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Review

Role and Regulation of β-Catenin Signaling During Physiological Liver Growth

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Wnt/β-catenin signaling plays key roles not only during development but also in adult tissue homeostasis. This is also evident in liver biology where many temporal roles of β-catenin have been identified during hepatic development, where, in hepatic progenitors or hepatoblasts, it is a key determinant of proliferation and eventually differentiation to mature hepatocytes, while also playing an important role in bile duct homeostasis. β-Catenin signaling cascade is mostly quiescent in hepatocytes in an adult liver except in the centrizonal region of a hepatic lobule. This small rim of hepatocytes around the central vein show constitutive β-catenin activation that in turn regulates expression of genes whose products play an important role in ammonia and xenobiotic metabolism. Intriguingly, β-catenin can also undergo activation in hepatocytes after acute liver loss secondary to surgical or toxicant insult. Such activation of this progrowth protein is observed as nuclear translocation of β-catenin and formation of its complex with the T-cell factor (TCF) family of transcription factors. Expression of cyclin-D1, a key inducer of transition from the G_1 to S phase of cell cycle, is regulated by β-catenin–TCF complex. Thus, β-catenin activation is absolutely critical in the normal regeneration process of the liver as shown by studies in several models across various species. In the current review, the temporal role and regulation of β-catenin in liver development, metabolic zonation in a basal adult liver, and during the liver regeneration process will be discussed. In addition, the probability of therapeutically regulating β-catenin activity as a possible future treatment strategy for liver insufficiency will also be discussed.

Key words: Liver development; Liver regeneration; Proliferation; Glutamine synthetase; Cyclin-D1; Nonparenchymal cells; Zonation

WNT/β-CATENIN SIGNALING

Wnts are secreted glycoproteins that require posttranslation modifications for their biologic activity. Porcupine protein is important for acylation and glycosylation of Wnt proteins in the endoplasmic reticulum (1). Wntless protein in Golgi apparatus is absolutely critical for Wnt sorting and its secretion from a cell (2). Upon secretion of biologically active Wnt protein, it binds to receptor Frizzled and coreceptor LDL-related protein 5 (LRP5) or LRP6 on a target cell to initiate a signaling cascade in a cell. In fact, any of the 19 mammalian Wnt proteins can bind to any of the 10 Frizzled proteins to activate a diverse set of downstream signaling to eventually activate either the Wnt/β-catenin pathway or the planar cell polarity (PCP) pathway or the Wnt/calcium pathway. The divergence and its molecular basis are beyond the scope of the current review and work in progress (3). However, activation of the Wnt/β-catenin pathway that entails formation of a β-catenin–T-cell factor (TCF)/lymphoid enhancement factor (LEF) complex leading to target gene expression is overall well understood. In its simplest form, Wnt activation results in inactivation of β-catenin degradation complex composed of adenomatous polyposis coli gene product (APC), axin, glycogen synthase kinase 3β (GSK3β), and casein kinase 1 (CK1). This implies that β-catenin cannot be phosphorylated at specific residues in exon 3, and in its hypophosphorylated form, β-catenin is released from the complex, accumulates in the cytoplasm, and eventually translocates to the nucleus to bind to the TCF/LEF family of transcription factors (TFs) to induce target gene expression (Fig. 1). Various target genes of the Wnt/β-catenin pathway have been identified, although these are context and tissue specific. In the absence of the Wnt signal, β-catenin protein is phosphorylated at serine 45 (Ser45) by CK1, which primes it for sequential phosphorylation by GSK3β at Ser33, Ser37,

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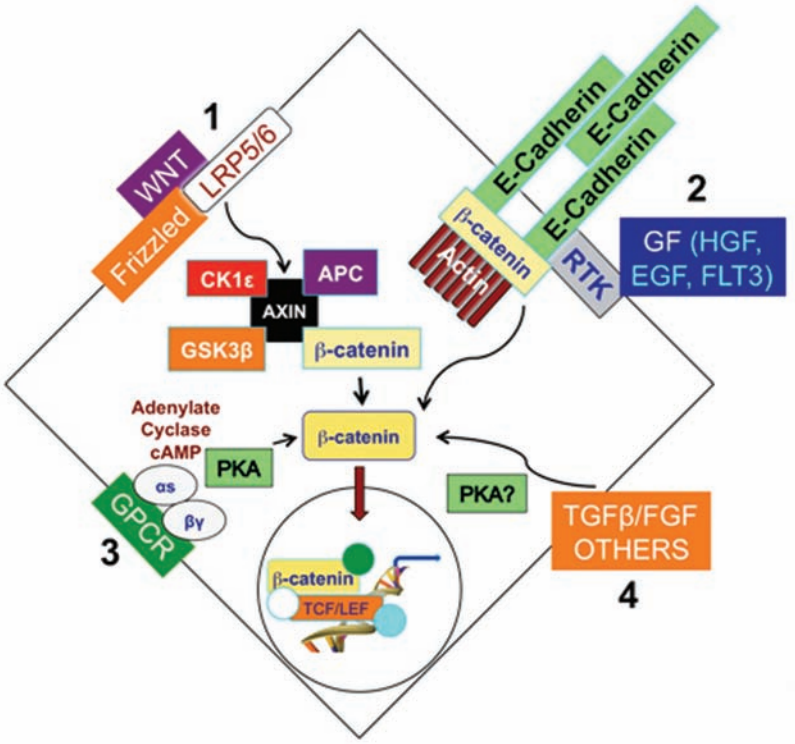

Figure 1. Various mechanisms leading to β-catenin activation in a cell. While Wnt signaling (1) is the chief upstream effector of β-catenin, which allows its stabilization by inhibiting its degradation complex, E-cadherin–β-catenin complex at adherens junctions (2) is susceptible to various receptor tyrosine kinases that allow tyrosine phosphorylation-dependent β-catenin activation. Protein kinase A (3) through G protein-coupled receptor activation has been shown to directly phosphorylate β-catenin at serine 552 and serine 675 and induce its activation. Last, growth factors such as TGF-β and FGFs have been shown to activate β-catenin (4) through less well understood mechanisms that may or may not involve protein kinase A (LRP5/6, LDL-related protein 5 or 6; CK1ε, casein kinase 1 ε; GSK3β, glycogen synthase kinase 3β; APC, adenomatous polyposis coli gene product; GF, growth factors; HGF, hepatocyte growth factor; EGF, epidermal growth factor; PKA, protein kinase A; GPCR-G protein-coupled receptor; TGF-β, transforming growth factor-β; FGF, fibroblast growth factor; TCF, T-cell factor; LEF, lymphoid enhancement factor).

and threonine-41 (Thr41) (4). These phosphorylation events are sufficient for β-transducin repeat-containing protein (βTrCP) to recognize β-catenin for its eventual degradation by ubiquitin–proteosome. It is relevant to mention that in around 10–40% of all hepatocellular cancer (HCC) patients, the *CTNNB1* gene displays mutations affecting exon 3 that allow β-catenin to escape phosphorylation and degradation and hence leads to an activation of the Wnt/β-catenin signaling [reviewed in (5)]. Several liver-specific targets of the Wnt pathway, such as glutamine synthetase (GS), cyclin-D1, lect2, VEGF-A, EpCAM, and others, may be playing roles in HCC tumor biology, which is the basis of rationalizing anti-β-catenin therapies in a subset of such patients (6–11).

β-CATENIN AS PART OF ADHERENS JUNCTIONS

In addition to being the chief downstream effector of Wnt signaling, β-catenin is also part of the adherens junctions (AJ) where it forms a bridge between the cytoplasmic tail of E-cadherin and actin cytoskeleton (Fig. 1). This AJ assembly provides lateral anchoring between cells to maintain intercellular adhesion. While the regulation of the AJ assembly is beyond the scope of the current review, a few relevant points need special emphasis. The association of β-catenin–E-cadherin is known to occur in endoplasmic reticulum, and special tyrosine phosphorylation sites in E-cadherin facilitate this association to β-catenin (12). This interaction masks the PEST sequence (proline: P; glutamic acid: E; serine: S; and threonine: T) in E-cadherin protein, preventing its degradation and allowing its successful transport to the membrane. At the membrane, tyrosine phosphorylation of β-catenin at residues Y142, Y489, and Y654 by different kinases including HGF/Met, EGFR, Fer, Src, and others [reviewed in (13)] can induce dissociation of β-catenin from E-cadherin to dismantle cell–cell junctions and may also lead to activation of β-catenin signaling in the nuclei. A classic example relevant to hepatocytes is the ability of hepatocyte growth factor (HGF) to induce nuclear translocation of

β-catenin by phosphorylating it as tyrosine 654 (Tyr654) and Tyr670 (14,15).

β-CATENIN REGULATION BY WNT-INDEPENDENT SIGNALING CASCADES

Activation of β-catenin can occur not only in response to Wnt signaling, but also via additional mechanisms (Fig. 1). As discussed in the preceding section, β-catenin–E-cadherin complex is susceptible to receptor tyrosine kinase (RTK) signaling and several growth factors such as HGF, epidermal growth factor (EGF), Fms-related tyrosine kinase 3 (Flt3), and others can cause tyrosine phosphorylation-dependent activation of β-catenin (14, 16,17). Other growth factors, specifically those relevant in carcinogenesis, such as transforming growth factor-β (TGF-β), have also been shown to induce β-catenin activity, although mechanisms are not fully clear (18). Similarly, protein kinase A (PKA) has been shown to induce β-catenin activation via serine phosphorylation of β-catenin at residues Ser552 and Ser675 (19,20). Thus, multiple non-Wnt-based mechanisms can also activate β-catenin signaling.

WNT SIGNALING IN HEPATIC DEVELOPMENT: TEMPORAL ROLE AND REGULATION

Wnt/β-catenin signaling is a major player in multiple developmental processes. Global deletion of β-catenin in mice leads to embryonic lethality with a notable defect in gastrulation (21). Past the gastrulation stage, β-catenin continues to play notable roles in regulating both patterning and organogenesis (22). In fact, it has now been shown to be key modulator of anteroposterior endodermal patterning. Signaling factors in the form of fibroblast growth factor (FGF), bone morphogenic protein (BMP), and Wnt secreted from the adjacent mesoderm dictate patterning of endoderm into the foregut, midgut, and hindgut along the anterior–posterior axis [reviewed in (23)]. In the posterior endoderm, FGF4 and Wnt proteins emanating from surrounding mesoderm have now been shown to suppress foregut fate and promote hindgut fate; however, in the anterior endoderm, the suppression of these very signaling pathways determines the foregut fate. Suppression of Wnt signaling by secreted Frizzled-related protein 5 (sFRP5) maintains the foregut fate in the anterior endoderm and allows for subsequent liver development (24,25). Liver development proper initiates with hepatic competence of the ventral foregut endoderm, meaning that special domains within this developing tissue gain the ability to respond to inductive signals for formation of a primitive liver bud. This is mostly brought about by pioneer TFs including forkhead family member FOXA1 and GATA4 [reviewed in (23)]. The pioneer TFs have the ability to bind to compacted chromatin to loosen it spatiotemporally for subsequent TF and cofactor recruitment and liver-specific gene expression. Interestingly, these hepatic competence factors are not limited in their expression to ventral foregut endoderm but also in nonhepatic-producing dorsal endoderm. *Wnt8a* overexpression in this region in zebrafish has been shown to induce ectopic hepatoblasts and eventually ectopic liver in the posterior endoderm (26). This suggests that very tight and temporal regulation in the form of initial repression and then activation of Wnt signaling may be of essence in normal initiation of hepatic development. This paradigm is further ascertained by the fact that hepatic induction that follows hepatic competence also requires *wnt2bb*-mediated β-catenin-dependent activation at least in zebrafish (27). *Wnt2bb* mutants have been reported to have very small or no liver buds. Prox1 expression that indicates hepatoblasts is greatly reduced in this mutant, and overexpression of the dominant-negative TCF blocked liver formation. Gain-of-function studies in zebrafish showed that overexpression of *Wnt2bb* or *Wnt8a* (as mentioned above as well) in entire tissues induced ectopic hepatoblast and hepatocyte formation in the posterior endoderm that normally gives rise to the intestine (28). Xenopus studies also showed that activation of β-catenin signaling in the endoderm (at 30-somite stage) resulted in ectopic liver marker expression in the posterior endoderm, suggesting a positive role of Wnt/β-catenin signaling in liver specification (25). A mouse model in which Wnt/β-catenin signaling is activated or inactivated in the foregut endoderm after anteroposterior endoderm patterning but prior to liver specification will be necessary to define the role of Wnt/β-catenin signaling in liver specification. We have performed foxa3-cre-driven β-catenin deletion that was evident at E9.5 in mouse hepatoblasts (29). However, this did not affect the hepatoblast compartment, and HNF4α-positive hepatoblasts were seen unequivocally in these conditional knockout embryos. While this may imply that Wnt/β-catenin signaling is dispensable for hepatic induction in mice, it is a technical challenge to abolish β-catenin expression at the right time and at the right place.

The role of β-catenin in hepatic morphogenesis is indisputable. In fact, highest expression of β-catenin during normal mouse liver development corresponds to embryonic days 10–14 followed by a gradual decline (30,31). β-Catenin at this stage is observed in the cytoplasm and nuclei of a subset of hepatoblasts. The exact upstream effectors of β-catenin during this stage have not been systematically elucidated. One study has shown that hepatic sinusoidal endothelial cells are a source of Wnt9a that may be regulating β-catenin activation in hepatoblasts in a paracrine fashion (32). FGFs (1, 4, 8, and 10) have also been implicated in activation of β-catenin during different stages of hepatic development both in vivo and ex vivo (33,34). HGF/Met signaling, which is indispensable

to liver development, is also known to induce β-catenin activation and may be contributing to its activation in hepatoblasts (35).

The first-ever study that implicated β-catenin's role in hepatic development utilized embryonic liver cultures from day 10 mice embryos, which were cultured for 72 h in the presence of β-catenin antisense phosphomorpholinos (PMO) (36). This led to a notable β-catenin knockdown leading to defect in hepatoblast expansion due to a detriment in proliferation and survival and defect in hepatocyte maturation. Foxa3-cre-mediated β-catenin ablation in vivo resulted in embryonic lethality at mid to late gestation (29). Major subsets of the conditional knockouts die at around the E16–E17 stage and exhibit a notable liver hypoplasia. Hepatic morphogenesis in these embryos is vastly affected with multitude of effects. A significant decrease in hepatoblast proliferation was evident that coincided with a diminished expression of *Ccnd1*. Concomitantly, increased cell death was evident, and β-catenin-deficient livers showed a notable increase in oxidative stress. This was due to decreased expression of multiple genes regulating the redox state of a cell, including several glutathione S-transferases. Simultaneously, hepatoblasts failed to mature, did not develop a cuboidal morphology, and expressed low levels of key liver-enriched transcription factors, including hepatocyte nuclear factor 4-α (HNF4α) and CCAAT enhancer-binding protein-α (C/EBPα).

The role of Wnt/β-catenin signaling in bile duct development has also been reported. In fact, bile duct development was aberrant in foxa3-cre-derived β-catenin knockout (KO) embryos. Inactivation of APC protein in developing livers led to, untimely, premature and excessive β-catenin activation in hepatoblasts, which resulted in excessive bile duct formation at the cost of hepatocyte differentiation (37). β-Catenin antisense PMO treatment of embryonic liver cultures also led to a dramatic decrease in cytokeratin-19-positive cholangiocytes (36). In another study by our lab, we demonstrated that Wnt3a promoted only bile duct differentiation and survival in ex vivo embryonic liver cultures containing predominantly hepatoblasts (38). More recently, the role of Wnt5a in modulating bile duct differentiation of hepatoblasts was also reported. Wnt5a is a unique Wnt, which has been shown to either promote or inhibit β-catenin activity, especially during development based on its spatiotemporal expression and the expression of its disparate receptors in the cells receiving signals (39). Wnt5a was normally expressed in the midgestational liver in mice in predominantly mesenchymal cells. Its genetic loss led to enhanced bile duct differentiation of hepatoblasts. In contrast, Wnt5a treatment of hepatoblast cultures in vitro led to decreased bile duct differentiation by enhancing phosphorylated calcium/calmodulin-dependent protein kinase II (CaMKII) activation (40). While they do not address the impact of Wnt5a on β-catenin signaling, Wnt5a-mediated CaMKII activation has been shown to antagonize β-catenin signaling (41) through mechanisms such as TGF-β activated kinase 1 (TAK1)–Nemo-like kinase (NLK) (42) or Siah2 and APC (43).

More recently, we have identified yet another layer of regulation of β-catenin function during hepatic development. We reported the presence of a truncated form of β-catenin (75 kDa) in addition to the full-length form (97 kDa) at specific hepatic developmental stages (44). The truncated form at low levels is initially evident at around embryonic day 12.5 but becomes the predominant species from E16.5 to the perinatal stage, following which the full length again becomes the dominant species. Using antibodies directed against amino versus carboxy terminal of β-catenin protein and tandem mass spectrometry peptide sequencing of immunoprecipitated 75-kDa β-catenin from E18.5 mouse livers, we identified the truncated β-catenin to be a product of calpain cleavage at amino acid 96. β-Catenin truncation coincided with stages of hepatocyte maturation, and while the upstream regulator of this phenomenon is unknown, the truncated β-catenin is transcriptionally active, and RNA-seq analysis identified several novel targets, many of which are histone H3 and H2 proteins. Since these proteins are involved in nucleosome structure and function, which is pertinent for histone–DNA interactions, and chromatin compaction, truncated β-catenin may eventually influence the epigenetic regulation of gene expression. Another analysis using Genomatix search for common TF-binding sites in the promoters of novel truncated-β-catenin targets identified enrichment for sites for binding Hif1α, Klf4, Egr1, and NF-κB. We speculate that β-catenin truncation may lead to a conformational change that may be permissive to specific TF binding, thus allowing for specific target gene expression compared to the full-length form (Fig. 2). It is also worth mentioning that at later stages during development, while truncated β-catenin localizes to hepatocytes, the full-length form is evident in biliary epithelial cells only. At this time, the significance of this observation is unclear, suffice to say that role and regulation of β-catenin in bile duct development remains to be further elucidated.

Thus, it is likely that tightly regulated β-catenin activity governed by spatiotemporal expression of pro- and anti-β-catenin Wnt proteins during hepatic development, as well as specific posttranslational modifications in β-catenin, whose upstream regulation is not fully clear at this time, may all eventually contribute to its diverse functions in hepatoblast expansion, hepatoblast survival, bile duct specification, and hepatocyte maturation [reviewed in (45)].

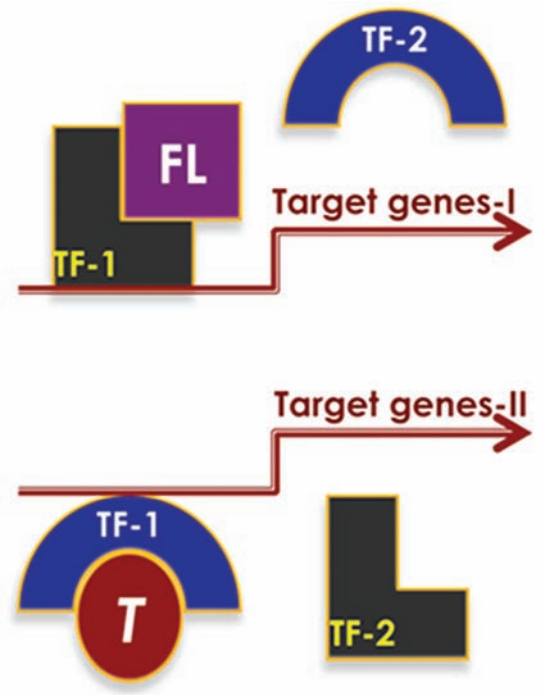

Figure 2. A schematic depicting hypothesis of how truncated β-catenin may have distinct functions than full-length form during liver development. We speculate that conformational change in β-catenin due to calpain-mediated cleavage that yields truncated β-catenin (*T*) may be permissive to its interactions with distinct transcription factor (TF2) compared to full-length β-catenin (*FL*) that interacts normally with TF1. This switch may correspond to disparate functions of the two forms during normal liver development (see text for more details).

β-CATENIN SIGNALING IN POSTNATAL HEPATIC GROWTH SPURT

The growth of liver continues following birth. In fact, several hepatocytes are seen in the S phase of cell cycle at day 5 after birth to around day 20. An increase in total β-catenin protein levels in whole cell lysates was observed between days 5 and 20 after birth followed by a decline at all later stages. Increased levels of active β-catenin (hypophosphorylated β-catenin at Ser37/Thr41), enhanced β-catenin–TCF complex, and increased cyclin-D1 expression were also observed during these times. Increased E-cadherin–β-catenin interactions were evident at days 5–20 followed by a modest decrease that was maintained at all later stages and may represent a mechanism to regulate excessive β-catenin activation. Met–β-catenin complex was not formed in the liver until day 25 after birth and may be due to ongoing HGF/Met signaling, which is known to phosphorylate β-catenin at Tyr654 and Tyr670, leading to its activation. Thus, multiple mechanisms lead to β-catenin stabilization and activation in the liver at days 5–20 after birth, which in turn leads to increased cyclin-D1 levels and eventually contributes to cell proliferation and postnatal liver growth spurt. When β-catenin was conditionally deleted using α-*Fetoprotein-Albumin-Cre*, a 28% or 17.5% decrease in liver weight to body weight ratio was observed in knockouts in males and females, respectively, and coincided with a notable deficit in hepatocyte proliferation. Hence, β-catenin is a key contributor to postnatal hepatic growth.

β-CATENIN SIGNALING IN ADULT LIVER: ROLE IN PERICENTRAL GENE EXPRESSION

Hepatocytes show remarkable heterogeneity based on their location within the hepatic lobule. This is due to a diverse set of functions ascribed to hepatocytes based on their proximity to portal blood. This phenomena of metabolic zonation accounts for efficient hepatocyte functioning within the lobule, especially in performing functions such as protein, glucose, and xenobiotic metabolism (46). When an adult liver is stained for β-catenin, two types of localization patterns are evident (Fig. 3). Most hepatocytes across the entire hepatic lobule exhibit membranous β-catenin, which reflects its association to E-cadherin at the AJ. However, in the vicinity of central veins, the pericentral hepatocytes display cytoplasmic and even nuclear localization of β-catenin, although nuclear localization is harder to appreciate. It should be emphasized that nuclear localization of β-catenin in pericentral hepatocytes is not anywhere close in quality and quantity to the staining evident in hepatic tumors with *CTNNB1* mutations (47). Nonetheless, cytoplasmic and nuclear β-catenin indicates basal activity in a normal adult liver in the centrizonal area of the hepatic lobule as also reported in β-catenin–TCF reporter mice or TOPGAL mice (48,49). Several pericentral target genes of β-catenin have been now identified that play key roles in ammonia metabolism and xenobiotic metabolism. The studies in transgenic mice overexpressing active β-catenin were the first to demonstrate increased expression of glutamine synthetase (GS), ornithine aminotransferase (OAT), and glutamate transporter GLT-1 (6). Another study showed that β-catenin-mutated, GS-positive tumors in mice showed a noteworthy increase in the expression of several cytochrome P450s (CYPs), especially Cyp2e1 and Cyp1a2. Eventually, this was all verified in hepatocyte-specific β-catenin conditional KO mice, which showed loss of GS and a notable decrease in the expression of Cyp2e1 and Cyp1a2 (9,50). In fact, when challenged with high doses of acetaminophen, an over-the-counter antipyretic that requires Cyp2e1 and Cyp1a2 for its bioactivation to a toxic intermediate, β-catenin KO mice are protected (50,51).

How is β-catenin being directed to regulate pericentral gene expression? In an important study, Benhamouche and colleagues showed active β-catenin to be expressed in the pericentral hepatocytes, while its major negative

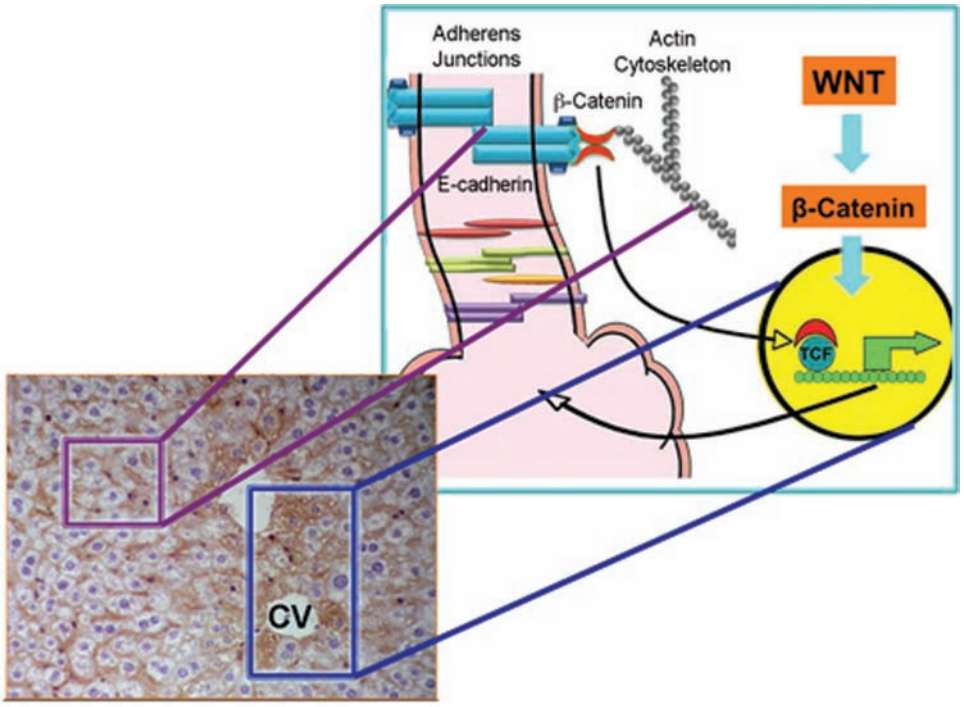

Figure 3. β-Catenin immunohistochemistry in normal adult mouse liver. Predominantly, β-catenin is observed staining hepatocyte membrane only in both the midzonal (purple box) and periportal regions of a hepatic lobule, where it interacts with E-cadherin and constitutes the adherens junctions. Around the central vein (CV), β-catenin, in addition to being membranous, is also cytoplasmic and nuclear (blue box) where it acts as a downstream effector of the Wnt signaling pathway and regulates pericentral target gene expression.

regulator APC was expressed in the periportal zone (52). Through modulation of APC expression using hepatocyte-specific APC-null mice, the authors were able to transform the entire livers to perivenous phenotype, which resulted in perturbations of periportal ammonia metabolism and associated mortality. Conversely, overexpression of Wnt inhibitor dickkopf 1 (Dkk1) in hepatocytes led to conversion of the entire liver to periportal phenotype. What are the upstream effectors of β-catenin that dictate its activation in pericentral hepatocytes? The studies with Dkk1 overexpression and abrogation of pericentral gene expression of GS supports the role of Wnt signaling in regulating β-catenin activity in these hepatocytes. However, whether hepatocytes are the source of Wnts to act in an autocrine manner or whether these arise from nonparenchymal cells to act in a paracrine fashion remains undetermined at this time. Use of cell-specific cre-recombinase mice and *wntless*- or *porcupine*-floxed mice may be highly relevant to determine the identity of cell types that are the source of Wnt proteins and eventually to establish the identity of specific Wnts that may be regulating centrizonal β-catenin activation under physiological conditions. These findings are of key relevance, as modulation of Wnt signaling may be key to obtaining functional perivenous hepatocytes after stem cell differentiation. Indeed, Colletti and colleagues showed that spontaneous differentiation of liver stem cells led to their differentiation to periportal hepatocytes, and only after β-catenin activation using a small molecule GSK3β inhibitor 6-bromoindirubin-3′-oxime (BIO) led to their conversion to pericentral hepatocytes (53).

How does nuclear β-catenin transactivate pericentral gene expression? As mentioned earlier, β-catenin does not bind DNA but has the capability to bind the TCF family of TF to induce their activation and target gene expression. An elegant study published recently by Dr. Colnot's group has performed a very careful and comprehensive CHIP-seq analysis using hepatocyte-specific APC KO livers and hepatocyte-specific β-catenin KO livers (54). For *Glul*, gene encoding GS, they show TCF4 and β-catenin occupying at least six chromatin regions within the 44-kb promoter region. In fact, four of these regions were enriched for Wnt-responsive elements. Similar occupation was also identified for pericentral genes such as Cyp1a2 and constitutive androstane receptor (CAR). Simultaneously, in silico analysis revealed DNAse1-hypersensitive sites and histone 3 lysine 27 acetylation-marked sites in regions occupied by TCF/β-catenin in the upstream sequences of the target genes. Further analysis by the authors actually identified 308 genes that appear to be normally regulated by β-catenin in an adult liver and appear to be mostly related to metabolic functions

including those involved in bile acid, cholesterol, and drug metabolism functions.

How β-catenin regulates pericentral versus periportal gene expression is an intriguing mechanism as well. Colletti et al. made an exciting observation that HNF4α binds to the promoters of genes expressed in the periportal area. They showed that TCF/LEF could bind to not only their own Wnt responsive elements in target gene promoters but also to HNF4α-response elements. They also showed that LEF could interact with HNF4α. More recently, Gougelet and colleagues showed that there was no zonal preference of HNF4α localization in the liver (54). However, they showed that β-catenin could also bind to HNF4α. Furthermore, expression of HNFα could inhibit β-catenin-dependent TOPflash reporter activity, whereas active β-catenin expression could inhibit HNF4α reporter activity. This shows that HNF4α- and β-catenin-dependent transcriptional regulation is antagonistic, and this balance may be of essence in establishing zonation that demarcates periportal from perivenous hepatocytes. The upstream effectors dictating such equilibrium remain elusive at this time.

β-CATENIN SIGNALING IN LIVER REGENERATION

The liver is an organ with a variety of disparate functions that are indispensable to survival. On top of it, it is the gateway to portal circulation, which is notorious for carrying an array of toxins, bacterial products, and nutrients that need to be "calibrated" to physiological levels for maintaining homeostasis. That may be the reason why liver is uniquely endowed with an ability to regenerate. The cellular and molecular basis of liver regeneration (LR) is much better understood now, and the model of surgical resection in rodents called the two-thirds or partial hepatectomy (PH) has lent itself well to elucidate such mechanisms. All cell types in the liver including hepatocytes, cholangiocytes, Kupffer cells, stellate cells, and sinusoidal endothelial cells undergo proliferation to restore lost liver mass as liver regenerates. More importantly, these cells are the source of various growth factors and cytokines that act in concert to orchestrate this precisely regulated model of hepatic growth. While several molecules have been implicated in normal LR in the PH model, a currently accepted dogma is the extent of molecular redundancy that enables LR to go through despite knockdown or ablation of a single signaling cascade. Such redundancy may be paramount to daily hepatic health owing to its strategic location and critical functions. That is one reason why a notable delay in LR by 24 h in the hepatocyte-specific β-catenin KO mice underscores its unprecedented role in hepatic growth after PH.

In 2001, β-catenin was discovered for the first time to be playing a role in rat LR after PH (55). In this model, β-catenin was shown to translocate to the nucleus within 1–5 min after surgery and remained in hepatocyte nuclei for at least 24 h. β-Catenin stabilization occurred secondary to its stabilization, as its degradation complex, comprised of APC and GSK3β, was inhibited minutes after PH. β-Catenin gene expression was induced only after 6 h in this model. A follow-up study showed that inhibition of β-*catenin* gene expression using PMO antisense oligonucleotides at the time of PH in rats led to decreased recovery of liver weight to body weight ratio (LW/BW) as a function of reduced hepatocyte proliferation (56). This observation was further validated in mice when hepatocyte-specific β-catenin conditional KO were subjected to PH studies (9,57). There was a clear and demonstrable decrease in hepatocyte proliferation at 40 h after PH in these animals. Similar deficits in LR have also been reported in zebrafish when dominant-negative TCF was expressed in liver (58). To test if the converse was true and β-catenin activation could impart a regenerative advantage to the liver, we investigated LR in transgenic mice expressing ser45-mutant β-catenin in hepatocytes. When subjected to PH, these mice showed accelerated hepatocyte proliferation such that most hepatocytes were PCNA positive before their wild-type counterparts (59). Similarly, another study in APC (Min/+) mice that displayed β-catenin activation also showed a regeneration advantage, as did zebrafish overexpressing Wnt8a or APC (–/–) zebrafish when subjected to hepatectomy (58).

Additionally, β-catenin signaling was identified to play a role in LR induced after toxicant-induced hepatic injury (51). Acetaminophen (APAP) at a sublethal dose results in centrizonal necrosis that is followed by enhanced proliferation of the surviving hepatocytes (60,61). β-Catenin activation was evident almost at the time of APAP injury, but lethal doses failed to induce β-catenin activation, suggesting that β-catenin increase is a reparative and not injury-inducing response. Moreover, β-catenin activation coincided with ensuing hepatocyte proliferation. However, the role of β-catenin in APAP-induced LR could not be directly tested using the β-catenin KO mice since these animals lack cyp2e1 and cyp1a2, the enzymes responsible for APAP bioactivation (50,61). Following chemical induction of these P450s in β-catenin KO, injury could be induced by APAP. At equitoxic doses of APAP in β-catenin KO and controls, the β-catenin KO mice showed a clear deficit in hepatocyte proliferation demonstrating a role of β-catenin signaling in LR prompted by APAP-induced liver injury (51). Is β-catenin signaling relevant in LR in patients? Based on our observation of β-catenin activation after APAP injury in mice, we examined β-catenin status in APAP overdose patients. APAP overdose patients can either continue to deteriorate, requiring liver transplantation, or undergo spontaneous LR, eliminating the need for transplantation (62). Interestingly, β-catenin activation

identified by its nuclear translocation in hepatocytes, correlated well with proliferation index in APAP overdose patients, which showed spontaneous regeneration and thus did not require liver transplantation (51). On the other hand, patients lacking nuclear β-catenin showed decreased numbers of hepatocytes in S phase, failure to regenerate, and required liver transplantation.

How does β-catenin regulate cell proliferation after PH? As mentioned previously, β-catenin can bind to TCF4 to then induce target genes, and there are several targets of the Wnt signaling pathway that regulate cell proliferation. However, many of the targets are tissue and context dependent and might not be specific to hepatocyte proliferation. Based on many observations over the last decade, one common molecule that coincides with β-catenin activation during hepatocyte proliferation is cyclin-D1. Cyclin-D1 is indeed a known target of Wnt signaling and identified initially in colorectal cancer (63). However, in liver, while initially β-catenin-mutant HCC was shown to have increased expression of cyclin-D1 (64), the transgenic mice overexpressing mutant β-catenin in hepatocytes did not show such increase (6). Intriguingly though, activation of β-catenin after PH always precedes cyclin-D1 expression and appearance of cells in the S phase of cell cycle. A significant reduction in cyclin-D1 expression was also observed in hepatocyte-specific β-catenin KO mice where hepatocytes displayed around a 24-h delay in S-phase transition. Conversely, β-catenin-active transgenic mice or zebrafish show higher cyclin-D1 expression leading to accelerated hepatocyte proliferation after hepatectomy in these models (58,59). Further studies have directly validated the regulation of cyclin-D1 by the β-catenin–TCF complex during LR process (65). Our unpublished studies have shown nuclear translocation of β-catenin as early as 1 h after PH in mice and formation of β-catenin–TCF4 complex at 3 h onward. It was intriguing to note that while β-catenin–TCF4 complex is formed and transcriptionally active as shown by occupancy of this complex on cyclin-D1 promoter (65), TOPGAL mice, when subjected to PH, did not show reporter activity (57). This demonstrates the limitation of using models like TOPGAL transgenic mice as faithful indicators of Wnt signaling in an adult liver. At the same time, one must entertain the complexity of transcriptional regulation of cyclin-D1. While several TF binding sites, including those of NF-κB, AP-1, HNF4α, CREB, TCF/LEF, and others, have been identified, the exact composition of the complex driving *Ccnd1* expression, the key G_1- to S-phase transition protein in LR (66,67), remains an enigma. A recent study also showed Wnt activation after PH in a sulfatase 2-dependent manner to activate β-catenin–TCF complex that led to GLI1 TF expression, which in turn induced *Ccnd1*. Thus, β-catenin-dependent cyclin-D1 expression may be direct and indirect; however, it is indeed a key component required for its expression in hepatocytes during LR.

Since β-catenin is unequivocally important in the normal regenerative response of the liver to either surgical or toxicant insult, it is of essence to determine the upstream effectors of β-catenin that regulate its temporal activation during the LR process. The Wnt signaling pathway as well as Wnt-independent mechanisms can activate β-catenin. What regulates β-catenin during LR is not completely understood. An important study by Dr. Rafii's group showed the importance of sinusoidal endothelial cells that released HGF and Wnt2 after PH in a vascular endothelial growth factor receptor-2 (VEGFR2) and an inhibitor of differentiation/DNA binding (Id1)-dependent manner (68). Wnt2 is highly relevant in activating β-catenin signaling, and HGF can also activate β-catenin in addition to stimulating other mitogenic cascades relevant in LR. Additionally, as previously mentioned, sulfatase 2, a heparan sulfate 6-O-endosulfatase that releases growth factors from extracellular storage sites, was shown to be essential for Wnt3a release after PH that in turn led to activation of β-catenin, GLI1 expression, and upregulation of cyclin-D1 (69). Yet another recent study showed an important role of long noncoding RNA (lncRNA), which was termed as an lncRNA associated with LR or lnc-RNA-LALR1 (70). The authors showed lncRNA-LALR1 to induce Wnt/β-catenin/cyclin-D1 axis after PH by downregulating Axin1 expression. Last, hepatic stellate cells were reported to secrete delta-like homolog-1 (Dlk1) that caused stellate cell activation by PPARγrepression (11). When Dlk-1-neutralizing antibody was injected 6 hr after PH in mice via tail vein, it led to a significant decrease in hepatocyte proliferation especially at days 1 and 2. This coincided with a decrease in *Wnt10b* expression in hepatocytes, a modest decrease in total and Akt-dependent Ser552–β-catenin protein, and decreased cyclin-D1. The authors of the study conclude that stellate cell-derived DLK1 actually induced the cascade by activating DLK1–Wnt10b–β-catenin axis in hepatocytes to contribute to LR. Thus, several mechanisms have already been suggested, and eventually it may be multiple mechanisms in tandem that may be upstream of β-catenin/cyclin-D1 during the LR process. It is further apparent that there is a notable participation of nonparenchymal cells, which may be the chief sources of various Wnt proteins or other factors that may influence β-catenin activation in hepatocytes (Fig. 4). However, a systematic study that addresses the role of Wnt factors versus other mechanisms during LR and also the most relevant cell source of such factors that converge on to β-catenin is still missing. Recently, LRP5/6-double floxed mice have been reported (71). These animals can be used to generate hepatocyte-specific LRP5/6 conditional KO mice and thus will be invaluable in investigating if β-catenin could be regulated by Wnt signaling or Wnt-independent mechanisms

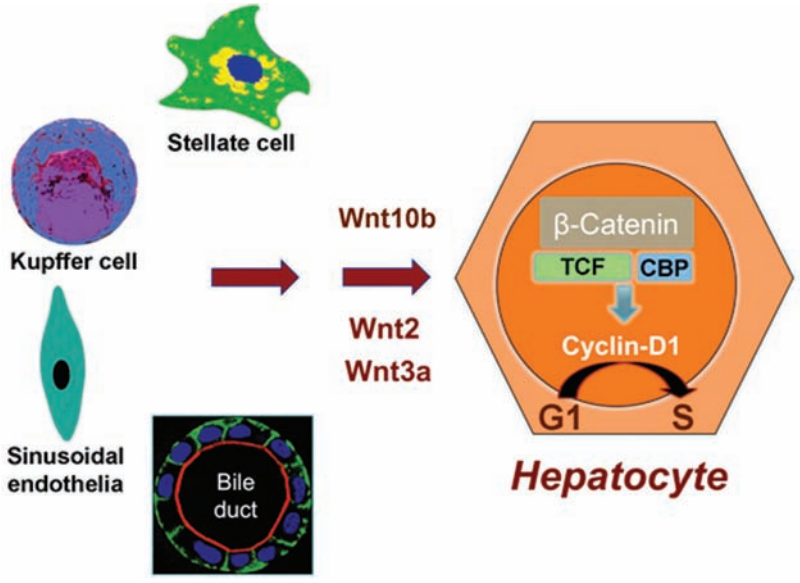

Figure 4. A schematic representing cellular basis of Wnt/β-catenin activation during liver regeneration process. Nonparenchymal cells are the source of several key Wnt proteins that, in a paracrine manner, stimulate β-catenin activity in neighboring hepatocytes, which in turn induces cyclin-D1 expression to facilitate G_1- to S-phase cell cycle transition that is essential for hepatocyte proliferation during liver regeneration process (TCF, T-cell factor; CBP, CREB-binding protein).

during LR since LRP5/6 are only necessary for Wnt signal transduction to β-catenin. If β-catenin is solely under the control of Wnt signaling, these mice should phenocopy hepatocyte-specific β-catenin KO after PH in their LR kinetics. To address the cellular participation as source of Wnt ligands during LR will be more complex since multiple Wnt genes are expressed in several cell types in an adult mouse liver basally (72). Recently, models such as *Wntless*-floxed mice (71,73) or *porcupine*-floxed mice (74), which when bred to cell-specific *cre-recombinase* transgenic mice will eliminate Wnt secretion or activation, respectively, from specific cells will be of relevance to identify the cell source and eventually the identity of specific Wnt proteins that may play a role in LR after PH.

Could β-catenin activation be modulated exogenously to enhance LR and possibly utilized as a therapeutic modality for select patients of liver insufficiency? Liver insufficiency due to acute or chronic liver disease may eventually progress to end-stage liver disease (ESLD) for which no satisfactory treatment other than liver transplantation is available. However, liver transplantation is limited by a dearth of organs available for transplantation, recurrence of the disease, and associated morbidity. Regenerative therapies may be a plausible alternative in the setting of liver insufficiency or ESLD if successful activation of pertinent signaling pathways can be achieved by meaningful modalities (75). As proof of concept, we performed Wnt1 gene therapy by delivering naked DNA through hydrodynamic tail vein injection in mice prior to PH (59). This technique led to transient overexpression of Wnt1 in hepatocytes. When these mice were subjected to PH, there were greater numbers of cyclin-D1 and PCNA-positive hepatocytes that also appeared notably earlier than control plasmid-injected mice. This study, along with regenerative advantages in β-catenin-overexpressing mice and zebrafish, provide proof of concept that β-catenin activation may be a therapeutic target in the setting of ESLD.

We have recently identified a novel means to activate β-catenin signaling that may have therapeutic relevance. Triiodothyronine or T3 has been shown to induce hepatocyte proliferation in rats and mice, although the mechanism remains unclear (76). Since T3 induces cyclin-D1 expression, we investigated if β-catenin may be involved in the process (77). We have now identified T3 to induce β-catenin activation (48). Since T3 induces PKA activation (78), we now showed that T3-induced PKA activation led to Ser675 phoshorylation of β-catenin to then induce cyclin-D1 expression (48).Thus, T3 administration may be a clinically relevant means to transiently induce β-catenin activation for regenerative therapies in the liver. There are other Wnt agonists that may activate β-catenin for therapeutic purposes. BIO inhibits GSK3β to induce β-catenin activation and has been used to maintain pluripotency of stem cells (79). It may be worthwhile to directly address its role in stimulating LR. Other Wnt agonists like 2-amino-4-[3,4-(methylenedioxy) benzylamino]-6-(3-methoxyphenyl) pyrimidine act in a GSK3β-independent mechanism. This small molecule has been used recently to activate β-catenin in the hepatic

ischemia/reperfusion (I/R) model of hepatic injury. When injected right before or after I/R, this agonist increased β-catenin activity as observed by increased Axin2 levels, which in turn decreased I/R-induced hepatocyte injury and significantly improved liver histology. Additionally, increased cell proliferation, decreased inflammatory cytokines, and decreased apoptosis was observed, which eventually led to improved survival in animals.

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