Research Article

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Hepatocyte γ-catenin compensates for conditionally deleted β-catenin at adherens junctions

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Background & Aims: Wnt/β-catenin signaling is important in liver physiology. Moreover, β-catenin is also pivotal in adherens junctions (AJ). Here, we investigate hepatocyte-specific β-catenin conditional null mice (KO) for any alterations in AJ and related tight junctions (TJ).

Methods: Using gene array, PCR, Western blot, immunohistochemistry, immunofluorescence, and co-immunoprecipitation, we compare and contrast the composition of AJ and TJ in KO and littermate wild-type (WT) control livers.

Results: We show association of E-cadherin with β-catenin in epithelial cells of WT livers, which is lost in the KOs. While total levels of α-catenin, E-cadherin, and F-actin were modestly decreased, KO livers show increased γ-catenin/plakoglobin. By co-immunoprecipitation, E-cadherin/β-catenin/F-actin association was observed in WT livers, while the association of E-cadherin/γ-catenin/F-actin was evident in KO livers. γ-Catenin was localized at the hepatocyte membrane at baseline in the KO liver. While *γ-catenin* gene expression remained unaltered, an increase in serine- and threonine-phosphorylated, but not tyrosine-phosphorylated γ-catenin was observed in KO livers. A continued presence of γ-catenin at the hepatocyte membrane, without any nuclear localization, was observed in liver regeneration after partial hepatectomy at 40 and 72 h, in both KO and WT. Analysis of TJ revealed lack of claudin-2 and increased levels of JAM-A and claudin-1 in KO livers.

Conclusions: β-Catenin adequately maintains AJ in the absence of β-catenin in hepatocytes; however, it lacks nuclear localization. Moreover, β-catenin/claudin-2 may be an important mechanism of crosstalk between the AJ and TJ.

Introduction

The Wnt/β-catenin signaling pathway plays an important role in liver pathobiology, regulating zonation, metabolism, development, and regeneration [1]. Its aberrant activation, due to mutations in the *β-catenin* gene (*CTNNB1*) or components of its degradation complex such as axin results in stabilization and nuclear translocation of β-catenin that induces target genes, critical to tumor growth and survival. β-Catenin is also important in cell–cell adhesion [2,3], and is a constituent of adherens junctions (AJ) where it bridges E-cadherin to the actin cytoskeleton [4]. Loss of E-cadherin/β-catenin association due to tyrosine-phosphorylation of E-cadherin and β-catenin by receptor-tyrosine kinases, such as epidermal growth factor receptor, hepatocyte growth factor receptor, and others results in diminished intercellular adhesion, commonly seen in tumors [5–8]. Tight junctions (TJ) between the basolateral and apical domains of hepatocytes contribute to paracellular adhesion, cell polarity, and serve as occluding barriers around the biliary canaliculi [9,10]. TJ are multiprotein complexes composed chiefly of claudins, junctional adhesion molecules (JAM), and occludin [11]. Anomalies in the TJ have been associated with an imperfect blood-bile barrier and are implicated in various hepatic pathologies [12,13]. A cross-talk between AJ and TJ, and other plasma domains is reported [14].

The major goal of the current study was to understand the junctional organization in recently reported β-catenin conditional knockout mice (KO) generated by interbreeding transgenic mice homozygous for floxed *β-catenin* gene (Ex1-6) and transgenic mice expressing Cre-recombinase under the *Albumin* promoter [15]. The KO mice exhibit lower liver weight to body weight ratio, a modest intrahepatic cholestasis, and anomalous biliary canalicular architecture [16]. No gross defects in cell polarity or adhesion are evident in KO livers. We wanted to explore the KO hepatic phenotype or lack thereof, through identification of compensatory mechanisms in AJ and TJ, which enable the

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Abbreviations: KO, liver-specific-β-catenin conditional knockout; WT, wild-type or controls; WB, Western blot; IF, immunofluorescence; AJ, adherens junctions; TJ, tight junctions; *JUP*, *γ-catenin* gene; JAM, junctional adhesion molecules; WCL, whole cell lysates; CAL, cytoskeleton associated lysates; RIPA, radioimmunoprecipitation assay; PVDF, polyvinylidene difluoride; IHC, immunohistochemistry; IP, immunoprecipitation; PCR, polymerase chain reaction; ZO, zona occludens.

maintenance of a near normal liver histology in the absence of β-catenin. Here, we identify an important compensatory role of γ-catenin (plakoglobin) at AJ along with altered organization of TJ in the KO livers.

Materials and methods

Animals and surgery

All animal studies were approved by the University of Pittsburgh IACUC office. Homozygous floxed β-catenin mice (C57BL/6 strain), and albumin-cre transgenic mice were bred as previously described [15]. Mice with genotype *Ctnnb1*$^{lox/lox}$; *Alb-Cre*$^{+/-}$ are referred to as knockouts (KO). Littermates with floxed β-catenin genotypes were used as wild-type controls (WT). Livers from age- and sex-matched 90–120 days-old KO and WT ($n \geqslant 3$) were paraffin embedded, cryo-fixed, and frozen at -80 °C until use. Partial hepatectomy on KO and WT is described elsewhere [15].

Protein extraction

Whole-cell lysates (WCL), were obtained from the WT and KO livers using radio-immunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology) containing fresh protease and phosphatase inhibitor cocktails (Sigma) as described previously [15]. Cytoskeleton-associated proteins were also extracted as described by Hugh *et al.* [17] to obtain Triton-insoluble or cytoskeletal-associated lysates (CAL). Protein assays were performed using BCA (Pierce).

Western blot analysis

At least 3–4 independent livers from KO or WT group were used and representative Western blots (WBs) are shown. 18–50 μg of protein were resolved on 5%, 7.5%, or 12% Tris–HCl precast gels (Bio-Rad Laboratories) using SDS–PAGE. The resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes and signal detected by Super-Signal West Pico or Femto Chemiluminescent Substrate (Pierce). Primary antibodies used were against β-catenin (BD Transduction Laboratories); γ-catenin, E-cadherin (Cell Signaling); α-catenin (Abcam); E-cadherin (BD Transduction Laboratories); claudin-1, claudin-2 (Invitrogen); γ-catenin, JAM-A, occludin, GAPDH, p-Tyr, p-Ser, p-Thr (Santa Cruz); and actin (Chemicon). Horseradish-peroxidase-conjugated secondary antibodies were purchased from Chemicon. Densitometry was performed on scanned autoradiographs using Image J software (NIH). For statistical analysis of differences in protein expression between KO and WT livers, signal from each lane for a protein was normalized to GAPDH expression in that lane. The mean (±standard deviation or SD) normalized expression values of any protein in WT and KO livers were compared for statistical significance by the two-tailed student *t* test and $p < 0.05$ was considered significant.

Immunoprecipitation (IP)

IP studies were performed with 500 μg of WCL or CAL as described elsewhere [8]. Antibodies used for IP were rabbit anti-γ-catenin (Cell Signaling), mouse anti-E-cadherin (BD Transduction Labs) or goat β-catenin conjugated A/G agarose beads (Santa Cruz).

Immunohistochemistry (IHC) and immunofluorescence (IF)

IHC was performed for β-catenin (Santa Cruz) or Ki-67 (Dako) as detailed elsewhere [15]. For IF, staining was performed using rabbit (cell signaling) or goat (Santa Cruz) anti-γ-catenin or E-cadherin (mouse monoclonal, BD biosciences) and Alexa 488-conjugated donkey anti-rabbit (molecular probes) and Cy3-conjugated donkey anti-mouse (Jackson immunoresearch) secondary antibodies, as described elsewhere [8]. Sections were counterstained with DAPI and imaged with Olympus FV1000 Confocal Microscope with Fluoview 1. 7A software.

Microarray analysis

Livers from three KO and WT were used for Affymetrix gene array analysis as previously published [15]. The signals from KO and WT livers were compared and presented as fold-change.

Table 1. Primer sequences.

Primer Name	Sequence
JUP Fwd	5'-ACGCCATTGATGCGGAGGGC-3'
JUP Rev	5'-CCCAGGCAGCTGGGTCATGC-3'
GAPDH Fwd	5'-ACCCAGAAGACTGTGGATGG-3'
GAPDH Rev	5'-CACATTGGGGGTAGGAACAC-3'
PBGN Fwd	5'-ATGTCCGGTAACGGCGGC-3'
PBGN Rev	5'-CAAGGCTTTCAGCATCGCCACCA-3'
Cyclophilin-A Fwd	5'-CCCCACCGTGTTCTTCGACA-3'
Cyclophilin-A Rev	5'-TCCAGTGCTCAGAGCTCGAAA-3'

Real-time PCR was also performed with another *JUP* primer (SA Biosciences); two liver-specific reference genes (porphobilinogen synthase or *PBGN* and *cyclophilin-A*), and two reference genes-glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*); and phosphoglycerate kinase 1 (*PGK*) from www.realtimeprimers.com.

Real-time PCR

RNA was extracted from KO and WT mice using Trizol (Invitrogen) subjected to real-time PCR analysis as described elsewhere [18]. Comparative ΔΔCT was used for analysis of the data, and calculations were made without the StepOne software. The real-time PCR primer sequences are available in Table 1.

Statistics

The statistical analysis was performed using the Microsoft Office Excel 2007 software.

Results

β-Catenin is conditionally deleted in the parenchymal cells from KO mice livers

To confirm β-catenin loss in KO livers, β-catenin expression levels were assessed. β-Catenin protein levels were significantly decreased in the WCL and CAL in KO livers as compared to WT ($p < 0.0005$) (Fig. 1A and B). Some remnant expression evident in KO livers was due to the presence of β-catenin in non-parenchymal cells, unaffected by albumin-cre expression, and to a very small number of hepatocytes that spontaneously escape Cre-mediated deletion [19]. Indeed, IHC in KO tissues shows loss of membranous β-catenin in hepatocytes and cytoplasmic β-catenin in cholangiocytes, whereas endothelial cells remained β-catenin positive (Fig. 1C). IP studies for β-catenin were performed next. When blotted for E-cadherin, the association between β-catenin and E-cadherin was detected only in WT livers (Fig. 1D). In the KO livers, E-cadherin was not pulled down by β-catenin, confirming loss of β-catenin in the epithelial compartment.

Assessment of adherens junction components in β-catenin KO livers

To investigate gene expression changes in the components of AJ, we analyzed microarray data from KO and WT livers [15]. KO livers showed altered expression of *α-catenin* (+2.4), *E-cadherin* (+2.6), and *γ-catenin* (+1.5) relative to WT (Table 2).

WB was performed to substantiate the array analysis results. No significant differences in E-cadherin, α-catenin, and F-actin protein levels were observed in CAL and WCL from KO and WT livers (Fig. 2A and B). Interestingly, a significant increase in γ-catenin protein expression was observed in KO extracts (Fig. 2A and B).