Published in final edited form as:
J Surg Res. 2010 December ; 164(2): 276–285. doi:10.1016/j.jss.2009.10.033.

β-Catenin regulates mesenchymal progenitor cell differentiation during hepatogenesis

Tove Berg, **Stijn De Langhe**, **Denise Al Alam**, **Sarah Utley**, **Joaquin Estrada**, and **Kasper Wang**
Saban Research Institute, Childrens Hospital Los Angeles, Keck School of Medicine, University of Southern California

Abstract

Background—Understanding the pathways regulating mesenchymal progenitor cell fate during hepatogenesis may provide insight into postnatal liver injury or liver bioengineering. While β-Catenin has been implicated in the proliferation of fetal hepatic epithelial progenitor cells, its role in mesenchymal precursors during hepatogenesis has not been established.

Materials and Methods—We used a murine model of conditional deletion of *β-Catenin* in mesenchyme using the *Dermo1* locus (*β-Catenin*^{Dermo1}) to characterize the role of *β-Catenin* in liver mesenchyme during hepatogenesis.

Results—Lineage tracing using a *LacZ* reporter indicates that both hepatic stellate cells and pericytes derive from mesenchymal *Dermo1* expressing precursor cells. Compared to control littermate livers, *β-Catenin*^{Dermo1} embryonic livers are smaller and filled with dilated sinusoids. While the fraction of mesenchymally-derived cells in *β-Catenin*^{Dermo1} embryos is unchanged compared to littermate controls, there is an increase in the expression of the mesenchymal markers, DESMIN, α-SMA, and extracellular deposition of COLLAGEN type I, particularly concentrated around dilated sinusoids. Analysis of the endothelial cell compartment in *β-Catenin*^{Dermo1}/*Flk1*^{lacZ} embryos revealed a marked reorganization of the intrahepatic vasculature. Analysis of various markers for the endodermally-derived hepatoblast population revealed marked alterations in the spatial expression pattern of pan-CYTOKERATIN but not E-CADHERIN, or ALBUMIN. *β-Catenin*^{Dermo1} phenocopies mesenchymal deletion of *Pitx2*, a known regulator of hepatic mesenchymal differentiation both during both organogenesis and postnatal injury.

Conclusions—Our data implicate Mesenchymal β-Catenin signaling pathway in the differentiation of liver mesenchymal progenitor cells during organogenesis, possibly via Pitx2. Hepatic Mesenchymal β-Catenin signaling, in turn, modulates the development of both endothelium and endodermally-derived hepatoblasts, presumably via other downstream paracrine pathways.

Corresponding Author: Kasper S. Wang, M.D. Developmental Biology Research Program The Saban Research Institute Childrens Hospital Los Angeles Keck School of Medicine University of Southern California office: (323) 669-2338 fax: (323) 666-3466 kwang@chla.usc.edu.
Other authors' email addresses: tberg@chla.usc.edu, dalalam@chla.usc.edu, sutley@chla.usc.edu, joestrada@chla.usc.edu

Keywords

hepatogenesis; Wnt; β-Catenin; stellate cell; Pitx2; Dermo1

INTRODUCTION

In the adult liver, mesenchymally-derived Hepatic Stellate Cells (HSC) are located in the space of Disse, just beneath the fenestrated endothelium that lines and separates the vascular sinusoids from hepatocytes. Under normal conditions, HSC serve as a reservoir for vitamin A and lipid storage and can be identified by their expression of DESMIN. During liver injury, HSCs transdifferentiate toward a myofibroblastic phenotype producing extra-cellular matrix (ECM) which contribute to the fibrogenic response to injury, which is also referred to as an activated state (1). These cells can be identified with a loss of vitamin A storage and DESMIN expression, as well as induced expression of α-SMOOTH MUSCLE ACTIN (αSMA) and ECM proteins such as COLLAGEN Type I. Understanding the underlying molecular mechanisms for how differentiation of HSCs occur is, therefore, of great importance in the treatment and prevention of hepatic fibrosis after liver injury. The specific role for Wnt/β-Catenin signaling in transdifferentiation of HSC is unclear. Kordes *et al* showed that Wnt signaling maintains HSC in a quiescent state, whereas Cheng *et al* showed that antagonism of Wnt signaling inhibits HSC activation in culture (2,3). Since tissue repair and regeneration commonly recapitulate ontogeny, studying the role of β-Catenin signaling in mesenchymal cells during hepatogenesis may provide insight into how HSC are regulated postnatally during injury.

Mesenchymal-to-epithelial instructions are a critical component of hepatogenesis. A close interaction between endodermal progenitor cells from the foregut endoderm, as well as instructive signals from surrounding mesenchymal septum transversum, are all essential components of proper liver induction and formation. Lineage tracing experiments using the *MesP1-Cre*$^{+/-}$*/Rosa26*$^{+/-}$ (herein called *MesP1*^{LacZ}) mice to specifically drive *LacZ* expression in the mesenchyme surrounding the foregut endoderm, indicate that the mesoderm surrounding the liver bud give rise to liver mesenchymal cells, such as submesothelial cells and their derivates, hepatic stellate cells (HSC) and perivascular smooth muscle cells (pericytes). These mesenchymal cells all co-express DESMIN and αSMA so that the only histologic distinction between HSC and pericytes during hepatogenesis is the proximity of perivascular mesenchymal cells to the sinusoids (4). However, relatively little is known about the molecular mechanisms controlling mesenchymal progenitor cell regulation and differentiation during liver organogenesis (5).

The Wnt family of ligands regulate stem cell renewal and differentiation through the stabilization and nuclear translocation of the transcription factor β-Catenin. Delanghe *et al* showed that knocking out *β-Catenin* specifically in the mesenchyme leads to defects in multiple organ systems with severe cardiac and vasculogenesis-related defect (6). These conditional knockouts also largely phenocopy null mutation of the homeobox gene *Pitx2* in terms of arrest in turning of the body axis and defective body wall closure, partial right pulmonary isomerism, cardiac tract abnormalities, and facial abnormalities such as defective development of the mandibular and maxillary facial prominences and regression of the stomodeum (7). Furthermore, loss of *β-Catenin* in the lung leads to reduced number of mesenchymal parabronchial smooth muscle progenitor cells and impaired differentiation of endothelial cells. Together these observations indicate a role for β-Catenin in the amplification and differentiation of mesenchymal progenitor cells during organogenesis, possibly through the Pitx family of transcription factors.

Wnt/β-Catenin signaling is critical for hepatogenesis. The lack of β-Catenin during hepatogenesis results in increased hepatocyte cell death and decreased proliferation as well as decreased expression of the transcription factors CCAAT-Enhancer Binding Protein-α and Hepatocyte Nuclear Factor-4α, both of which are important for the function of mature hepatocytes (8). Additionally, organ cultures with knockdown of *β-Catenin* utilizing antisense methodology leads to loss of biliary epithelial markers (9). Conversely, stabilization of β-Catenin inhibits hepatoblast expansion and hepatocyte differentiation while promoting biliary differentiation (10). Together these studies indicate an important role for β-Catenin in proliferation as well as differentiation of hepatoblasts. The role of β-Catenin signaling in mesenchymal precursor cells during liver organogenesis, however, is less clear.

Herein, we investigate the role of β-Catenin signaling in differentiation of mesenchymal cells in the developing liver by using transgenic mice expressing *Cre recombinase* under the control of the mesenchyme-specific *Dermo1* promoter to establish a conditional knock out *β-Catenin* in the mesenchyme of embryonic liver.

MATERIALS AND METHODS

Mutant embryos

β-Catenin$^{f/f}$,*Cmv*$^{Cre/+}$, *Rosa26R*$^{+/+}$, and *Flk1*$^{lacZ/+}$ mice were obtained from Jackson Laboratory (USA). *Dermo1*$^{Cre/+}$ were a gift from Dr. David Ornitz (11). Utilizing Cre recombinase genetics, *β-Catenin*$^{+/-}$ mice were obtained by crossing *β-Catenin*$^{f/f}$ mice with *Cmv*$^{Cre/+}$ mice, wherein *Cre* is expressed in the germ line resulting in knockout of *β-Catenin*. Next, *β-Catenin*$^{+/-}$ mice were crossed with *Dermo1*$^{Cre/+}$ mice to obtain double heterozygous males (*Dermo1*$^{Cre/+}$/*β-Catenin*$^{+/-}$), which were then crossed with *β-Catenin*$^{f/f}$ females to generate mesenchymal specific abrogation of *β-Catenin* (herein called *β-Catenin*^{Dermo1}). Analysis of 327 embryos from F1 intercrosses revealed that the *Dermo1*$^{Cre/+}$/*β-catenin*$^{f/-}$ conditional knockout (herein called *β-Catenin*^{Dermo1}) is lethal at embryonic day E13.5-E14.5 due to the severe cardiac and vasculogenesis-related defects as previously published (6). Littermates not expressing *Cre* were used as controls.

In addition, *β-Catenin*$^{f/f}$ mice were intercrossed with *Rosa26R*$^{+/+}$ to generate *β-Catenin*$^{f/f}$/*Rosa26R*$^{+/+}$ homozygous mice. *β-Catenin*$^{f/f}$/*Rosa26R*$^{+/+}$ mice were then crossed with *Dermo1*$^{Cre/+}$/*β-Catenin*$^{+/-}$ *mice* to generate *β-Catenin*^{Dermo1}/*Dermo1*$^{LacZ/+}$ mice. Littermates were used as controls. To generate *β-Catenin*^{Dermo1}/*Flk1*$^{Lacz/+}$ mice, *β-Catenin*$^{f/f}$ mice were intercrossed with *Flk1*$^{Lacz/+}$ mice. Progeny were then intercrossed with *Dermo1*$^{Cre/+}$/*β-Catenin*$^{+/-}$ mice.

Finally, *Pitx2*^{Dermo1} were generated in a similar fashion described above as with the *β-Catenin*^{Dermo1} mice using *Pitx2*$^{f/f}$, *Pitx2*$^{+/-}$, and *Dermo1*$^{Cre/+}$*mice*. Four conditional knockouts (*Pitx2*^{Dermo1}) from two litters were analyzed for each experiment. Similar to *β-Catenin*^{Dermo1}, mesenchymal deletion of *Pitx2* in *Pitx2*^{Dermo1} mice is embryonic lethal by E14.5. Littermates not expressing *Cre* were used as controls.

PCR was performed to validate genotype. All animal experiments were performed in accordance NIH guidelines.

Localization of β-galactosidase (LacZ) activity

Livers from E11.5-P1 embryos were fixed with 4% paraformaldehyde, and LacZ expression was characterized by β-galactosidase activity using X-gal solution as described by Kelly *et al* (12). For histology, E13.5-P1 livers were further embedded in 3% low melting Agarose and cut into 500 μM sections using Vibrotome (Leica VT 1000S) from Leica, IL, USA.

Samples were then stained with X-gal solution, embedded in paraffin, and sectioned at 5μm thickness as previously described (13). Liver samples were then stained overnight with X-gal solution at 37°C, washed in PBS, fixed for 1h with 4% PFA at 4°C and washed in PBS. Samples were dehydrated and embedded in paraffin for immunohistochemistry or immunofluorescence as described below.

Histology, immunohistochemistry, and histological analysis

Embryos were isolated from the embryonic stages described. After fixation in 4% PFA, dehydration and paraffin embedding, 5 μm sections were cut and samples deparaffinated in Histochoice (Sigma-Aldrich, MO, USA) followed by rehydration through ethanol to water. Antigen retrieval was performed by microwaving the slides in Unmasking solution (Vector Laboratories, CA, USA) for 4 minutes x3. Immunohistochemistry was carried out by using EnVision+ Dual Link System HRP (DAB+) (DacoSytomation, CA, USA) according to instructions from the manufacturer. For immunofluorescence, tissues were treated identically as for immunohistochemistry but after incubated with the primary antibody, the slides were incubated with secondary antibodies for 1.5 hours at room temperature conjugated with FITC or Cy3 from Jackson laboratories as indicated. Slides were then mounted with Vectashield containing DAPI (Vector Laboratories, CA, USA) and photomicrographs were taken. Primary antibodies used were α-Albumin (1:200) (DakoCytomation, CA, USA), α-Desmin (1:100) (DakoCytomation, CA, USA), α-Pan Cytokeratin (1:100) (Sigma-Aldrich, MO, USA)., α-Smooth Muscle Actin (1:100) (Sigma-Aldrich, MO, USA), α-Collagen (Karlan, AZ, USA). Overnight incubation at 4°C was used for all primary antibodies. Controls excluding the primary antibody were in all cases negative.

The number of cells staining positive for DESMIN, E-CADHERIN, ALBUMIN, pan-CYTOKERATIN and LacZ were quantified and normalized to high powered field or the total number of nuclei in a high powered field. Quantification was performed on 3 nonsequential sections and averaged for each animal. Areas positive for LacZ staining were quantified using ImageJ (NIH) and normalized to the total area analyzed per section. Data were expressed as mean ± standard deviation. Student's *t*-test was used to assess statistical significant differences between groups. Significant difference was defined as $P < 0.05$.

RESULTS

Dermo1$^{cre/+}$ mice, expressing *Cre* recombinase under the control of *Dermo1* (*Twist2*) promoter, have been previously used to conditionally knock out genes in the mesenchyme (9,10). Liver mesenchymal specificity of *Dermo1*$^{cre/+}$ was tested in a cross with *Rosa26R* mice to generate *Dermo1*^{LacZ} mice, which express *LacZ* after driver line specific Cre-mediated excision of the loxP flanked nontranscribed neo cassette (14). X-gal staining for *LacZ* expression (or β-galactosidase activity) confirmed activity of the *Dermo1*^{cre} construct as early as embryonic day (E) 11.5 during hepatogenesis (Figure 1A–C). From E11.5 through E18.5, *LacZ* expression was detected scattered throughout the liver parenchyma in fibroblast-like cells (arrows) as well as in cells adjacent to blood vessels (arrowhead). Mesenchymal specificity of the *Dermo1* driver line was confirmed by co-localization of *LacZ* expression with DESMIN, a marker for mesenchymally-derived cells (1). Double positive fibroblast-like cells represent hepatic stellate cells, and double positive perivascular cells represent pericytes (Figure 1D and E, arrow and arrowhead respectively). Co-expression of *LacZ* and DESMIN in *Dermo1*^{LacZ} mice indicates that both hepatic stellate cells and pericytes derive from a common *Dermo1* expressing precursor cell, similar to published observations with *MesP1*^{LacZ} (4).

To investigate the role of β-Catenin in the mesenchymal compartment in the liver during liver organogenesis, we generated conditional knockout mice lacking *β-Catenin* expression in the mesenchyme (*β-Catenin*^{Dermo1}). As previously reported, these conditional knockout embryos are lethal by E13.5–14.5 due to cardiac and vasculogenesis-related defects (6). We consistently observed that fetal livers from *β-Catenin*^{Dermo1} at E13.5 and E14.E were smaller than livers from littermate controls (Figure 2A, B). Histologic analysis revealed larger and more numerous vessels in the *β-Catenin*^{Dermo1} livers compared to controls (Figure 2C, C', D and D'). This defect in the *β-Catenin*^{Dermo1} liver is consistent with the previously described defect in maturation of endothelial cells, which results in leaky blood vessels throughout the embryo (10). Pools of blood could also be detected within the livers of conditional knockout mice at E13.5 (Figure 2D, arrowhead). X-gal staining of *β-Catenin*^{Dermo1}*/Dermo1*$^{LacZ/+}$ revealed in no change in the fraction of total cells that are *LacZ* expressing compared to littermate controls (0.250±0.077 in control mice vs. 0.300±0.125 positive cells per total number of cells in *β-Catenin*^{Dermo1} mice; p-value not significant (NS); Figure 2E, F).

To further analyze the effect of *β-Catenin* knockout in the mesenchyme on mesenchymal cellular differentiation, immunohistological analyses were performed for different mesenchymal markers. Expression of DESMIN was increased in the *β-Catenin*^{Dermo1} livers compared to that of littermate controls (17.8±3.6 vs. 13.6±4.5 DESMIN+ cells/hpf, n=13, $p < 0.05$, Figure 3A–B, arrows). This was primarily due to increased DESMIN expression in perivascular cells adjacent to larger and more numerous intraparenchymal blood vessels. When DESMIN expressing cells were normalized to total cells instead of area in order to account for more area in β-Catenin Dermo1 mouse livers occupied by larger and more numerous blood vessels, the increase in DESMIN positive cells was still noted in the conditional knockout mice compared to wildtype littermates (0.17±0.06 vs. 0.10±0.04, n=13, $p<0.05$). Qualitative assessment of increased α-SMA expression paralleled DESMIN expression detected along the intraparenchymal blood vessels of the conditional knockout livers of *β-Catenin*^{Dermo1} mice (Figure 3C–D). Increased expression of both DESMIN and α-SMA, together with increased deposition of Collagen type I (Figure 3E–F), indicate a possible defect in mesenchymal differentiation in the *β-Catenin*^{Dermo1} conditional knockout livers.

The defect in vascular development identified histologically was further analyzed in *β-Catenin*^{Dermo1}*/Flk1*^{LacZ} mice. *Flk1* encodes the receptor of Vascular Endothelial Growth Factor-2, which is expressed in relatively immature vasculature (14). *β-Catenin*^{Dermo1}*/ Flk1*^{LacZ} mice, therefore, enable labeling of the fetal hepatic vasculature in the setting of mesenchymal deletion of *β-Catenin.* Staining for *LacZ* expression indicated an alteration in the spatial expression pattern of *Flk1* in the *β-Catenin* conditional knockout mice compared to their control littermates (Figure 3G–H) with increased expression of *Flk1* in regions of dilated blood vessels. However, morphometric analysis of total *LacZ* expression for the entire liver was unchanged (21.8±3.4 vs. 19.3±15.1, p-value NS) indicating a redistribution of *Flk1* expression *β-Catenin*^{Dermo1} livers. From these data, we infer that knockout of *β-Catenin* in hepatic mesenchymal cells leads to the dilated fetal intrahepatic vasculature potentially through altered perivascular signaling.

We then sought to determine if inactivation of *β-Catenin* in the mesenchyme affects the cells of endodermal origin. Immunohistochemistry for E-CADHERIN, a marker of cells of endodermal lineage, revealed no qualitative difference in spatial distribution of expression (Figure 4A, B and insets A', B'). Quantitative analysis of number of E-CADHERIN positive cells per area did not show any difference between control littermates and *β-Catenin* conditional knockout mice (201±26 vs. 224±29 E-CADHERIN positive cells/hpf,

respectively; n=18; p-value NS). These data imply that conditional deletion of *β-Catenin* in the mesenchyme does not affect the quantity of endodermally-derived cells.

To further investigate differentiation of cells of the endodermal compartment, we performed immunofluorescence against ALBUMIN (marker of hepatocytes) and Pan-CYTOKERATIN (marker of bile duct epithelium). Co-localization of ALBUMIN and Pan-CYTOKERATIN identifies hepatic epithelial progenitor cells, or hepatoblasts, with the capacity to differentiate into hepatocytes or cholangiocytes. Hepatoblasts co-expressing ALBUMIN and Pan-CYTOKERATIN were identified in both control mice (Figure 4C, arrow) and *β-Catenin*^{Dermo1} embryos (Figure 4D, arrow). There were no significant differences in the total number of cells positive for either ALBUMIN or pan-CYTOKERATIN (Control vs. *β-Catenin*^{Dermo1}: 74.6±17.5 vs. 74.7±20.0 ALBUMIN+ cells/hpf respectively, p-value NS; 153±29 vs. 158±27 Pan-CYTOKERATIN+ cells/hpf, respectively, p-value NS, n=11). There was a trend toward an increase in the number of ALBUMIN+Pan-CYTOKERATIN+ cells in *β-Catenin*^{Dermo1} livers although this was not significant (60.8±19.0 vs. 66.5±6.4 cells/hpf, p-value NS, n=6). While the total number of Pan-CYTOKERATIN+ cells (yellow hepatoblasts plus red cholangiocytes) per high-powered field was not reduced, the number of Pan-CYTOKERATIN only positive cells (red stained cells in the merged images, cholangiocyte differentiated) was reduced in *β-Catenin*^{Dermo1} embryo livers compared to littermate controls (Control vs. *β-Catenin*^{Dermo1}: 84.9±21.2 vs. 41.1±21.2 Pan-CYTOKERATIN positive cells/hpf, respectively, $p<0.05$, n=6). Furthermore, there was a more clustered appearance to the distribution of all Pan-CYTOKERATIN positive cells (cholangiocytes plus hepatoblasts) in control mice compared to *β-Catenin*^{Dermo1} littermates (Figure 4E–F and insets). Collectively, these data may indicate a possible delay in cholangiocyte differentiation in the *β-Catenin*^{Dermo1} livers.

Since *β-Catenin*^{Dermo1} mice grossly phenocopy *Pitx2*$^{-/-}$ mice and the conditional deletion of *β-Catenin* in the mesenchyme leads to progressive loss of PITX2 during lung organogenesis (6), we wanted to investigate the relationship between mesenchymal *β-Catenin* and *Pitx2* during hepatogenesis. Since *Pitx2*$^{-/-}$ mice exhibit a phenotype consisting of smaller livers with dilated sinusoidal spaces (15) very similar to our observed *β-Catenin*^{Dermo1} phenotype, we sought to determine if expression of *Pitx2* specifically expressed in the mesenchyme is critical during hepatogenesis. We, therefore, bred *Pitx2*^{Dermo1} mice, which lack *Pitx2* expression in the *Dermo1*-expressing mesenchyme. Littermates lacking the *Dermo1-Cre* transgene were used as controls. A total of four conditional knockouts at E14.5 and their littermate controls were obtained from two different litters. We observed that in comparison to wildtype livers, E14.5 *Pitx2*^{Dermo1} livers were smaller and were filled with dilated blood vessels similar to *β-Catenin*^{Dermo1} livers (Figure 5A and B). The similarity in dilated vasculature of the *Pitx2*^{Dermo1} and that of the previously reported *Pitx2*$^{-/-}$ livers (15) indicates that it is specifically the mesenchymal expression of *Pitx2* that is responsible for the vascular phenotype. Similar to *β-catenin*^{Dermo1} mice, *Pitx2*^{Dermo1} livers also exhibit increased expression of DESMIN and αSMA (Figure 5C–F). Similar to *β-catenin*^{Dermo1} mice, *Pitx2*^{Dermo1} mice demonstrate no obvious changes in epithelial differentiation in terms of ALBUMIN and Pan-CYTOKERATIN expression (data not shown). These data indicate that β-Catenin activation may signal through downstream Pitx2 in the mesenchyme during hepatogenesis as it does during lung organogenesis.

DISCUSSION

Although Wnt/β-Catenin signaling plays a role in adult HSC, its role in embryonic hepatic mesenchyme is less clear. In this study, we identified a role for β-Catenin in the differentiation of the liver mesenchymal cells using transgenic mice expressing Cre recombinase under the mesenchyme-specific promoter *Dermo1* to specifically knock out *β-*

Catenin in the mesenchymally-derived cells. Delanghe *et al* previously showed that conditional deletion of *β-Catenin* in the *Dermo1*-expressing mesenchyme during lung organogenesis blocks differentiation of mesenchymal progenitor cells into parabronchial smooth muscle cells and also inhibits vascular endothelial maturation (7). The authors also show that mesenchymal deletion of *β-Catenin* coincides leads to the loss of *Pitx2* expression. Furthermore, the overall phenotype of *β-Catenin*^{Dermo1} mice also phenocopies *Pitx2* null mice, further indicating a link between β-Catenin and Pitx2 signaling. Indeed, other studies show that the β-Catenin signaling pathway both induces *Pitx2* expression and also directly activates PITX2 (6,7).

We show that *β-Catenin*^{Dermo1} embryos develop smaller livers filled with peripherally-located, dilated and leaky sinusoids, identical to the phenotype previously described for *Pitx2* null embryos (15,16). Moreover, the histologic phenotype of the previously described complete knockout of *Pitx2* is recapitulated with the mesenchymal deletion of *Pitx2* in our study, indicating that expression of *Pitx2*, specifically in the mesenchyme in particular, is important during hepatogenesis. Additionally, increased expression of DESMIN, α-SMA, and Type I COLLAGEN, particularly in the region of aberrant hepatic vasculogenesis in both the conditional knockout of *β-Catenin* and *Pitx2* indicates a similar defect in mesenchymal differentiation. We, thus, conclude that Pitx2 is downstream of β–Catenin activation in the mesenchyme during hepatogenesis.

We hypothesized that altered mesenchymal-β–Catenin signaling would affect hepatic vasculogenesis. Semela *et al* detailed a link between pericyte signaling and endothelium by showing *in vitro* that hepatic pericytes promote tubular formation of endothelial cells through Platelet-derived Growth Factor (PDGF) and Ephrin-B2 pathways (17). In this study, we show disrupted intrahepatic vasculogenesis with abrogation of mesenchymal β–Catenin signaling through the formation of dilated and leaky sinusoids. We further showed marked increases in expression of *Flk1* in endothelial cells surround these aberrantly formed sinusoids. It is unclear, however, whether loss of mesenchymal-β–Catenin signaling directly affects either PDGF or Ephrin-B2 pathways.

There are numerous examples of mesenchymal-epithelial interactions. During lung organogenesis, parabronchial smooth muscle cells promote the branching morphogenesis of the adjacent epithelial lining through paracrine pathways (18). We previously showed that embryonic HSCs secrete Fibroblast Growth Factor-10 to promote proliferation and survival of adjacent hepatoblasts (13). In this study, we show that mesenchymal deletion of *β-Catenin* results in a significant reduction in Pan-CYTOKERATIN-positive cholangiocytes. Since hepatoblasts as well as both cholangiocytes and hepatocytes can still be identified in *β-catenin*^{Dermo1} mice, it is clear that bilineage differentiation of hepatoblasts still occurs. One plausible explanation for the observation that cholangiocytes are diminished and disorganized is that mesenchymal inactivation of β–Catenin signaling results in a delay or defect in cholangiocyte differentiation (Figure 6). A number of mesenchymally-derived growth factors have been identified as affecting hepatoblast proliferation and differentiation. In particular, the Fibroblast Growth Factors secreted by hepatic stellate cells affect both proliferation and differentiation toward a cholangiocyte cell fate (13,19). The mechanism involved here warrant further investigation.

In summary, we show that mesenchymal β-Catenin activation is not essential for the formation of hepatic stellate cells and pericytes as both cell types are present with inactivation of β-Catenin. However, mesenchymal β-Catenin activation is necessary for proper mesenchymal differentiation as inactivation of *β-Catenin* in the mesenchyme leads to increased collagen deposition, as well as increased DESMIN and α-SMA expression near sinusoids and blood vessels. Furthermore, absence of *β-Catenin* in the mesenchyme during

liver organogenesis leads leaky and dilated sinusoids characterized by altered expression of the endothelial marker *Flk1*. Similarities between the livers of conditional *β-Catenin* knockout mice and conditional *Pitx2* knockout mice is consistent with previous findings that mesenchymal β-Catenin signals through Pitx2 in the lung mesenchyme suggesting that β-Catenin/Pitx2 signaling might be important for maintenance of hepatic mesenchymal progenitor cells. We speculate that absence of *β-Catenin* or *Pitx2* in the hepatic mesenchyme might lead to a switch in mesenchymal differentiation towards a perivascular or a myofibroblast like phenotype thus affecting the blood vessel differentiation and formation as well as endoderm differentiation toward a cholangiocyte cell fate.

Acknowledgments

Financial support: This study was supported in part by the Research Center for Alcoholic Liver and Pancreatic Diseases (P50 AA11999) funded by the National Institute on Alcohol Abuse and Alcoholism (KSW). This work was also supported by National Institutes of Health grants K08 AA016290 (KSW) as well as grants from the Saban Research Institute Career Development (KSW).

Abbreviations

CKO	Conditional Knock Out
ECM	Extra-Cellular Matrix
Pitx2	paired-like homeodomain 2
HSC	Hepatic Stellate Cells
FGF10	Fibroblast Growth Factor 10
FGFR2b	Fibroblast Growth Factor Receptor 2 IIIb
E11.5	embryonic day 11.5
Hnf4α	Hepatocyte nuclear factor 4α
DAPI	4',6-Diamidino-2-phenylindole
β-galactoside	
SMA	Smooth muscle actin
CK19	Cytokeratin 19

REFERENCES

1. Friedman SL. Mechanisms of hepatic fibrogenesis. Gastroenterology 2008;134:1655–1669. [PubMed: 18471545]
2. Kordes C, Sawitza I, Haussinger D. Canonical Wnt signaling maintains the quiescent stage of hepatic stellate cells. Biochem Biophys Res Commun 2008;367:116–123. [PubMed: 18158920]
3. Cheng JH, She H, Han YP, Wang J, Xiong S, Asahina K, Tsukamoto H. Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis. Am J Physiol Gastrointest Liver Physiol 2008;294:G39–49. [PubMed: 18006602]
4. Asahina K, Tsai SY, Li P, Ishii M, Maxson RE Jr. Sucov HM, Tsukamoto H. Mesenchymal origin of hepatic stellate cells, submesothelial cells, and perivascular mesenchymal cells during mouse liver development. Hepatology 2009;49:998–1011. [PubMed: 19085956]
5. Duncan SA. Mechanisms controlling early development of the liver. Mech Dev 2003;120:19–33. [PubMed: 12490293]
6. De Langhe SP, Carraro G, Tefft D, Li C, Xu X, Chai Y, Minoo P, et al. Formation and differentiation of multiple mesenchymal lineages during lung development is regulated by beta-catenin signaling. PLoS ONE 2008;3:e1516. [PubMed: 18231602]

7. Ai D, Liu W, Ma L, Dong F, Lu MF, Wang D, Verzi MP, et al. Pitx2 regulates cardiac left-right asymmetry by patterning second cardiac lineage-derived myocardium. Dev Biol 2006;296:437–449. [PubMed: 16836994]
8. Tan X, Yuan Y, Zeng G, Apte U, Thompson MD, Cieply B, Stolz DB, et al. Beta-catenin deletion in hepatoblasts disrupts hepatic morphogenesis and survival during mouse development. Hepatology 2008;47:1667–1679. [PubMed: 18393386]
9. Monga SP, Monga HK, Tan X, Mule K, Pediaditakis P, Michalopoulos GK. Beta-catenin antisense studies in embryonic liver cultures: role in proliferation, apoptosis, and lineage specification. Gastroenterology 2003;124:202–216. [PubMed: 12512043]
10. Decaens T, Godard C, de Reynies A, Rickman DS, Tronche F, Couty JP, Perret C, et al. Stabilization of beta-catenin affects mouse embryonic liver growth and hepatoblast fate. Hepatology 2008;47:247–258. [PubMed: 18038450]
11. Yu K, Xu J, Liu Z, Sosic D, Shao J, Olson EN, Towler DA, et al. Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. Development 2003;130:3063–3074. [PubMed: 12756187]
12. Kelly RG, Brown NA, Buckingham ME. The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. Dev Cell 2001;1:435–440. [PubMed: 11702954]
13. Berg T, Rountree CB, Lee L, Estrada J, Sala FG, Choe A, Veltmaat JM, et al. Fibroblast growth factor 10 is critical for liver growth during embryogenesis and controls hepatoblast survival via beta-catenin activation. Hepatology 2007;46:1187–1197. [PubMed: 17668871]
14. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995;376:62–66. [PubMed: 7596435]
15. Zhang HZ, Degar BA, Rogoulina S, Resor C, Booth CJ, Sinning J, Gage PJ, et al. Hematopoiesis following disruption of the Pitx2 homeodomain gene. Exp Hematol 2006;34:167–178. [PubMed: 16459185]
16. Kieusseian A, Chagraoui J, Kerdudo C, Mangeot PE, Gage PJ, Navarro N, Izac B, et al. Expression of Pitx2 in stromal cells is required for normal hematopoiesis. Blood 2006;107:492–500. [PubMed: 16195330]
17. Semela D, Das A, Langer D, Kang N, Leof E, Shah V. Platelet-derived growth factor signaling through ephrin-b2 regulates hepatic vascular structure and function. Gastroenterology 2008;135:671–679. [PubMed: 18570897]
18. Bellusci S, Grindley J, Emoto H, Itoh N, Hogan BL. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development 1997;124:4867–4878. [PubMed: 9428423]
19. Sekhon SS, Tan X, Micsenyi A, Bowen WC, Monga SP. Fibroblast growth factor enriches the embryonic liver cultures for hepatic progenitors. Am J Pathol 2004;164:2229–2240. [PubMed: 15161655]

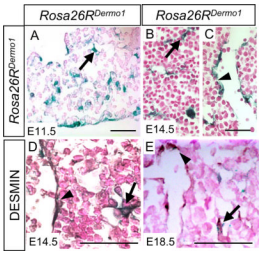

Figure 1.

Spatial and temporal expression pattern of *LacZ* in *Rosa26*^{Dermo1} livers embryos during hepatogenesis at E11.5 (A) and E14.5 (B and C). Co-localization with the mesenchymal marker DESMIN (brown) and β-galactosidase activity (blue) at E14.5 (D) and E18.5 (E). Arrows indicates fibroblast-like cells with β-galactosidase activity distributed throughout the liver parenchyma and arrowheads indicate cells with β-galactosidase activity lining the sinusoids. Bars denote 25 μm.

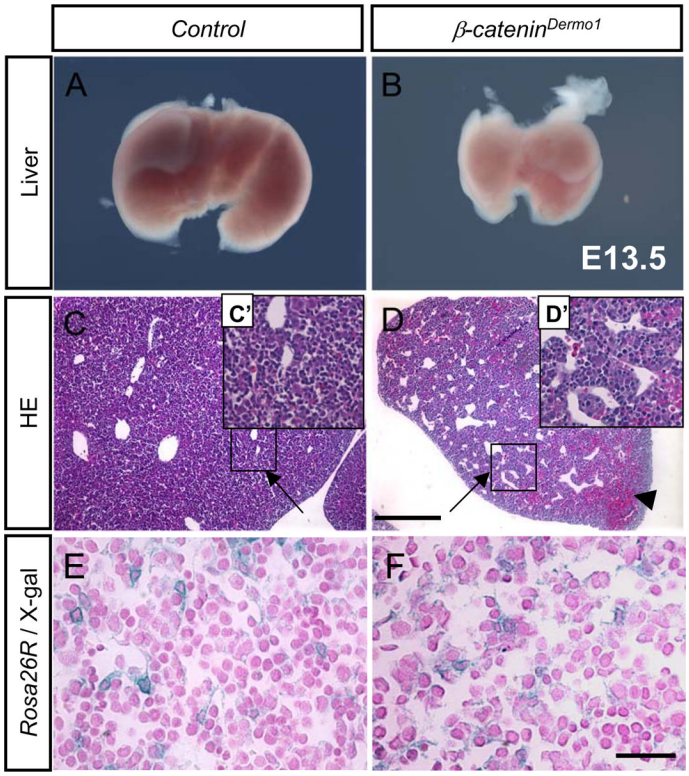

Figure 2.
Phenotype analysis of mesenchymal *β-Catenin* deletion in E13.5 *β-Catenin*^{Dermo1} embryo livers. *β-Catenin*^{Dermo1} livers are smaller than those of control littermates (A, B). Hematoxilin/Eosin staining shows disturbed architechture in the *β-Catenin*^{Dermo1} with dilated sinusoids (arrow) and abnormally pooled blood (arrowhead) (C, D, and insets C', D'bars denote 200 μm.). *LacZ* expression in *β-Catenin*^{Dermo1};*Rosa26R* and littermate controls (E and F, bars denote 50 μm).

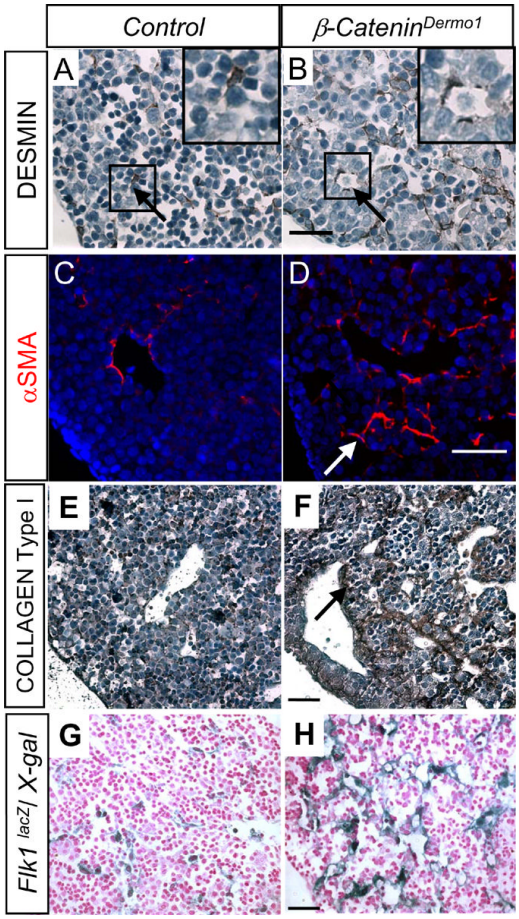

Figure 3.

Immunohistochemical analysis of mesenchymal markers in E14.5 *β-Catenin*^{Dermo1} embryo livers. Increased staining for DESMIN (A, B and insets A', B') αSMA (C, D arrow) along dilated sinusoids in *β-Catenin*^{Dermo1} livers compared to control littermates. Increased deposition of COLLAGEN Type I in *β-Catenin*^{Dermo1} livers compared to control littermate (E, F arrow). Increased *LacZ* expression in E14.5 *β-Catenin*^{Dermo1};*Flk1*^{LacZ} livers outlining the dilated blood vessels in *β-Catenin*^{Dermo1} livers compared to control livers (G, H). Bars denote 50μm.

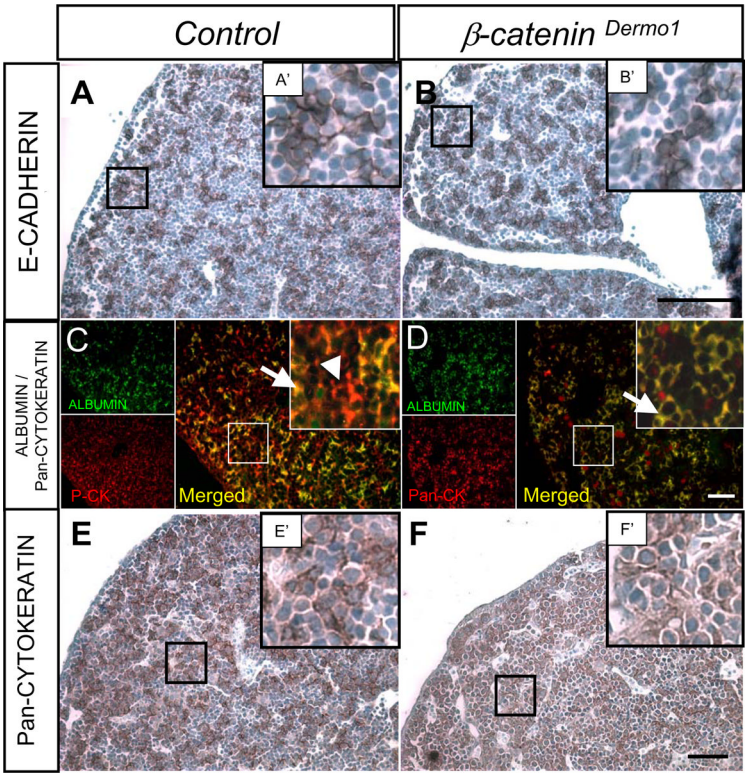

Figure 4.

Immunohistochemical analysis of epithelial markers E14.5 *β-Catenin*^{Dermo1} embryo livers. Staining for E-CADHERIN in the E14.5 *β-Catenin*^{Dermo1} livers (B, B') and littermate control (A, A'). Immunofluorescent staining for ALBUMIN (green), Pan-CYTOKERATIN (red) and merge (arrow, yellow) (C, D). Arrowhead denotes Pan-CYTOKERATIN only positive cells in the control compared to *β-Catenin*^{Dermo1}. Immunohistochemical staining for pan-CYTOKERATIN mark bile duct epithelial cells and hepatoblasts (brown) (E, F' and insets E', F'). Bars denote 50 μm

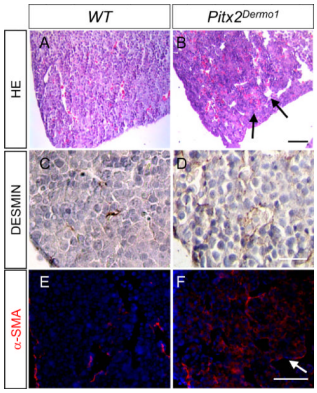

Figure 5.

Phenotype analysis of E14.5 *Pitx2*^{Dermo1}. Hematoxylin and Eosin staining of *Pitx2*^{Dermo1} mice (B) and control littermates (A). Bar indicates 50 μm. Arrow indicates dilated sinusoids in the conditional knockout. Immunohistochemical staining for mesenchymal DESMIN (brown) (C, D) Bar denotes 25 μm. Immunofluorescent staining for mesenchymal αSMA (red) (E, F) show increased staining in conjunction with dilated blood vessels in *Pitx2*^{Dermo1} livers compared to control littermates (E, F arrow). Bar denotes 50 μm (C, E).

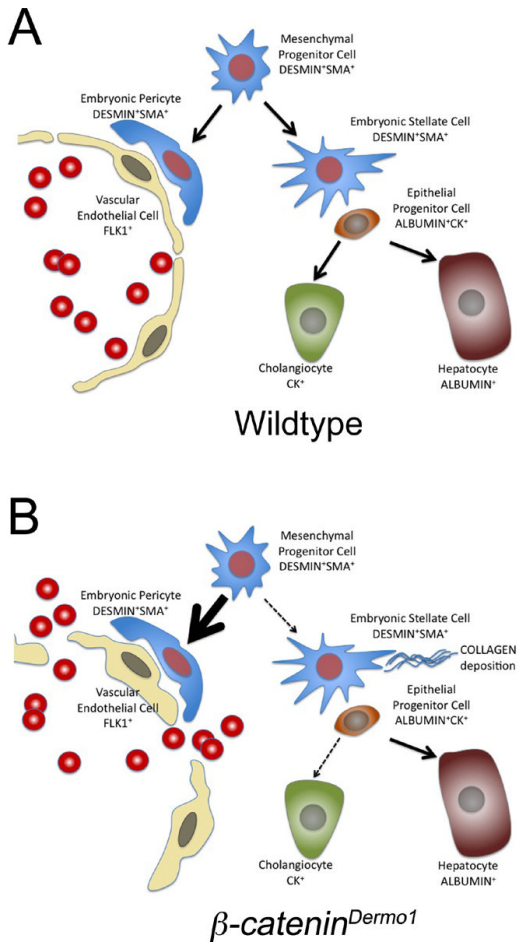

Figure 6.
Schematic for hypothesis model. (A) Wildtype and (B) *β-Catenin*^{Dermo1} livers.