Research Article

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# Constitutive androstane receptor (Car)-driven regeneration protects liver from failure following tissue loss

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**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. Reactivation 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.
**Lay summary**: The unique regenerative capacity of liver has its natural limits. Following tissue loss that is too excessive, such as through extended resection in the clinic, liver failure may develop. This 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; reactivation of Car through pharmacological means is sufficient to prevent or rescue the SFSS.




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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.

## 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 48 h [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 steatosis, 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 not completely 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 with surgical damage, we have introduced a modified version of extended hepatectomy in mice (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, steatosis 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 was further 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 that an impaired regenerative capacity may be sufficient to cause SFSS.

Regenerative deficits are indeed a consistent finding in models of resection- or transplantation-induced SFSS [8–10,12]. The road to impaired hepatocyte proliferation however remains poorly 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 at 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 and methods

### *Animals*

Animals aged 8–10 weeks were kept on a 12 h day/night cycle with free access to food and water. Male wild-type (wt) mice (C57BL/6, Harlan) were used unless otherwise stated. *Car* knockout animals (9103-M, C57BL/6-*Nr1i3tm1.1Arte*) and corresponding wt 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 C57BL/6 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 48 h), 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 Fig. 1A for TCP effects in the absence of surgery). TCP was dissolved in DMSO (5 mg/ml), mixed with prewarmed PBS (final volume 100 µl) and given by oral gavage (1–3 mg/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 5 mg/ml) was i.p. injected with prewarmed PBS (final volume 100 µl) into humanized *CAR* (huCAR) mice at 50 mg/kg directly before hepatectomy and then daily until harvest. For *ex vivo* liver slice cultures, 250 nM TCP and 1 or 100 µM 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 h before hepatectomy. The lack of significant toxicity was ascertained through the assessment of liver injury markers.

### *Immunochemistry and tissue microarray*

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

### *Western blotting*

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

### *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 Supplementary material. 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 (C57BL/6) and one human subject (diagnosed with colorectal liver metastasis, tissue from an unaffected lobe). Biopsies were embedded in 10 ml liquid (2% wt/vol) ultralow-melting-point agarose dissolved in Krebs-Henseleit buffer (KHB, 5 mM NaCl, 118 mM KCl, 1.1 mM $MgSO_4{\cdot}7H_2O$, 1.2 mM $KH_2PO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2{\cdot}2H_2O$, 25 mM D-Glucose, 9 mM HEPES in ultrapure water), cut with a vibratome into 200 µm thick slices and kept in KHB. Five to six slices were then plated on 0.4 µM inserts (30 mm 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 14 mM D-glucose (Sigma), 30 nM insulin (Gibco Life Technologies), 100 nM glucagon (Sigma), 1 nM corticosterone (Sigma), 1 nM Egf (Sigma) plus 5% FCS. The tissue cultures were kept in a standard cell incubator (37 °C, 5% $CO_2$ and 95% humidity) and the medium was changed daily. Integrity of explants was assessed by H&E staining (i.e. the presence of a nucleus and a normal cell structure on histology). Experiments were limited to 24/48 h cultures due to inconsistent tissue integrity at later times. Experiments with liver biopsies from three mice and one human subject were run in triplicates.

### *Statistical analysis*

Data are presented as mean ± SD. Differences between the groups were assessed by a two-tailed *t* test assuming unequal variance. In general, at least 5 mice/group