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Hepatic Prominin-1 expression is associated with biliary fibrosis

Marie V. Nguyen^a, Jessica A. Zagory^a, William H. Dietz^a, Alex Park^a, Michael Fenlon^a, Menghan Zhao^a, Jiabo Xu^a, Ingrid Lua^b, Nirmala Mavila^c, Kenji Asahini^b, and Kasper S. Wang^{a,c}

^aDivision of Pediatric Surgery, Developmental Biology, Regenerative Medicine and Stem Cell Program, The Saban Research Institute, Children's Hospital Los Angeles, CA

^bKeck School of Medicine, University of Southern California, Los Angeles, CA

^cCedars Sinai Medical Center, Los Angeles, CA

Abstract

Background—Intrahepatic biliary fibrosis, as seen with cholestatic liver injuries such as biliary atresia, is mechanistically distinct from fibrosis caused by hepatocyte toxicity. We previously demonstrated the expansion of cells expressing the stem/progenitor cell marker PROMININ-1 (PROM1), within regions of developing fibrosis in biliary atresia. Thus, we hypothesized that *Prom1* expression is biliary fibrosis-specific.

Methods—Gene expression of *Prom1* was analyzed in adult mice undergoing either cholestatic bile duct ligation (BDL) or hepatotoxic carbon tetrachloride (CCl₄) administration by quantitative polymerase chain reaction. Lineage tracing of *Prom1*-expressing cells and *Collagen-1a* (*Col1a*)-expressing cells was performed after BDL in *Prom1*^{cre-ert2-lacZ};*Gfp*^{Isl} and *Col1a*^{Gfp} transgenic mice, respectively.

Results—*Prom1* expression increased significantly after BDL compared to sham (6.6±0.9 fold change at 2 weeks, p<0.05) but not with CCl₄ (−0.7±0.5-fold change, NS). Upregulation of *Prom1* was observed histologically throughout the liver as early as five days after BDL in *Prom1*^{cre-ert2-lacZ} mice by LacZ staining in non-hepatocyte cells. Lineage tracing of *Prom1*-expressing cells labeled prior to BDL in *Prom1*^{cre-ert2-lacZ};*Gfp*^{Isl} mice, demonstrated increasing colocalization of GREEN FLUORESCENT PROTEIN (GFP) with biliary marker CYTOKERATIN-19 within ductular reactions up to 5 weeks after BDL consistent with biliary transdifferentiation. In contrast, rare colocalization of GFP with mesenchymal marker α-SMOOTH MUSCLE ACTIN in *Prom1*^{cre-ert2-lacZ};*Gfp*^{Isl} mice and some colocalization of GFP

Corresponding author: Kasper S. Wang, MD, FACS FAAP, Associate Professor of Surgery, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, 4650 Sunset Blvd, Mailstop #100, Los Angeles, CA 90027, Office: 1-323-361-2338, kwang@chla.usc.edu.

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with PROM1 in *Col1a^{Gfp}* mice, indicate minimal contribution of *Prom1* progenitor cells to the pool of collagen-producing myofibroblasts.

Conclusion—During biliary fibrosis, *Prom1*-expressing progenitor cells transdifferentiate into cells within ductular reactions. This transdifferentiation may promote fibrosis.

Introduction

Biliary atresia (BA), a congenital progressive fibro-obliteration of the bile ducts, is the leading cause of pediatric end-stage liver disease and liver transplantation in the United States [1]. While surgical bypass of the atretic extrahepatic biliary tree is essential for survival, the extent and progression of intrahepatic biliary fibrosis are key determinants of transplant-free survival [2]. Thus, characterizing the pathogenesis of biliary fibrosis associated with biliary atresia remains essential in efforts to impact survival for infants with BA.

Prominin-1 (Prom1), or CD133, is a penta-transmembrane glycoprotein expressed by collagen-producing hepatic stellate cells and portal fibroblasts as well as by stem/progenitor cells in a variety of tissues including the liver [3]. The function of CD133 is unknown, although several studies indicate its role in maintaining the topology of the plasma membrane, in effect regulating the state of cellular differentiation [4] [5] [6]. *Prom1*-expressing hepatic progenitor cells possess great regenerative and malignant potential [7] and may contribute to the populating of tissue-engineered liver constructs [8]. We have also previously demonstrated the expansion of a population of cells expressing PROM1 within periportal regions of evolving biliary fibrosis in both the rodent rotavirus model of BA as well as in infants with BA [9]. These cells exhibit characteristics of progenitor cells with expression of both epithelial and mesenchymal markers such as CYTOKERATIN-19 (CK19) and α -SMOOTH MUSCLE ACTIN (α SMA), respectively.

Thus, we hypothesized that PROM1-expressing cell expansion is a biliary fibrosis-specific phenomenon. To test this hypothesis, we compared *Prom1* gene expression patterns in adult mouse models of cholestasis/biliary fibrosis by bile duct ligation (BDL) and hepatocyte-specific carbon tetrachloride (CCl₄) injury, as well as lineage tracing to demonstrate the progenitor cell nature of *Prom1*-expressing cells.

Methods

Animal experiments

Six-week old wildtype C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were injected subcutaneously with either CCl₄ (1 mL/kg body weight) in mineral oil (1:3 dilution), or control, every third day. Six-week old wildtype, *Prom1^{cre-ert2-lacZ};Gfp^{lsl}* (both from Jackson Laboratories), or *Col1a^{Gfp}* (a gift from David Brenner, UC San Diego) [10] transgenic mice underwent BDL as previously described [11]. Double transgenic offspring bred from *Prom1^{cre-ert2-nlacZ}* and *Gfp^{lsl}* mice were treated with tamoxifen (mg/g) in olive oil 3 weeks prior to BDL. Briefly, mice underwent laparotomy under isoflurane anesthesia. The extra-hepatic bile duct was isolated and doubly ligated. The peritoneal cavity was filled with saline for fluid resuscitation and the laparotomy incisions closed. While extra-hepatic bile

ducts of sham-operated controls did not receive ligation, all other procedural measures remained consistent. Thereafter, all mice were recovered and fed standard rodent chow and water *ad libitum*. With either liver injury model, mice were euthanized up to 6 weeks post-injury for collection of liver samples. All animal procedures were approved by the Children's Hospital Los Angeles Saban Research Institute's or the University of Southern California's Institutional Animal Care and Use Committee.

Immunohistochemistry

Livers were fixed in 4% paraformaldehyde (PFA, Poly Sciences Inc., Warrington, PA), embedded in paraffin and then sectioned. Immunofluorescence imaging was performed as previously described [12] using primary antibodies listed in Supplemental Table 1 and secondary antibodies conjugated either with anti-mouse Cy3/Cy5, anti-rat Cy3/Cy5, or anti-rabbit Cy3/Cy5 (Jackson Immuno Research Lab, West Grove, PA). Images were acquired using a Leica DM5500B immunofluorescence microscope with Leica Suite Advanced Fluorescence (LAS AF) 6000 software (Wetzlar, Germany) or a LSM700 confocal system with ZEN software (Carl Zeiss Microimaging, Thornwood, NY). Bright field images were acquired using a Leica DM1000 (DFC290) microscope interfaced with Leica Application Suite, Version 2.7.1R1 (Leica Microsystems, Switzerland). Sirius red staining was performed as previously described [9].

LacZ staining

Liver tissue was fixed with 2% PFA at 4°C overnight, then incubated in 20% sucrose at 4°C overnight and finally embedded in OCT. Frozen sections were washed in phosphate buffered solution (PBS) for 5 minutes and incubated in β -gal substrate (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40 in PBS) in the dark overnight at 37°C. Sections were washed in PBS for 5 minutes and counterstained with eosin. ImageJ® was used to quantify cell positivity for LacZ staining.

Quantitative polymerase chain reaction

Total RNA was isolated from whole and non-parenchymal cell fractions of snap-frozen mouse liver tissues and FACS-sorted cells using the Qiagen RNA isolation kit (Qiagen, Valencia, CA). Complementary DNA synthesis was performed using iScript Reverse Transcription Supermix (Bio-Rad, Irvine, CA), and quantitative real-time PCR (qPCR) was performed as previously described [11, 13], using intron-spanning and gene-specific primers and probes designed using the Roche Universal Probe Library Assay Design Center (Roche, Basel, Switzerland) (see Supplemental Table 2 for primer sequences and probes).

Results

Prominin-1 expression increases after BDL but not after CCl₄ induced liver injury

We previously reported the emergence and expansion of PROM1-expressing cells associated with evolving biliary fibrosis due to biliary atresia [9]. To further explore this observation, we compared cholestatic BDL with hepatotoxic CCl₄ up to 6 weeks post-injury. Notable in both models of liver injury, there is significant destruction of liver parenchymal with loss of

hepatocytes within lobules with loss of cellular definition and nuclear pyknosis, even without hematoxylin counterstaining (eosin only) (Figure 1a). By Sirius red staining, we observed comparable progression of fibrosis in both models (Figure 1a). Analysis of gene expression profiles demonstrated an increase in *Prom1* expression prior to 3 weeks post-BDL 6.6 ± 0.9 fold ($p < 0.05$) weeks and a 12 ± 4 fold ($p < 0.05$) after 3 weeks post-BDL (Figure 1b). In contrast, there was no observable change in *Prom1* expression with CCl_4 treatment (-0.7 ± 0.5 -fold change, NS). These data suggest a biliary-fibrosis specific upregulation of *Prom1*.

Spatial localization of *Prom1* expression was then performed by LacZ staining (eosin counterstain only) of livers from *Prom1^{cre-ert2-lacZ}* mice 5 days after BDL, where β -galactosidase expression marks active expression of *Prom1*. Rare cells positive for LacZ staining were observed 5 days after sham operation (Figure 2a). In contrast, 5 days after BDL, there were significantly more cells positive for LacZ within periportal regions near bile ducts and also within the liver parenchyma, along sinusoids where hepatic stellate cells reside (8.6 ± 1.2 vs 37.2 ± 8.8 , unpaired t-test, $p < 0.05$) (Figure 2b, 2b'). These data are consistent with the observed upregulation of *Prom1* expression by qPCR (Figure 1b).

Cell fate of *Prom1*-expressing cells after bile duct ligation

In order to determine if *Prom1*-expressing cells transdifferentiate during biliary fibrosis, we crossed *Prom1^{cre-ert2-lacZ}* mice with *Gfp^{lsI}* mice. Tamoxifen was administered to 4-week old *Prom1^{cre-ert2};Gfp^{lsI}* mice 3 weeks prior to BDL in order to induce expression of *Prom1*-expressing cell-specific Cre-driven recombination to permanently express GREEN FLUORESCENT PROTEIN (GFP) in their subsequent progeny. Three weeks after BDL, CK19^{positive} (pos) ductular reactions were observed in the periportal regions of hepatic lobules; few CK19^{pos} cells were co-positive for GFP, indicating derivation from cells that were originally expressing *Prom1* at the time of tamoxifen administration (Figure 3). Two weeks later, at 5 weeks post-BDL, nearly all cells in CK19^{pos} ductular reactions were GFP^{pos}. These data suggest that over time, ductular reactions increasingly derive from *Prom1*-expressing precursor cells.

Co-positivity of GFP with α -SMOOTH MUSCLE ACTIN (α SMA) was also noted in rare fibroblast-like cells that could represent aPFs; however, most α SMA^{pos} cells were α SMA^{neg} up to 5 weeks. At 5 weeks, co-positivity of GFP with NTPDase-II was observed in periportal regions but only within rare larger hepatocyte-like cells and not small fibroblast-like cells (Figure 4).

Three weeks following BDL in *Col1a^{Gfp}* mice, GFP^{pos} cells, which are actively expressing *Collagen-1a*, were observed within regions of evolving biliary fibrosis. Some PROM1^{pos}GFP^{pos} and PROM1^{pos}GFP^{neg} cells were observed but PROM1^{pos}CK19^{pos} ductular reactions were not (Figure 5). Collectively these data suggest that *Prom1*-expressing progenitor cells transdifferentiate predominantly into biliary ductular reactive cells and contribute minimally to the pool of collagen-producing myofibroblasts. Our data also suggest that ductular reactions which derive from *Prom1*-expressing progenitors, lose their expression of PROM1 over time.

Discussion

In this study, we demonstrated a biliary fibrosis-specific upregulation of *Prom1* expression after BDL. We further demonstrated evidence via histological lineage tracing, that ductular reactions within regions of evolving biliary fibrosis are increasingly comprised of cells deriving from *Prom1*-expressing progenitor cells. In contrast, we observed only a minimal contribution of *Prom1*-expressing cells to the pool of collagen producing myofibroblasts. Collectively, these data support the concept that *Prom1*-expressing progenitor cells transdifferentiate primarily into biliary ductular reactions, within regions of evolving biliary fibrosis, adjacent to collagen- expressing cells.

The underlying pathophysiology of biliary fibrosis, secondary to cholestatic liver injury, remains undetermined. Using *Col1a^{Gfp}* mice, Iwaisako et al. reported that during early biliary fibrogenesis following BDL, aPFs, which reside in periportal regions where biliary fibrogenesis originates, contribute more significantly to the population of collagen-producing myofibroblasts than hepatic stellate cells (HSCs), which reside in the hepatic sinusoids throughout the hepatic lobule [10]. Lua et al., however, observed that activated HSCs, not aPFs, are the predominant myofibroblasts following BDL [11]. Regardless of which cell population contributes most to the pool of collagen-producing myofibroblasts, our observations are consistent with *Prom1*-expressing progenitor cells transdifferentiating into cells comprising biliary ductular reactions within regions of biliary fibrosis during cholestatic liver injury. Furthermore, our data indicate that ductular reactions may lose expression of PROM1 over time following BDL. It is unclear how significant any downregulation of PROM1 in developing ductular reactions is, but we posit that this may be in response to terminal differentiation of a progenitor cell population.

The significance of *Prom1*-expressing biliary progenitor cell transdifferentiation remains unanswered by this study; however, there is evidence that *Prom1*- expressing hepatic progenitor cells (HPCs) may contribute to biliary fibrosis. Rountree et al. demonstrated that PROM1-expressing cells comprise ductular reactions that develop in the rodent model of cholestasis/biliary fibrosis induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet [14]. Moreover, expansion of a single PROM1^{pos}CD45^{neg} HPC clone isolated from liver tumors from *Albumin^{Cre};**Pten^{loxP}* mice could be passaged multiply maintaining an epithelial progenitor cell phenotype. However, spontaneous transformation of a subset of these PROM1^{pos} cells was associated with a more mesenchymal, fibroblastic phenotype and functionally with tumor metastases [15]. In this study, lineage tracing of *Prom1*- expressing precursor cells demonstrated qualitatively a large contribution to biliary ductular reactions within regions of collagen deposition. Although the direct contribution of *Prom1*-expressing progenitors to collagen-producing myofibroblasts was small, a role in biliary fibrosis cannot be excluded. Indeed, Nadler et al. demonstrated that cells within ductular reactions of biliary fibrosis in the murine model of biliary atresia express INTEGRIN $\alpha_v\beta_6$, a key mediator in the activation of profibrogenic Transforming Growth Factor- β [16]. We posit that the expression of *Prom1* may contribute to biliary fibrogenesis potentially, in part, through promotion of TGF β signaling activation in downstream progeny, but further analysis is required to validate this potential link.

We had previously demonstrated colocalization of PROM1 and α SMA during fibrogenesis associated with biliary atresia [9]. In this study, we demonstrated upregulation of *Prom1* gene expression within the liver following BDL but not with CCl₄ injury. Interestingly, by LacZ staining of *Prom1*^{cre-ert2-nlacZ} mice 5 days after BDL, we observed *Prom1* expression not only within the periportal regions of the hepatic lobule but also along hepatic sinusoids in a pattern consistent with that of HSCs. This spatial pattern of expression is not evident with lineage tracing of *Prom1*-expressing cells following BDL, suggesting not only transdifferentiation of *Prom1*-expressing progenitor cells, but also upregulation of *Prom1* expression in existing HSCs. Further analysis is required to first determine if indeed both quiescent HSCs and portal fibroblasts upregulate *Prom1* expression following BDL and second to determine the significance of *Prom1* expression during fibrogenesis.

In this study, we provide qualitative, histologic evidence of *Prom1*-expressing cell transdifferentiation during fibrogenesis associated with cholestasis following bile duct ligation. It is likely that there is significantly more biliary ductular transdifferentiation compared to myofibroblastic transdifferentiation; however, this study does not definitively prove this point given its descriptive nature. Further analyses are required to quantify and validate this point.

In summary, we demonstrate a link between Prominin-1 and biliary fibrosis and posit that PROM1 functionally promotes biliary fibrosis in part through the expansion of profibrogenic ductular reactions. As such, *Prom1*-expressing cells or signaling upstream or downstream of Prom1 could provide potential targets of therapeutic intervention of biliary fibrogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Collagen deposition demonstrated by sirius red staining with BDL and CCl₄ liver injuries (counterstained with eosin only). (B) Gene expression early and later after BDL and CCL₄. Scale bar = 50 μm. * denote $p < 0.05$.

Figure 2. LacZ staining (counterstained with eosin only) in *Prom1^{cre-ert2-lacZ}* mice 5 days after undergoing (A) sham operation or (B) BDL. Note the greater blue staining, which mark sites of *Prom1* expression, after BDL (B, and inset B') compared to sham. Scale bar = 50 μ m.

Figure 3. Lineage tracing of *Prom1*-expressing cells labeled with Green Fluorescent Protein, via tamoxifen injection 3 weeks prior to BDL, 3 and 5 weeks after BDL in *Prom1^{cre-ert2-lacZ};GFP^{Isl}* mice. CK19 (red). Arrowheads mark GFP^{pos}CK19^{pos} (yellow) cells. Scale bar = 25 μ m.

Figure 4.

Lineage tracing of *Prom1*-expressing cells labeled with Green Fluorescent Protein, via tamoxifen injection 3 weeks prior to BDL, 3 and 5 weeks after BDL in *Prom1^{cre-ert2-lacZ};GFP^{lsl}* mice. (A) NTPdase II (red), (B) αSMA (red). Arrowheads mark yellow co-positive cells. Note the presence of red single positive cells. Scale bar = 25 μm.

Figure 5.
Immunofluorescence co-localization of GFP, CK19, and PROM1 in *Coll1a^{Gfp}* mice 3 weeks after BDL. Arrowheads mark yellow cells co-positive for PROM1 and GFP (Collagen).
Scale bar = 75 μ m.