmgb1 supernatant levels. For *ex vivo* culture, three slices from each three mice, and five slices from one human liver biopsy were analyzed.

Figure 1

A Car activity following hepatectomy

B sHx in Car knockout mice

C TCP treatment of mice with eHx-induced liver failure

D TCP treatment of lethal liver failure

Figure 2

A *Foxm1b* knockdown before sHx

B *Foxm1b* knockdown before eHx + TCP

Figure 3

A Spontaneous hepatomegaly in huCAR mice following treatment with CITCO

B huCAR mice post sHx

C huCAR mice post eHx +CITCO

Figure 4