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Wnt- β -catenin Signaling Protects against Hepatic Ischemia and Reperfusion Injury in Mice

Nadja Lehwald^{*,1,2}, Guo-Zhong Tao^{*,1}, Kyu Yun Jang^{1,3}, Michael Sorkin⁴, Wolfram T. Knoefel², and Karl G. Sylvester^{1,5}

¹Department of Surgery, Division of Pediatric Surgery, Stanford University School of Medicine, Stanford, California

²Department of General, Visceral and Pediatric Surgery, School of Medicine, Heinrich Heine University, Duesseldorf, Germany

³Department of Pathology and Research Institute of Clinical Medicine, Chonbuk National University Medical School, South Korea

⁴Department of Surgery, Division of Plastic Surgery, Stanford University School of Medicine, Stanford, California

⁵The Lucile Packard Children's Hospital, Stanford, California

Abstract

Background & Aims—Ischemia and reperfusion injury are common causes of oxidative tissue damage associated with many liver diseases and hepatic surgery. The Wnt- β -catenin signaling pathway is an important regulator of hepatic development, regeneration, and carcinogenesis. However, the role of Wnt signaling in the hepatocellular response to ischemia-reperfusion (I/R) injury has not been determined.

Methods—Hepatic injury following ischemia or I/R was investigated in hepatocyte-specific, β -catenin-deficient mice, as well as Wnt1 overexpressing and wild-type (control) mice.

Results—Wnt- β -catenin signaling was affected by the cellular redox balance in hepatocytes. Following ischemia or I/R, mice with β -catenin-deficient hepatocytes were significantly more susceptible to liver injury. Conversely, mice that overexpressed Wnt1 in hepatocytes were resistant to hepatic I/R injury. Hypoxia inducible factor (HIF)-1 α signaling was reduced in β -

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Corresponding author: Karl G. Sylvester, M.D., Department of Surgery, Division of Pediatric Surgery, Stanford University School of Medicine, Hagey Laboratory for Regenerative Medicine, 257 Campus Drive, Stanford, CA 94305-5148, sylvester@stanford.edu, phone: (650) 804-0597, fax: (650) 725-5557.

*The first two authors contributed equally to this work.

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catenin-deficient liver but increased in hepatocytes that overexpressed Wnt1 under hypoxia and following I/R, indicating an interaction between β -catenin and HIF-1 α signaling in the liver. The mechanism by which Wnt signaling protects against liver injury involves β -catenin's role as a transcriptional co-activator of HIF-1 α signaling, which promotes hepatocyte survival under hypoxic conditions.

Conclusion—Cellular redox balance affects Wnt- β -catenin signaling, which protects against hypoxia and I/R injury. These findings might be used to develop strategies for protection of hepatocytes, regeneration of liver, and inhibition of carcinogenesis.

Keywords

TCF; LEF; liver damage; transplantation; reactive oxygen species; oxidative stress

Introduction

A common feature of many liver diseases as well as liver resection or transplantation is tissue hypoxia and reoxygenation that result in increased cellular reactive oxygen species (ROS), macromolecular damage and permanent hepatocellular injury^{1,2}. Since hepatocellular metabolism requires uninterrupted oxygen delivery for homeostasis, hepatocytes have evolved protective mechanisms in order to mitigate oxidative injury³.

The Wnt signaling pathway is an established molecular regulator of hepatic development, regeneration and carcinogenesis^{4–6}. The canonical Wnt signaling pathway is regulated through post-translational modifications of β -catenin. Wnt signaling is initiated through Wnt ligand binding to two membrane bound receptors, Frizzled, and the co-receptor Lipoprotein receptor Related Proteins 5 and 6 (LRP-5/6)^{7,8}. Wnt ligand binding disables GSK3 β preventing β -catenin N-terminal serine/threonine phosphorylation resulting in β -catenin accumulation and translocation to the nucleus where it activates the transcription complex T cell factor/lymphoid enhancer factor (TCF/LEF). Additional post-translational modifications (i.e. acetylation) of β -catenin have been described that significantly affect its activity as a transcriptional regulator^{9,10}. Previously, *in vitro* evidence has been provided suggesting that β -catenin can be diverted as a transcriptional activator from TCF/LEF to adaptive pathways like HIF-1 α (hypoxia inducible factor-1 α) in order to mediate the cellular response to oxidative stress^{11–14}. HIF-1, a key regulator of hypoxia, is a heterodimer consisting of a hypoxia-stabilized α -subunit (HIF-1 α) and a constitutively expressed β -subunit (HIF-1 β). Under hypoxic conditions, stabilized HIF-1 α translocates to the nucleus, dimerizes with HIF-1 β and binds hypoxia response elements (HRE) to activate target genes to promote angiogenesis and cellular metabolic changes^{15–17}.

Since both Wnt/ β -catenin and HIF-1 α signaling have established roles in regulating cell metabolism and survival, the present study was designed to investigate whether Wnt/ β -catenin signaling is required for an effective response to ischemia-reperfusion (I/R) injury in the liver. Herein, we demonstrate that hepatocyte-specific β -catenin knockdown mice are significantly more sensitive to liver I/R injury resulting in severe necrosis and apoptosis. Conversely, we show that in mice with hepatocyte-specific Wnt/ β -catenin stabilization, there is strong hepatic resistance to hypoxia and I/R injury. Together, these data provide the first

in vivo evidence that β -catenin is a key component of an effective, tissue-specific response to I/R and that the molecular mechanism is mediated by cellular redox balance.

Material and Methods

Animals

To investigate the role of Wnt/ β -catenin signaling in the liver, two hepatocyte-specific mouse models were created: for the β -catenin knockdown mouse, a previously described mouse possessing the liver enriched activator (LAP) promoter CEBP β driving tetracycline-transactivating (tTA) was crossed with a mouse possessing a tetracycline response element driving Cre (tetO-Cre)^{18,19}. Subsequent progeny were further bred with a β -catenin^{flox/flox} mouse⁶. The resultant triple transgenic mouse LAP-tTA/tetO-Cre/ β -catenin^{flox/flox} (LT2-KD) demonstrated responsiveness to the tetracycline analog doxycycline (dox) for β -catenin deletion. For wild-type controls (LT2-WT), their littermates (LAP-tTA⁻/tetO-Cre⁻/ β -catenin^{flox/flox}, LAP-tTA⁻/tetO-Cre⁻/ β -catenin^{wt/flox}, LAP-tTA⁺/tetO-Cre⁻/ β -catenin^{wt/wt}) were used. Dox-water was given in all breeding cages and removed at the age of 6–8 weeks. After dox-withdrawal for 4 weeks mice were used for experiments. For the conditional Wnt1 overexpressing mouse, a tetracycline-responsive tetO-Wnt1-Luc mouse was crossed to the LAP-tTA mouse described above^{18–20}. This double transgenic mouse LAP-tTA/tetO-Wnt1-Luc (Wnt1+) enables hepatocyte-specific activation of the transgene Wnt1-Luc in a dox-dependent manner. For experiments, sex-matched 6–8 weeks old littermates (LAP-tTA⁺/tetO-Wnt1-Luc⁺) were kept continually with (Wnt1-WT) or removed from dox-water (Wnt1-) for additional 3 weeks. Experiments were conducted under a protocol approved by Stanford University School of Medicine Institutional Animal Care and in accordance with NIH guidelines.

Liver Ischemia and Reperfusion Model

We used a murine model of 70% partial warm liver ischemia-reperfusion (75 minutes/6 hours) injury²¹. Details are provided in Supplementary data.

Cell Culture

Primary hepatocytes from 10–12 weeks old mouse livers were isolated using the two-step collagenase perfusion²². Hepatocytes were cultured in DMEM/F12 supplemented with 1% FCS.

The differentiated non-transformed mouse hepatocyte AML12 and human hepatocellular carcinoma HepG2 cell lines were cultured in either DMEM/F-12 or DMEM supplemented with 10% FCS²³ at 37°C with 5% CO₂. For hypoxia, cells were grown at 1% O₂ in a custom-designed incubator (XVivo Hypoxia Chamber; BioSpherix) for 24 hours. In order to simulate *in vivo* liver I/R, cells were incubated in 1% hypoxia for 24 hours followed by reoxygenation (H/R) for 2 hours.

Plasmid and Transfection

To activate canonical Wnt/ β -catenin signaling, we utilized the plasmid pcDNA3S33Y resulting in robust TCF-dependent transcriptional activation compared to wild-type β -

catenin²². Stable S33Y mutants conferring β -catenin gain-of-function were derived through retroviral transfection in AML12 hepatocytes.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared using the nuclear and cytoplasmic extraction kit (Thermo Scientific, Rockford, IL). DNA-protein binding reaction was performed using an Epo/HIF-1 α probe according to the manufacturer's instructions (Panomics, Fremont, CA).

Cell Proliferation and Viability Assays

BrdU ELISA and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN).

Apoptosis and Necrosis Assays

TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche Applied Science) according to the manufacturer's instructions. K18D237 antibody (AnaSpec, Fremont, CA) was used for detecting caspase-cleaved Keratin 18 (K18) by immunoblot²⁴. Caspase-Glo 3/7 assay (Promega, Madison, WI) was performed according to the manufacturer's instructions. Microscopic quantification analyses were performed in 10 high power fields (HPF) per specimen at 400 $\times$.

Luciferase Reporter Assay

Dual-Light Reporter Gene Assay (Applied Biosystems, Foster City, CA) was performed using the pMegaTOPFLASH, pMegaFOPFLASH, 5x-HRE or LacZ plasmids as previously described^{12,25}.

Determination of intracellular ROS levels

Dichlorofluorescein diacetate (DCF-DA; Invitrogen, Carlsbad, CA) was used to monitor intracellular ROS generation. After incubation with 10 μ mol/L DCF-DA for 30 minutes, cells were analyzed by flow cytometry²⁶. In addition, *in situ* ROS detection was performed by dihydroethidium (DHE; Invitrogen) labeling of frozen liver sections with 3 μ M DHE at 37°C for 30 minutes.

Statistics

All experiments were performed in at least triplicate unless otherwise indicated. Data are expressed as mean $\pm$ standard deviation, and evaluated by Student's t test. Significance was defined as $p < .05$; „n.s.“ indicates not significant.

Results

Oxidative stress inhibits β -catenin/TCF signaling in hepatocytes

Since Wnt/ β -catenin signaling and cellular redox balance are integral to liver homeostasis, we sought to determine the effect of ROS generation on Wnt signaling in hepatocytes. Exposure of AML12 cells to either hypoxia or H/R resulted in a significant decrease in β -

catenin/TCF reporter activity without a change in total β -catenin protein (Fig.1A) or transcript level (not shown). However, β -catenin/TCF target gene expression was significantly reduced in response to hypoxia (supplementary Fig.S1A) in-line with the observed signal activity repression (Fig.1A). Moreover, the hypoxia-induced reduction in TCF signaling appeared to be ROS dependent as the antioxidant N-acetylcysteine (NAC), a ROS scavenger, was able to decrease intracellular ROS (Fig.1B) and diminish the suppressive effect of hypoxia-derived ROS on β -catenin/TCF activity (Fig.1C). Together, these data demonstrate that β -catenin signal transduction is significantly impacted by cellular redox balance.

Conditional β -catenin knockdown sensitizes the liver to ischemia-reperfusion injury

Previous studies have reported heightened hepatic sensitivity to injury stimuli in the absence of β -catenin²⁷. However, the response to a clinically relevant source of ROS generation like hypoxia or I/R has not been evaluated. To determine β -catenin's role in the adaptive response to I/R *in vivo*, we developed a novel mouse model for conditional and regulatable β -catenin deletion from hepatocytes in order to overcome compensatory changes that may occur when β -catenin is deleted during liver development in cells with transcriptionally active albumin (Alb-Cre)⁶. The triple transgenic mouse LAP-tTA/tetO-Cre/ β -catenin^{flox/flox} (LT2-KD) was engineered to conditionally delete β -catenin in response to dox-withdrawal (Fig.2A; supplementary Fig.S2A)^{18,19}. Four weeks of dox-withdrawal resulted in a significant reduction in β -catenin expression in hepatocytes at mRNA and protein level (Fig. 2A). Untreated LT2-WT and KD mice did not show any significant difference in transaminases or liver histology (supplementary Fig.S2B, Fig.S2C). To determine if β -catenin knockdown exacerbates oxidative injury *in vivo*, we subjected LT2-KD mice and littermates (LT2-WT) to either liver ischemia or I/R respectively. Liver injury as assessed by serum transaminases was significantly increased in KD mice after ischemia and dramatically exacerbated after I/R compared to LT2-WT mice (Fig.2B). The genotypic difference in liver damage was specific for ischemia and I/R, as transaminases were similar in sham-treated LT2-WT and KD mice (supplementary Fig.S2D). Histology (Fig.2C) of KD livers revealed severe hypoxic injury around the central vein with hepatocyte swelling, necrosis and karyolysis, while signs of ischemia were substantially attenuated in wild-type mice. H&E staining after I/R showed extensive hepatocellular injury with wide necrosis, congestion and swelling in KD hepatocytes. Conversely, LT2-WT livers showed relatively well-preserved histological architecture with less hepatocyte damage. TUNEL staining and quantification showed more severe apoptosis in both ischemia and I/R-challenged KD livers (Fig.2C, Fig. 2D). Immunoblot for caspase-cleaved K18 also indicated increased hepatocellular apoptosis following I/R in LT2-KD mice (Fig.2D). Morphometry revealed necrosis in ~50% of I/R-subjected KD livers, while only ~25% of LT2-WT livers showed signs of necrosis (Fig.2E). DHE staining for the *in situ* detection of ROS (Fig.2C) revealed a pronounced increase in LT2-KD livers when compared to controls after I/R. As evidence of an overall increase in hepatocellular oxidative injury, LT2-KD livers demonstrated a uniform induction of the compensatory anti-oxidant genes GST, SOD1 and GPX1 following I/R (supplementary Fig.S2E). Given these phenotypic effects in the LT2-KD mice and a potential crosstalk between β -catenin and HIF-1 α that has been previously demonstrated *in vitro*¹¹⁻¹³, we

sought further evidence for the effect of β -catenin deletion on HIF signal transduction in response to hypoxia and I/R.

In response to ischemia, HIF-1 α protein significantly increased, whereas HIF-2 α expression seemed not inducible (Fig.2F). Intriguingly, a significantly impaired induction in HIF-1 α protein (Fig.2F, KD vs LT2-WT) and its target genes VEGF and HO-1 (supplementary Fig.S2F) was observed in β -catenin deficient hepatocytes after ischemia and/or I/R. Further, isolated LT2-KD hepatocytes demonstrated both reduced TCF and HIF signal activity under hypoxia (supplementary Fig.S3A, Fig.S3B) compared to LT2-WT. Together, these results demonstrate that β -catenin has a protective role during liver ischemia and I/R that dramatically reduces the degree of hepatocellular injury. These data further suggest that HIF-1 α signaling is impaired in the absence of β -catenin in response to ischemia or I/R.

Augmented β -catenin signaling protects mice from liver I/R Injury

To further investigate the role of Wnt/ β -catenin signaling in injury protection *in vivo*, we created a hepatocyte-specific Wnt1 overexpressing mouse responsive to doxycycline. Bioluminescent imaging showed increased Wnt1-Luciferase activity after dox-withdrawal with a maximum after 21 days (Fig.3A). Longer dox-withdrawal (>3 weeks) did not further increase Wnt1 expression. A significant increase in Wnt1 and Cyclin D1 protein together with a moderate increase in total β -catenin (Fig.3A) was observed in Wnt1+ livers along with several established canonical β -catenin downstream target genes after 3 weeks of Dox-withdrawal (supplementary Fig.S4A). Untreated Wnt1-WT and Wnt1+ mice did not show any difference in transaminases or liver histology (supplementary Fig.S4B, Fig.S4C). To determine if Wnt-mediated β -catenin gain-of-function has a protective effect against hypoxic injury *in vivo*, we subjected Wnt1+ and Wnt1-WT mice to hepatic ischemia and I/R. Hepatocellular injury as measured by serum transaminases was substantially attenuated in Wnt1+ mice after ischemia and I/R (Fig.3B) when compared to controls indicating significant protection against oxidative injury *in vivo*. Sham-treated mice demonstrated transaminases in the normal range (supplementary Fig.S4D). Liver histology (Fig.3C) showed remarkable hepatocyte protection against ischemia in Wnt1+ livers with well-preserved hepatocellular architecture. Ischemic Wnt1-WT livers revealed severe hepatocyte injury with swelling and cytoplasmic vacuolization. In response to I/R, Wnt1-WT livers demonstrated extensive hepatocyte damage with signs of liver congestion and degenerative changes. Conversely, the hepatocellular architecture in Wnt1+ livers was still well-preserved. Correspondingly, Wnt1+ mice were also more resistant to apoptosis (Fig.3C, Fig. 3D) and showed less necrosis (Fig.3C, Fig.3E) after ischemia and I/R. DHE staining (Fig. 3C) showed less ROS production in Wnt1+ livers after I/R when compared to controls. Interestingly and contrary to LT2-KD mice, notable HIF-1 α protein and its target genes VEGF (Fig.3F), Epo and Glut1 (supplementary Fig.S4E) were uniformly increased more in Wnt1+ livers after ischemia and/or I/R when compared to controls. However, HIF-2 α protein did not change in response to ischemia or between Wnt1-WT and Wnt1+ mice. The augmented response in HIF-1 α and correspondingly increased target gene expression is a potentially significant contributor to the observed increase in hepatocyte resistance to hypoxic injury. In further support of a Wnt1-mediated resistance to oxidative injury, a uniform decrease in anti-oxidant gene expression was also observed in Wnt1+ livers

following I/R (supplementary Fig.S4F). Taken together, these data demonstrate that Wnt1 overexpression confers significant hepatic protection against I/R injury through augmented HIF-1 α signaling and an overall reduction in redox imbalance.

Hepatocytes overexpressing β -catenin resist hypoxia-induced apoptosis through augmented HIF-1 α signaling

To further explore the mechanism by which Wnt/ β -catenin affords increased protection against hypoxia or H/R, mutant AML12 hepatocytes carrying an amino terminus phosphorylation resistant point mutation (S33Y) in β -catenin were created. S33Y mutants demonstrated increased β -catenin level, robust TCF signal transduction and increased proliferation compared to control cells (supplementary Fig.S5A, Fig.S5B). When cells were exposed to hypoxia, β -catenin mutants demonstrated death resistance and continued proliferation despite hypoxic stress (Fig.4A, supplementary Fig.S5B, Fig.S5C). Moreover, S33Y mutants showed reduced sensitivity to oxidative stress with lower intracellular ROS levels and less apoptosis compared to controls (Fig.4B, Fig.4C, Fig.4D). Reoxygenation after hypoxia resulted in increased apoptosis, but β -catenin mutants remain less susceptible to H/R-induced injury than controls (Fig.4C). HIF-1 α protein was not detectable under normoxia, but was stabilized by hypoxia and H/R (Fig.4C). Intriguingly, β -catenin mutants displayed increased HIF-1 α in response to both hypoxia and H/R compared to control hepatocytes. Taken together, these data demonstrate that β -catenin stabilization protects hepatocytes against hypoxic injury in association with diminished ROS production and augmented HIF-1 α signaling resulting in increased cell survival.

When exposed to hypoxia, TCF signal transduction in S33Y cells decreased comparable to controls despite a higher baseline elevation (Fig.4E). Interestingly, β -catenin mutants also demonstrated significantly increased HIF activity compared to control cells (Fig.4F). These results support the *in vivo* findings above and further extend these observations to demonstrate that augmented HIF-1 α signaling occurs with β -catenin stabilization, yet independent of the canonical β -catenin/TCF pathway. Moreover, these data suggest that the ROS-mediated dampening of β -catenin/TCF signaling is not dependent on phosphorylation of β -catenin.

In response to hypoxia, β -catenin diverts from TCF binding to HIF-1 α to support cell survival

Given the findings above, we next sought to determine if β -catenin complexes directly with TCF or HIF-1 α according to cellular conditions that may favor proliferation or survival respectively. To investigate whether the inhibition in TCF signaling is a result of a β -catenin switch as a transcriptional activator, co-immunoprecipitation assays were performed. The hepatocellular carcinoma cells HepG2 were utilized to investigate this phenomenon in a human cell line with clinical relevance and established β -catenin gain-of-function. Under normoxia, β -catenin was found to primarily complex with TCF but alternatively predominantly binds HIF-1 α in response to hypoxia (Fig.5A). To extend the *in vitro* crosstalk above, we further provide *in vivo* evidence that β -catenin binds to HIF-1 α in ischemic livers to promote injury protection as shown by co-immunoprecipitation (Fig.5B).

These results provide additional evidence that in Wnt1 overexpressing livers, β -catenin/HIF-1 α binding is significantly increased when compared to Wnt1-WT mice.

Next, we determined the effect of β -catenin gain-of-function mutation (β -catenin^{S33Y}) on HIF-1 DNA binding activity. EMSA was performed using a HRE probe, derived from the erythropoietin enhancer, along with nuclear extracts from control cells and β -catenin mutants treated with normoxia and hypoxia (Fig.5C). Significantly more HIF-1/HRE probe binding was observed in β -catenin^{S33Y} vs control lysates under hypoxia. This binding specificity of HIF-1 was confirmed by adding an unlabeled competitor probe that resulted in the disappearance of the band (not shown). To investigate if β -catenin/TCF signaling can be dampened by HIF-1 α directly, we stabilized HIF-1 α expression under normoxia by the hypoxia-mimetic cobalt chloride (CoCl₂) (Fig.5D). HIF stabilization by CoCl₂ under normoxia resulted in a significant reduction in TCF and increase in HIF-1 α signal activity and protein level in AML12 hepatocytes (Fig.5D). To further elucidate whether the protective effect against hypoxia-induced apoptosis is specific to HIF-1 α signaling, hepatocytes were treated with YC-1, a known HIF inhibitor. HIF-1 α inhibition under hypoxia significantly reduced HIF signal activity (supplementary Fig.S6A) and caused more apoptosis (Fig.5E).

Taken together, these data support a model in which β -catenin binds to HIF-1 α under oxygen-limiting conditions to support hepatocyte survival (Fig.6). Evidence is also provided to suggest that a phosphorylation independent event modulates β -catenin's role as a transcriptional activator in response to hypoxia.

Discussion

The hepatocellular response to a variety of stimulants involving oxidative stress is a common injury mechanism in nearly all liver pathologies^{1,2}. In this study, we utilized cellular hypoxia and tissue ischemia-reperfusion as clinically relevant models involving profound changes in hepatocyte redox balance to gain a better understanding of the critical molecular regulators of hepatic adaptation and potential liver protection strategies. The pleiotropic effects of Wnt signaling are well established with specific roles identified for the Wnt/ β -catenin axis during hepatic development, nutrient metabolism, regeneration and carcinogenesis^{4-6,28-30}. Despite abundant data demonstrating the predominance of biologic activity mediated by Wnt signal transduction through β -catenin and TCF/LEF, more recent evidence supports a diverse role for β -catenin as a transcriptional co-activator independent of TCF. Specifically, β -catenin has been previously shown to interact with HIF-1 α and FOXO transcription factors to mediate a cellular response to unfavorable environmental conditions *in vitro*^{11,12,14,31}. Since the liver is of singular metabolic importance and β -catenin as well as HIF signaling are known critical regulators of cellular metabolism, this study was designed to further investigate the *in vivo* relevance and molecular mechanisms governing possible β -catenin/HIF crosstalk. Moreover, as tissue hypoxia and Wnt signaling are both known to be critical mediators of diverse biologic processes from development to tissue regeneration and tumorigenesis, the findings reported herein may have far reaching implications for developing novel treatment strategies.

In this study, we report that β -catenin signaling through HIF-1 α is critical for an adequate *in vivo* tissue response in a model of hepatic ischemia-reperfusion injury. We submit that the severe hepatic damage observed in the β -catenin knockdown mouse in response to ischemia and I/R results from the deletion of β -catenin in adult mouse liver. In contrast to all other reports^{6,27,29,30} using the β -catenin^{flox/flox}/Alb-Cre mouse that results in perinatal β -catenin deletion, our novel β -catenin knockdown approach has the additional advantage of conditional control for the genetic deletion of β -catenin from mature hepatocytes in order to overcome any compensatory adaptive changes that may occur during liver development and post-natally. HIF-1 α is a key transcription factor involved in the cellular response to oxygen and nutrient stress signaling. HIF-1 α is stabilized under hypoxic conditions resulting in the upregulation of genes involved in angiogenesis, energy metabolism, adaptation, survival, and glycolysis^{15,16,32,33}. Crosstalk between Wnt/ β -catenin and HIF-1 α signaling has been previously described in different organ systems *in vitro*^{12,13,31,34}, but to date has not been shown *in vivo* liver injury. As previously reported by Kaidi and others^{12,13}, the current study complements previous findings that HIF-1 α can compete with TCF for binding β -catenin in response to hypoxia. Our *in vivo* findings further suggest that HIF-1 α signaling is specifically impaired and cellular redox balance disrupted as a result of β -catenin depletion from hepatocytes. We have extended the prior observations of others to critically demonstrate that β -catenin signal transduction is significantly impacted by cellular redox balance as shown by changes in ROS level and their modulation by the anti-oxidant NAC.

Taken together, our results demonstrate that an increase in cellular ROS levels that accompany numerous injury stimuli (oxidative, hypoxic, metabolic and genotoxic stress), and thereby relevant hepatic pathologic states, are critical intra-cellular mediators of β -catenin signaling in response to changing conditions. This provides a key mechanistic distinction to deepen our understanding of the proposed model (Fig.6) in which hepatocytes respond to changes in relevant stimuli to enact programs alternatively for proliferation (TCF) when conditions are favorable, or adaptation and survival (HIF-1 α) in response to limiting environmental conditions.

Another intriguing finding in this study was the observation that canonical phosphorylation changes in β -catenin did not appear to be the primary molecular mechanism by which Wnt signal transduction is altered in response to changing cellular conditions. Wnt pathway gain-of-function mutations, including commonly identified key β -catenin phosphorylation site mutations that were utilized herein, have been reported in human HCC and mouse liver tumor models^{28,35}. In light of our findings, the interaction of accumulated β -catenin with both TCF and HIF-1 α may represent a maladaptive response in which given an abundance of β -catenin, cellular proliferation proceeds despite unfavorable conditions, but also results in enhanced HIF-1 α signaling for increased survival as we have demonstrated. Since the phosphorylation resistant mutants (β -catenin^{S33Y}) were responsive to oxidative changes as demonstrated by decreased TCF activity comparable to cells with wild-type β -catenin, we speculate that alternative post-translational modifications (e.g. acetylation) regulating β -catenin are additionally responsible for modulating its activity as a transcriptional regulator^{9,10,13}. Additional studies beyond the scope of this report are needed to further define the post-translational mechanisms governing β -catenin activity in response to ROS.

To strengthen and extend our findings, we also sought to determine whether the Wnt pathway could be utilized to augment HIF-1 α signaling to promote cell survival in response to the same injury stimuli that β -catenin deficient mice were sensitized to *in vivo*. Wnt-1 is an established proto-oncogene and upstream regulator of β -catenin signaling^{36–38}. It has been previously reported that Wnt-1/ β -catenin activation via retroviral Wnt-1 injection promotes liver regeneration through enhanced hepatocyte proliferation following partial hepatectomy³⁵. However, to our knowledge Wnt pathway manipulation has not previously been tested for an ability to promote tissue resistance to oxidative injury, and specifically to I/R in the liver. Our findings that Wnt-1 overexpression results in activation of β -catenin/TCF signaling and relevant downstream target genes is also supported by previous experimental observations in other tissues^{39,40}. Interestingly, we observed that Wnt1-mediated β -catenin stabilization resulted in increased hepatocyte survival and strong hepatic resistance to oxidative injury. Intriguingly, we also detected a parallel increase in HIF-1 α protein, relevant HIF-1 α target genes and a corresponding decrease in the compensatory antioxidants SOD1 and GPX. Surprisingly, HIF-2 α did not change at protein level in response to I/R. Together, these findings support the important role of the Wnt axis as a key *in vivo* modulator of HIF-1 α signaling and redox balance for adaptation and cell survival.

In summary, this study provides the first *in vivo* evidence that β -catenin is a key component of a tissue-specific response to I/R and that the molecular mechanism appears to be mediated by cellular redox balance and β -catenin's role as a transcriptional activator. Our observations have significant clinical relevance since ischemia and reperfusion are common features of many liver diseases. In light of the data presented here, this study may further support the promise and clinical application of Wnt1 manipulation or other modulators of Wnt/ β -catenin signaling in clinically relevant settings involving a hepatic oxidative stress response to effect liver injury protection, repair and regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

B2M β 2-microglobulin

CoCl₂	Cobalt Chloride
DCF-DA	dichlorofluorescein diacetate
DHE	dihydroethidium
Epo	erythropoietin
FACS	fluorescence activated cell sorting
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glut1	glucose transporter 1
GPX1	glutathione peroxidase 1
GST	glutathione S-transferase
HCC	hepatocellular carcinoma
HO-1	heme oxygenase 1
HIF-1α	hypoxia inducible factor-1 α
HPF	high power field
H/R	hypoxia reoxygenation
HRE	hypoxia response element
I/R	ischemia reperfusion
LAP	liver enriched activator protein (a.k.a. CEBP/ β)
LEF	lymphoid enhancer factor
NAC	N-acetylcysteine
SOD1	superoxide dismutase 1
TCF	T cell factor
tetO-Cre	tetracycline response element driving Cre
tTa	tetracycline transactivating
VEGF	Vascular Endothelial Growth Factor

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Fig. 1. β -catenin/TCF signaling is impacted by cellular redox changes

- A.** β -catenin/TCF reporter activity significantly decreases in response to 1% hypoxia for 24 hours or H/R (24/2 hours) in AML12 hepatocytes without a change in total β -catenin protein.
- B.** Intracellular ROS levels in AML12 hepatocytes are significantly elevated by hypoxia as measured by DCF-DA flow cytometry. One hour pre-treatment with NAC (2 mmol/L) significantly prevents ROS production under hypoxia.

- C. One hour pre-treatment with NAC (2 mmol/L) prevents hypoxia-induced inhibition of β -catenin/TCF signaling in AML12 hepatocytes. RLU= relative light units.
* $p < 0.05$, ** $p < 0.01$.

Fig. 2. β -catenin knockdown mice are more susceptible to hepatic I/R injury

- A.** Schematic strategy for the generation of LT2-KD mouse. qRT-PCR demonstrates a significant reduction of β -catenin mRNA in LT2-KD livers after 4 weeks of dox-withdrawal. Immunoblot shows a remarkable knockdown at its protein level in LT2-KD hepatocytes.
- B.** Hepatocellular injury, as evidenced by elevated serum transaminases, is significantly increased in LT2-KD mice after ischemia and I/R.

- C.** Severe liver damage, increased apoptosis and elevated ROS is detected in LT2-KD mice after ischemia and I/R. Representative liver histology of sham, ischemia or I/R-treated livers. Apoptosis is measured by TUNEL staining after ischemia or I/R. Intracellular ROS levels are detected by DHE staining after I/R.
- D.** LT2-KD livers are more susceptible to I/R-induced apoptosis. Quantification of TUNEL-positive cells/10 HPF in ischemia or I/R-treated livers (see Fig.2C). Immunoblot shows more caspase-cleaved K18Asp237 in total liver lysates of LT2-KD mice after I/R.
- E.** β -catenin deficient livers show increased necrosis by quantification of necrotic areas in ischemia and I/R-treated livers (see Fig.2C).
- F.** Reduced HIF-1 α protein and its target gene (VEGF) mRNA expression in LT2-KD mice. VEGF mRNA expression was measured in sham, ischemia or I/R-treated livers by qRT-PCR. HIF-1 α induction after ischemia is significantly impaired in LT2-KD livers as shown by immunoblot (n=5). *p<0.05, **p<0.01.

Fig. 3. Wnt1 gain-of-function provides strong protection against hepatic I/R Injury

- A.** Bioluminescence imaging of Wnt1+Luciferase mice show increased liver-specific Luciferase activity upon Dox-withdrawal. Increased Wnt1 and β-catenin target gene (Cyclin D1) protein are expressed in liver lysates of Wnt1+ mice along with a moderate increase in total β-catenin after 3 weeks of dox-removal.
- B.** Reduced hepatocellular injury as evidenced by lower transaminases is detected in Wnt1+ mice after ischemia and I/R.

- C.** Minor hepatic damage with reduced apoptosis and lower ROS was determined in Wnt1+ livers after ischemia and I/R. Representative liver histology (H&E) of sham, ischemia or I/R-treated livers. Apoptosis was measured by TUNEL staining after ischemia or I/R. Intracellular ROS levels were detected by DHE staining after I/R.
- D.** Wnt1+ livers are more resistant to ischemia and I/R-induced apoptosis. Quantification of TUNEL-positive cells/10 HPF in ischemia and I/R-treated livers (see Fig.3C). Immunoblot of liver lysates demonstrates diminished levels of caspase-cleaved K18Asp237 in Wnt1+ mice after I/R.
- E.** Wnt1+ livers show less necrosis in ischemia and I/R-treated livers by quantification of necrotic areas (see Fig.3C).
- F.** HIF-1 α protein and its target gene (VEGF) expression are significantly induced in Wnt1+ livers. VEGF mRNA expression in sham, ischemia and I/R-treated livers was measured by qRT-PCR. HIF-1 α but not HIF-2 α is significantly augmented in Wnt1+ livers after ischemia as shown by western blot (n=5). *p<0.05, **p<0.01.

Fig. 4. β -catenin stabilized hepatocytes are resistant to hypoxia-induced apoptosis *in vitro* by augmented HIF-1 α signaling

- A.** S33Y mutant hepatocytes are stress-resistant and proliferate despite hypoxic stress as measured by BrdU ELISA assay.
- B.** Under hypoxia, β -catenin mutants show reduced intracellular ROS as determined by DCF-DA flow cytometry.

- C.** S33Y hepatocytes are highly resistant to hypoxia or H/R-induced apoptosis as determined by caspase-cleaved K18Asp237. β -catenin mutants show more HIF-1 α induction under hypoxia or H/R compared to controls.
- D.** Less apoptosis is detected in β -catenin mutants under hypoxia by Caspase-Glo 3/7 activity assay.
- E.** TCF reporter activity is decreased in S33Y mutants after hypoxia to a comparable degree as control cells despite a heightened β -catenin/TCF baseline activity.
- F.** Augmented HIF-1 α reporter activity is detected in β -catenin mutants under hypoxia. RLU= relative light units. * $p < 0.05$.

Fig. 5. β-catenin signaling protects hepatocytes against hypoxic injury through augmented HIF-1 signaling

- A.** β-catenin alternatively binds TCF (normoxia) or HIF-1α (hypoxia) depending on oxygen availability. Binding switch was verified by co-immunoprecipitation on HepG2 cell lysates after exposure to normoxia (N) or hypoxia (H) using anti-β-catenin antibody followed by immunoblotting for HIF-1α and TCF4.

- B.** Wnt1+ livers show more β -catenin/HIF-1 α binding as verified by co-immunoprecipitation in sham or ischemia-treated liver lysates using anti- β -catenin antibody followed by HIF-1 α immunoblotting.
- C.** Augmented HIF-1 α promotor binding in β -catenin mutants after 24 hours hypoxia. For EMSA, 5 μ g of nuclear extracts were used for HIF-1/HRE DNA-probe binding reactions.
- D.** β -catenin/TCF reporter activity can be reduced by HIF-1 α stabilization with CoCl₂ (150 μ mol/L) under normoxia or hypoxia treatment in AML12 hepatocytes. HIF-1 α induction was verified by HRE reporter assay and immunoblot. RLU= relative light units.
- E.** HIF-1 α inhibition under hypoxia by YC-1 pre-treatment (100 μ mol/L) for 1 hour results in more apoptosis in AML12 hepatocytes as measured by MTT assay and immunoblot for caspase-cleaved K18Asp237. *p<0.05.

Fig. 6. Working model explaining β -catenin's role in hepatocyte protection to I/R Injury

- A.** Under normoxic, favorable conditions (upper panel), β -catenin complexes with TCF to promote hepatocyte proliferation.
- B.** Under unfavorable conditions like hypoxia or I/R, β -catenin diverts from TCF and preferably associates with HIF-1 α to favor cell adaptation and survival (lower, left panel). However, in the absence of β -catenin (lower, middle panel), neither sufficient TCF nor effective HIF-1 α signaling can be activated leading to cell death and enhanced liver injury under hypoxic stress. With Wnt/ β -catenin signaling gain-of-function (lower, right panel), signaling through both TCF and HIF-1 α occurs resulting in increased resistance to I/R and continued proliferation.