

Homeobox Gene Prx1 Is Expressed in Activated Hepatic Stellate Cells and Transactivates Collagen $\alpha 1(I)$ Promoter

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Hepatic stellate cells (HSCs) are mesenchymal cells of the liver, which are normally in quiescent state and synthesize tracing amounts of extracellular matrix proteins. Upon fibrogenic stimulus, HSCs become activated and increase synthesis of type I collagen 50–100 fold. Prx1 and Prx2 are two homeobox transcription factors which are required for mesenchymal tissue formation during embryogenesis. The present study shows that Prx1 mRNA is expressed in *in vivo* and *in vitro* activated HSCs, but not in quiescent HSCs. Prx1 is also expressed in fibrotic livers, while it is undetectable in normal livers. Overexpression of Prx1a in quiescent HSCs cultured *in vitro* induced collagen $\alpha 1(I)$ mRNA and TGF $\beta 3$ mRNA expression. Prx1 transactivated TGF $\beta 3$ promoter 3 fold in transient transfection experiments. In the whole liver, Prx1a induced expression of collagen $\alpha 1(I)$, $\alpha 2(I)$, $\alpha 1(III)$ and α -smooth muscle mRNAs, which are the markers of activation of HSCs. Prx1 also increased expression of collagen $\alpha 1(I)$ mRNA after acute liver injury. This suggests that Prx1a promotes activation of HSCs and expression of type I collagen. Several regions in the collagen $\alpha 1(I)$ promoter were identified which mediate transcriptional induction by Prx1. The regions are scattered throughout the promoter and individually have modest effects; however, the cumulative effect of all sequences is >50 fold. This is the first description of the effects of Prx1 in HSCs and in the liver, and identification of the two Prx1 target genes, which play a pivotal role in development of liver fibrosis, is a novel finding for liver pathophysiology. *Exp Biol Med* 233:286–296, 2008

Key words: gene regulation; hepatic fibrosis; pair-related transcription factor

Introduction

Liver fibrosis is an aberrant wound-healing response to chronic liver injury and is the 8th most common cause of death in the USA. Liver fibrosis is characterized by the excessive accumulation of extracellular matrix proteins (ECM), mainly type I collagen, which is produced by hepatic stellate cells (HSCs) (7, 39). HSCs represent 15% of the total cell number in the liver and are located within the space of Disse in the liver sinusoids (18). HSCs have a quiescent phenotype in the normal liver, and their role is to store vitamin A and regulate contractility of the sinusoids. Upon liver injury, quiescent HSCs become activated, a process in which they lose vitamin A droplets, upregulate synthesis of extracellular matrix proteins and differentiate into myofibroblast-like phenotype (16, 19, 27, 28). When isolated from the normal liver and cultured *in vitro*, HSCs spontaneously transdifferentiate from the quiescent phenotype to the activated myofibroblasts, a process resembling HSC activation in fibrosis (12, 20, 23).

The activation of HSCs is accompanied by changes in gene expression: the genes encoding ECM, cytoskeleton and components of protein synthesis machinery are preferentially upregulated during activation of HSCs (33). Expression of type I collagen increases 50–70 fold in activation of HSCs (58). Increased collagen synthesis is initiated and maintained by profibrotic cytokines like TGF- β family members and connective tissue growth factor (CTGF) (9, 16, 27), which are produced in response to the profibrotic insult (17, 49). Upon a prolonged insult, collagen synthesis fails to respond to negative feedback regulation and proceeds to uncontrolled fibrosis. Deposition of the crosslinked type I collagen fibrils, which are resistant to proteolytic degradation, is responsible for alteration of the normal liver extracellular matrix (fibrosis) and change in organ architecture (cirrhosis) (2, 45).

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To date, only a few transcription factors were found that were individually able to promote or inhibit the activation of HSCs (42), such as c-myc, JunD, KLF-6 and PPAR- γ (10, 43, 50, 56). On the other hand, transcription of type I collagen genes has been excessively studied (3, 6, 29, 46, 53, 54). Two features of transcription of collagen $\alpha 1(I)$ gene have emerged: 1. coordinated activity of multiple factors is required for regulation (15, 21, 31), and 2. collagen $\alpha 1(I)$ promoter has a modular architecture with characteristic elements active in some cell types, while the other elements are functional in other cell types (3, 5, 29, 54, 55). Promoter sequences of collagen $\alpha 1(I)$ gene bind several ubiquitous transcription factors like Sp1, NF1, AP1, CBF1 and BTEB (11, 37, 46, 51, 52, 55). DNA sequences between -2.3 and -1.7 kb were required for $\alpha 1(I)$ promoter expression in bone and tooth (5). Sequences that control expression in tendon are distributed between -3.5 and -1.7 kb of the promoter, with sequences downstream of -1.7 kb still capable of directing expression to this tissue (29). In another study transgenic mice harboring 900 bp of the $\alpha 1(I)$ proximal promoter expressed the transgene at relatively low levels almost exclusively in the skin. In mice containing 2.3 kb of $\alpha 1(I)$ proximal promoter, the transgene was also expressed at high levels in osteoblasts and odontoblasts, but not in other type I collagen-producing cells (54). Transgenic mice harboring 3.2 kb of the proximal promoter showed an additional high level expression of the transgene in tendon and fascia fibroblasts (54). These data strongly suggest a modular arrangement of separate cell specific cis-acting elements that can activate the mouse $\alpha 1(I)$ collagen gene in different type I collagen-producing cells. Distal DNase I hypersensitive sites were also described in the $\alpha 1(I)$ (35) and $\alpha 2(I)$ gene locus (1) as potential binding sites for enhancer-like or locus controlling region factors, but the functional significance of these sites is not clear.

Two homeobox transcriptional factors, Prx1 (also known as PRRX1, mhox, k2, Pmx and rHOX) and Prx2 (also known as PRRX2 and S8), have not been associated with transcription of type I collagen genes, although Prx1 colocalized with procollagen I in the developing chick vascular system (4). Prx1 and Prx2 are developmental regulators expressed in mesenchymal cells derived from neural crest (8, 38, 41, 44, 60). Prx1 knock-out mice die perinatally, presumably due to severe cleft palate (44). They exhibited defects of skeletogenesis, which include abnormalities in development of craniofacial, limb, and vertebral skeletal structures. The role of Prx1 in cartilage and bone development is mediated by Prx1-targeted genes, but so far only two target genes have been identified, α -smooth muscle actin (α SMA) (25, 62) and tenascin C (30, 47). Prx2 null mice showed no skeletal defects and were viable normally. However, the abnormalities were more pronounced in Prx1 and Prx2 double knock-out mice (41, 60), suggesting nonredundant roles of Prx1 and Prx2.

There are no reports on expression or function of Prx1 and Prx2 genes in the liver or HSCs. In this study, we show that Prx1 and Prx2 were upregulated in activated rat and

human HSCs and that Prx1 was upregulated *in vivo* in rat fibrotic livers. Two novel Prx1 target genes were identified: collagen $\alpha 1(I)$ and TGF $\beta 3$. Overexpression of Prx1a in quiescent HSCs stimulated expression of collagen $\alpha 1(I)$ mRNA, while *in vivo* it stimulated expression of collagen $\alpha 1(I)$, $\alpha 2(I)$ and $\alpha 1(III)$ mRNAs. Multiple regions in the collagen $\alpha 1(I)$ promoter were identified which confer response to Prx1a.

Materials and Methods

Isolation and Culture of Rat HSCs. Rat HSCs were isolated from rat livers by perfusion with collagenase and pronase, followed by centrifugation over Nycodenz gradient, as described (61). The purity of HSCs was determined by staining with anti-desmin antibody, as described (33), and was $>95\%$. For *in vitro* activation, HSCs were cultured in uncoated plastic dishes in DMEM supplemented with 10% FBS. Total RNA was extracted at days 3, 4, 5 and 8 after HSC isolation. cDNA of culture activated human HSCs and mouse HSCs activated *in vivo* were a kind gift of Dr. Bernd Schnabl (Columbia University, NY).

Construction of Adenovirus. Coding regions of mouse Prx1a and Prx1a- Δ OAD were cloned into pAD-TRACK-CMV vector. Transcription of the cloned genes is driven by the constitutive CMV promoter within this vector. As a control, a noncoding DNA was cloned into the same vector (26). This vector also contains a green fluorescent protein (GFP) expression cassette as a transcription unit that is independent of the cloning of the test genes. The viral genomes were reconstructed by recombination in *Escherichia coli* between the pADTRACK constructs and pADEasy-1 plasmid. Viruses were produced as described (26) and purified by adenovirus purification kit (Invitrogen, Mountain View, CA). The resulting viruses expressed both mPrx1a and GFP, or mPrx1a- Δ OAD and GFP.

Transduction of Rat HSCs with Adenovirus-es. Two days after isolation the control adenovirus and adenovirus expressing Prx1a or Prx1a- Δ OAD were added to rat HSCs at multiplicity of infection (MOI) of 500. This results in transduction of $>90\%$ of HSCs (58). Total RNA was prepared from the HSCs 3 days after the infection with adenoviruses (5 days after isolation).

Animal Model of Liver Fibrosis. Sprague-Dawley rats were obtained from Charles River. Rats (~ 200 g) were injected intraperitoneally with CCl $_4$ (2 μ l/g in 50% mineral oil) twice a week for 4 weeks. Control rats received only mineral oil. After 4 weeks of treatment, the livers were harvested for histology and mRNA extraction. Acute liver injury was induced by a single injection of CCl $_4$, and the livers were analyzed after 3 days. All animals received humane care according to the criteria outlined by the National Institute of Health.

Injection of Adenoviruses into Mice. Control adenovirus and adenovirus expressing mPrx1a were injected

at 10^{10} pfu/kg through the tail vein into mice. Livers were harvested at 5 or 7 days after the adenoviral injection, and poly(A)+ RNA was prepared using a direct poly(A)+ RNA isolation kit (Sigma, St. Louis, MO).

RT-PCR Analysis. Total RNA of quiescent and activated rat HSCs was extracted using an RNA isolation kit (Eppendorf, Westbury, NY). Poly(A)+ RNA of normal rat livers, CCl₄-treated livers, normal mouse liver and mouse livers injected with adenoviruses was extracted by a direct poly(A)+ RNA isolation kit (Sigma, St. Louis, MO). RT-PCR was performed with 50 ng of total RNA or 20 ng of poly(A)+ RNA using rTth reverse transcriptase (Boca Scientific, Boca Raton, FL) in the presence of 2.5 μ Ci of [α -³²P]dCTP, according to the previously used protocol (33, 58). PCR products were resolved on a sequencing gel and visualized by autoradiography. The number of cycles was adjusted to be in the linear range of the reaction. The gene specific primers and the sizes of the expected PCR products are listed in Table 1. Primers for rat and mouse GAPDH were described before (58). Primers of r-Prx1a and r-Prx1b were gifts from Dr. Michael J. Kern (Medical University of South Carolina, Charleston, SC) and are described in (48).

Western Blot Analysis. Total protein was prepared by standard procedure from HEK293 cells infected with adenoviruses. Anti-Prx1 antibody was a gift from Dr. Michael J. Kern and was used at 1:1000 dilution. Western blot was performed according to the published protocol (48).

Reporter Gene Constructs and Transient Transfections. mPrx1a and mPrx1a- Δ OAR expression plasmids were kind gifts of Dr. Michael J. Kern. Mouse collagen α 1(I) promoter sequence between -6000 and -3200 was amplified by PCR from mouse genomic DNA with primers containing the MluI and SmaI restriction sites and cloned into MluI and SmaI sites of SV40-pGL3 promoter vector (Promega, Madison, WI). The sequence between -3200 and -1 was amplified with primers having MluI and XhoI sites and cloned into the corresponding sites of pGL3 basic vector (Promega, Madison, WI). The reporter gene containing 3000 nt of mouse TGF β 3 promoter was identically constructed.

HEK293 cells were transiently transfected with 1 μ g of the reporter plasmid, 1 μ g of Prx1 expressing plasmid or pCDNA3 empty vector (as a control) and 0.2 μ g of β Gal expressing plasmid per 32 mm dish. As a control, the reporter genes were replaced with the pGL3 promoter and pGL3 basic vectors. HEK293 cells were grown in DMEM supplemented with 10% FBS. On the next day, cells were transfected with plasmids by TransIt reagent (Mirus Bio., Madison, WI). Forty-eight hours later, the cells were harvested, and luciferase and β -galactosidase assays were performed as described (32). The experiments were performed in three independent transfections, and fold induction by Prx1 over the empty vector was calculated after normalization to the expression of β Gal. Error bars represent ± 1 SD.

Table 1. Primers Used in RT-PCR Reactions^a

r-Prx1 (288 nt)	F: CTTCTCCGTCAGTCACCTGC R: CGTGCAAGATCTTCCCGTAC
r-Prx2 (164 nt)	F: CCACATTCAACAGCAGCCAG R: TTCGTTCCGGCGAACTTGG
r-collagen α 1(I) (150 nt)	F: TGAGCCAGCAGATTGAGAAC R: TGATGGCATCCAGGTTGCAG
r-collagen α 2(I) (241 nt)	F: CTCACCTCTGAAGGCTCTAG R: CTCTTAACCAGACATGCTTG
r-collagen α 1(III) (167 nt)	F: AGGCCAATGGCAATGTAAAG R: GGCCTTGCGTGTGTTGATATT
r-smooth muscle actin (359 nt)	F: ACAGAGAGAAGATGACGCAG R: GGAAGATGATGCAGCAGTAG
r-actin (201 nt)	F: CGTGCGTGACATTAAAGAGAAGC R: TGGATGCCACAGGATTCCATACC
h-Prx1 (116 nt)	F: CAATGACCCTGGAATGTAAAG R: GATAGTGTGTCCGCTCAAAG
h-Prx2 (139 nt)	F: GTGTTTCGAGCGCACGCACTA R: CCAGCATGGCCCTTTCATTC
h-collagen α 1(I) (150 nt)	F: TGAGCCAGCAGATCGAGAAC R: TGATGGCATCCAGGTTGCAG
h-actin (213 nt)	F: GTGCGTGACATTAAGGAGAAG R: GAAGGTAGTTTCGTGGATGCC
mPrx1 (164 nt)	F: GCGGAGAAACAGGACAACAT R: ACTTGGCTCTTCGGTTCTGA
m-collagen α 1(I) (188 nt)	F: AGAGGCGAAGGCAACAGTCG R: GCAGGGCCCAATGTCTAGTCC
m-collagen α 2(I) (120 nt)	F: CTTCTGCTGCTAGCAACATGC R: TCAACACCATCTCTGCCTCG
m-collagen α 1(III) (125 nt)	F: ACGTAAGCACTGGTGGACAG R: AGCTGCACATCAACGACATC
m-smooth muscle actin (195 nt)	F: GAGTAATGGTTGGAATGGGC R: TCTGTCAGCAGTGTCCGATG
m-desmin (212 nt)	F: GTGAAGATGGCCTTGGATGT R: TGTGTAGCCTCGCTGACAAAC
m-actin (201 nt)	F: CGTGCGTGACATCAAAGAGAAGC R: TGGATGCCACAGGATTCCATACC
GFP (187 nt)	F: ACGTAAACGGCCACAAGTTC R: AAGTCGTGCTGCTTCATGTG
r-TGF β 1 (182 nt)	F: TGCTTCAGCTCCACAGAGAA R: TGGTTGTAGAGGGCAAGGAC
r-TGF β 2 (234 nt)	F: GCAGGATAATTGCTGCCTTC R: AGGATGGTCAGTGGTTTCAG
r-TGF β 3 (185 nt)	F: CTCTCTGTCACTTGACCA R: GCATCTCTCCAGCAACTCC
m-TGF β 3 (162 nt)	F: GATGAGCACATAGCCAAGCA R: GTGACATGGACAGTGGATGC

^a F, forward primer; R, reverse primer. The size of the PCR product is shown in parentheses.

Results

In the previous work we compared gene expression between culture-activated HSCs and quiescent HSCs using DNA microarrays (33). Prx1 and Prx2 showed dramatic increase in expression in activated HSCs compared to quiescent HSCs: 26 fold and 194 fold for Prx1 and 45 fold and 84 fold for Prx2 in two independent microarray experiments (Fig. 1A). To confirm the upregulation of Prx1 gene in *in vivo* activated HSCs, HSCs were isolated from bile duct ligated and normal mouse livers. Figure 1B shows barely detectable expression in HSCs isolated from

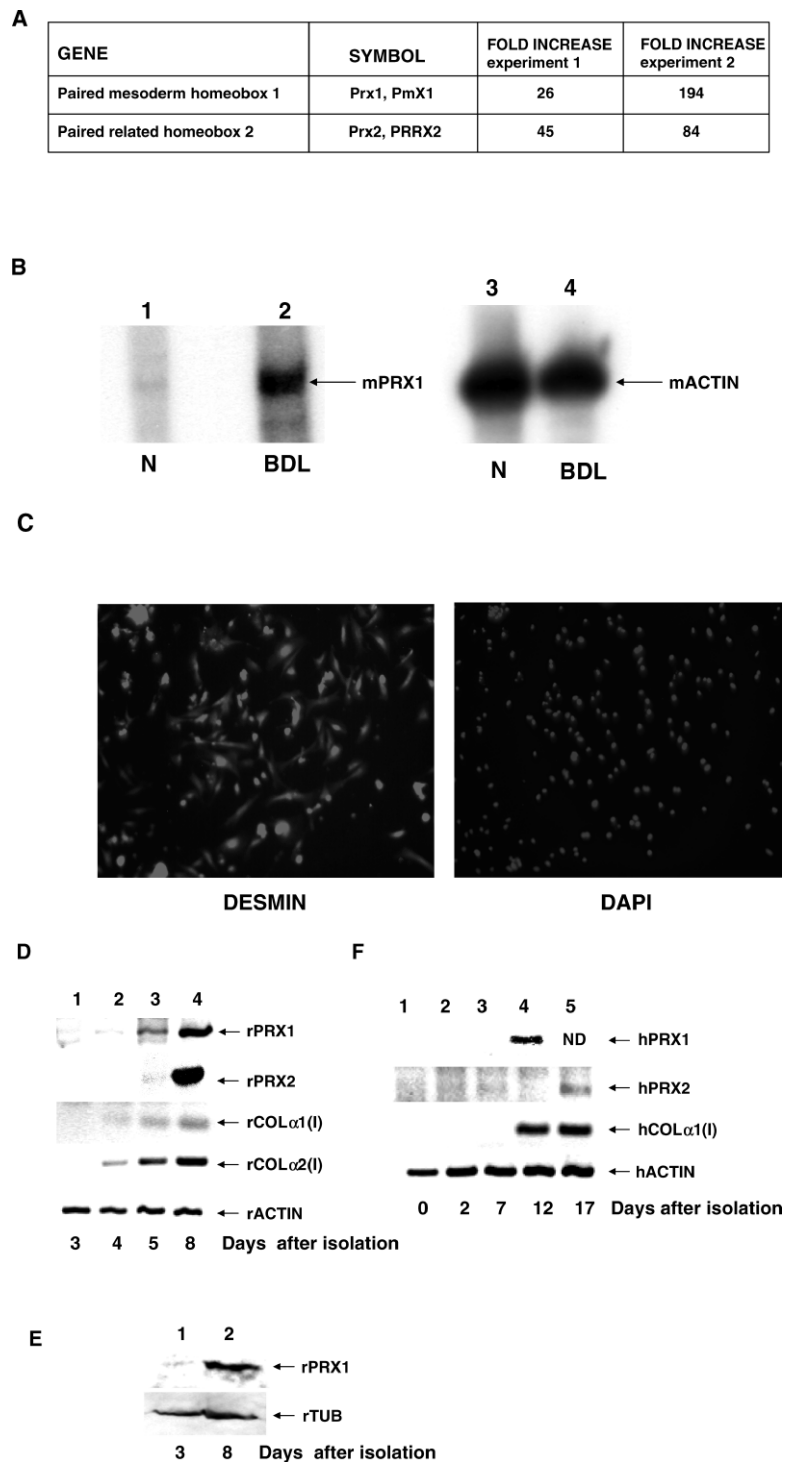


Figure 1. Expression of Prx1 and Prx2 in activation of HSCs. (A) Upregulation of Prx1 and Prx2 expression in culture activated rat HSCs, as assessed by DNA microarray analysis in two independent experiments. Fold increase in activated HSCs compared to quiescent HSCs is shown. The *P* values for activated vs. quiescent HSCs for each gene were <0.0001 (33). (B) Expression of Prx1 in *in vivo* activation of mouse HSCs. HSCs were isolated from three bile duct ligated mouse livers (BDL), and expression of Prx1 was compared to that in HSCs isolated from three normal livers (N) by RT-PCR. Migration of the specific PCR product is shown. Expression of actin is shown as a loading control (lanes 3 and 4). (C) Purity of isolated rat HSCs. Rat HSCs were cultured for 3 days after isolation. The cells were immunostained with anti-desmin antibody (left panel) and counterstained with DAPI (right panel). (D) Expression of Prx1 and Prx2 in culture activation of rat HSCs. Total RNA was extracted at the indicated time points after isolation and culturing of HSCs and analyzed by RT-PCR. Expression of collagen $\alpha 1(I)$ (rCOL $\alpha 1(I)$) and collagen $\alpha 2(I)$ (rCOL $\alpha 2(I)$) mRNAs was shown as a marker of activation of HSCs. β -actin was used as a loading control. (E) Prx1 protein expression in quiescent and activated rat HSCs. Fifty μ g of total protein from rat HSCs cultured for the indicated time period was analyzed by western blot with anti-Prx1 specific antibody (rPRX1) and with anti-tubulin antibody (rTUB) as a loading control. (F) Expression of Prx1 in isolated human HSCs from day 0 to day 12 in culture and Prx2 from day 0 to day 17 in culture. Total RNA was extracted at the indicated time points and analyzed by RT-PCR. Expression of collagen $\alpha 1(I)$ (hCOL $\alpha 1(I)$) mRNA was shown as a marker of activation of HSCs. β -actin was used as a loading control. ND, assay not done with this sample.

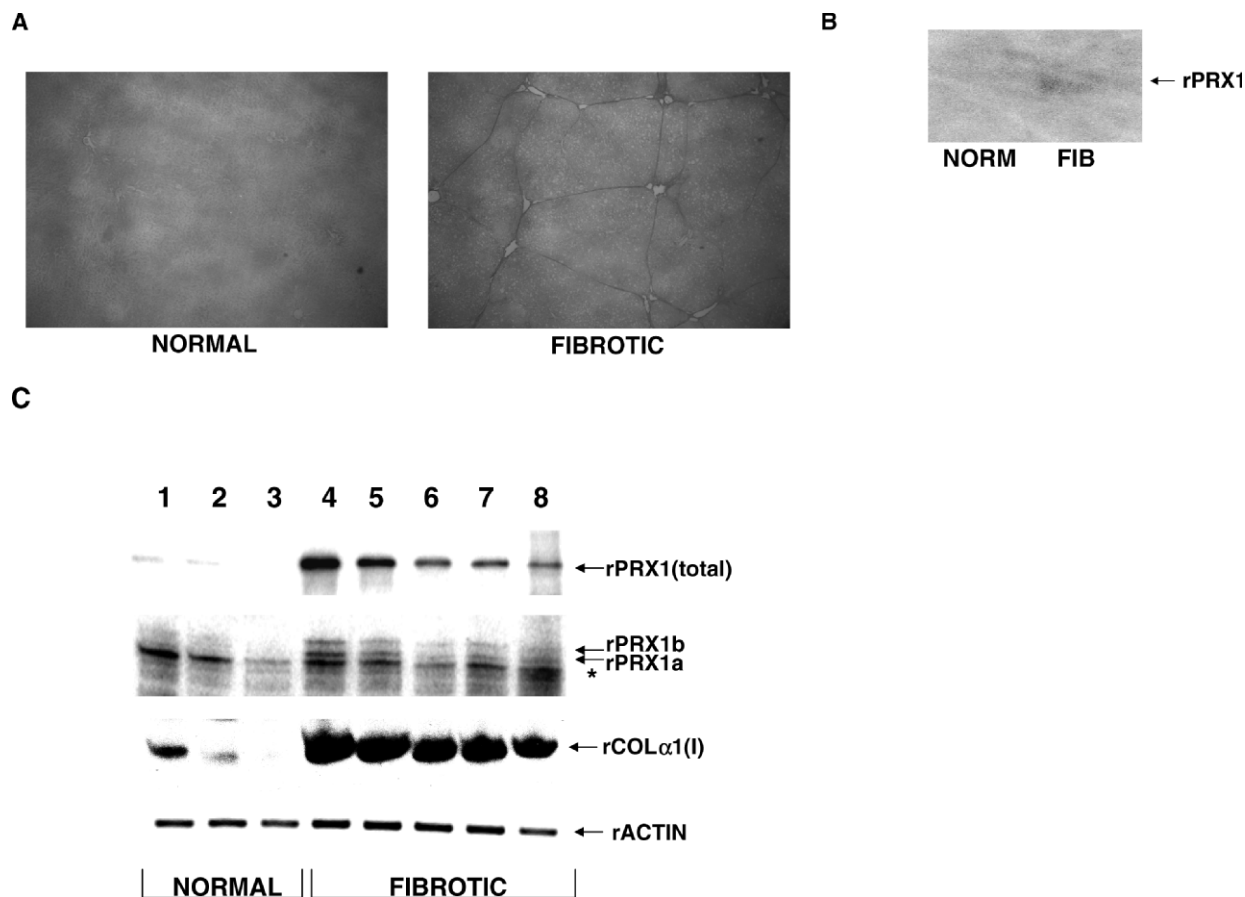


Figure 2. Expression of Prx1 in *in vivo* model of liver fibrosis. (A) Histology of liver fibrosis. Liver fibrosis was induced by chronic administration of CCl₄. Histology sections were stained with Sirius red, and representative fibrotic (FIBROTIC) and control (NORMAL) livers are shown. (B) Expression of Prx1 protein in the livers shown in A. Western blot was performed with 600 μ g of total liver proteins and probed with anti-Prx1 specific antibody (rPRX1). (C) Liver fibrosis was induced in five rats by chronic administration of CCl₄. Expression of total Prx1 and its alternatively spliced isoforms, Prx1a and Prx1b, was analyzed by RT-PCR using common Prx1 primers (rPRX1 total) and primers which can distinguish between Prx1a and Prx1b as two PCR products separated by 70 nt (rPrx1a arrow and rPrx1b arrow). Asterisk indicates a nonspecific PCR product seen using the isoform specific primers. Expression of collagen α 1(I) (rCOL α 1(I)) mRNA was shown as a marker of fibrosis, and β -actin was a loading control.

normal livers (lane 1) and much higher expression in HSCs isolated from fibrotic livers (lane 2). Expression of the loading control, actin, was equal in both samples (lanes 3 and 4).

Rat HSCs were used to assess the temporal profile of Prx1 expression during culture activation of HSCs. Figure 1C shows that isolated rat HSCs were >95% pure, as assessed by immunostaining for the HSC marker desmin. These cells were subjected to culture activation and analyzed at the time points indicated (Fig. 1D). There was no detectable Prx1 mRNA in rat HSCs at day 3 after isolation (Fig. 1D, lane 1), when the cells still had a quiescent phenotype. The expression of Prx1 was first detectable at day 4 (lane 2) and continued to increase from day 5 to day 8 (lanes 3 and 4). The expression pattern paralleled that of collagen α 1(I) mRNA and collagen α 2(I) mRNA. In rat HSCs Prx2 mRNA was induced later than Prx1 and was detectable only after 8 days of culturing (lane 4). In agreement with expression of its mRNA, Prx1 protein

showed high expression in activated HSCs (Fig. 1E, lane 2) and undetectable expression in quiescent HSCs (lane 1).

We also confirmed that Prx1 and Prx2 are upregulated in culture activation of human HSCs. No expression could be detected in quiescent human HSCs (Fig. 1F, lanes 1–3), and the first detectable expression was at day 12 after isolation (lane 4). This coincided with expression of collagen α 1(I) mRNA, expression of which was also delayed compared to culture activated rat HSCs (Fig. 1D). Expression of Prx2 mRNA was upregulated later than that of Prx1 and seen after 17 days of culturing (lane 5). Based on these results, we concluded that there is no expression of Prx1 or Prx2 mRNA in quiescent HSCs, whereas activated HSCs express both genes.

Next, we determined if Prx1 is upregulated in liver fibrosis induced by chronic CCl₄ administration (7). All CCl₄ treated rat livers showed bridging fibrosis, as determined by staining with Sirius red (the representative image is shown in Fig. 2A). Expression of Prx1 protein was detectable in the fibrotic liver, while it was absent in normal

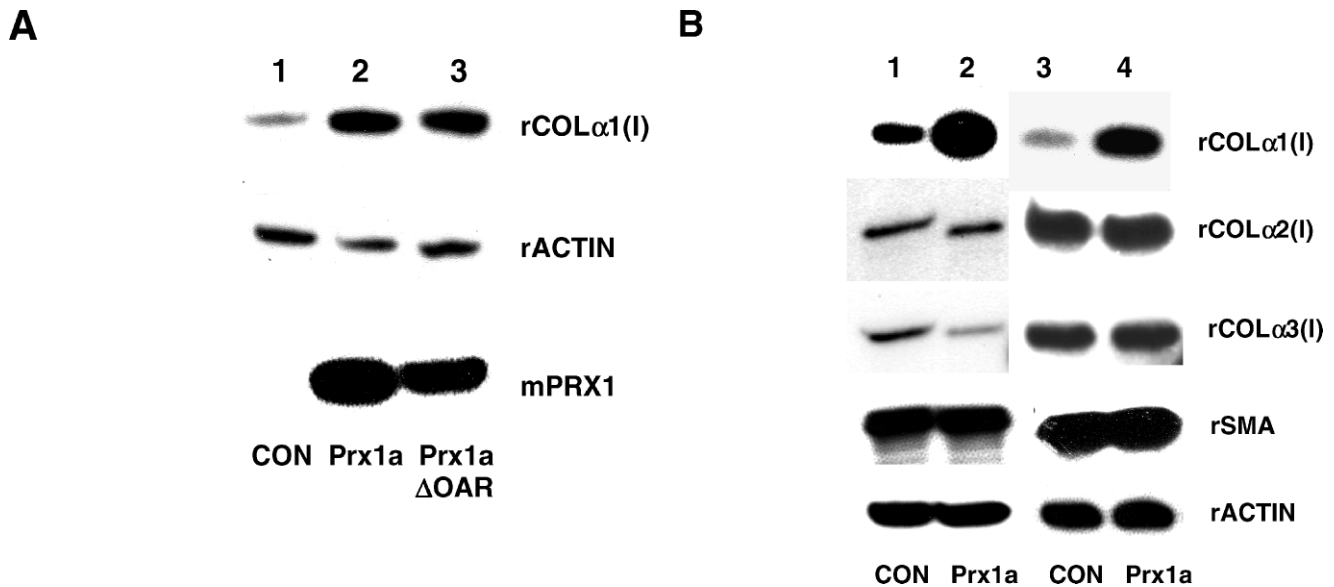


Figure 3. Effects of ectopic expression of Prx1a in quiescent HSCs on expression of collagen $\alpha 1$ (I) mRNA. (A) Effect of Prx1a on expression of collagen $\alpha 1$ (I) (rCOL $\alpha 1$ (I)). Control adenovirus (CON) and adenovirus expressing Prx1a or Prx1a- Δ OAR were added at MOI of 500 to isolated rat HSCs after 2 days in culture. After 3 additional days, mRNA level of endogenous collagen $\alpha 1$ (I) (rCOL $\alpha 1$ (I)) was assessed by RT-PCR. Actin is shown as a loading control (rACTIN). The expression of Prx1 was from the virus and, since the mouse Prx1 gene was delivered by the virus, it is labeled as mPrx1. It is included here as a marker of viral delivery. (B) Prx1 increases expression of collagen $\alpha 1$ (I) mRNA. Two additional independent experiments as in A are shown. Besides collagen $\alpha 1$ (I) (rCOL $\alpha 1$ (I)), expression of collagen $\alpha 2$ (I) (rCOL $\alpha 2$ (I)), collagen $\alpha 1$ (III) (rCOL $\alpha 1$ (III)) and α -smooth muscle actin (rSMA) mRNAs was analyzed by RT-PCR.

liver (Fig. 2B). To corroborate the finding that Prx1 expression is increased in liver fibrosis, five fibrotic livers were analyzed for expression of total Prx1 mRNA, as well as for expression of two alternatively spliced variants of Prx1 mRNA, Prx1a and Prx1b. All five fibrotic livers showed significant upregulation of total Prx1 mRNA (Fig. 2C, compare lanes 1–3 and 4–8). Both Prx1 isoforms were upregulated and contributed to the increase in the total level. In addition, the total level of Prx1 expression in five fibrotic livers correlated to the level of collagen $\alpha 1$ (I) expression.

To analyze the effects of Prx1 in HSCs, we overexpressed Prx1a in quiescent HSCs. A deletion mutant of Prx1a, Prx1a- Δ OAR, in which 29 amino acids from the C-terminus were deleted, showed greatly enhanced transcriptional activity in a previous study (47). Therefore, this mutant was also included in this analysis. Prx1a and Prx1a- Δ OAR were cloned into an adenovirus vector, while adenovirus expressing a noncoding mRNA was constructed as a control. The viruses expressed the genes using CMV promoter, resulting in a high level of Prx1 protein 1 day after viral transduction (not shown). The adenoviruses were used to transduce rat HSCs 2 days after isolation, when HSCs have no expression of endogenous Prx1 (Fig. 1), and the effects were analyzed at day 5 after isolation (3 days after the viral infection). Expression of collagen $\alpha 1$ (I) mRNA was stimulated several fold by overexpression of Prx1a, as compared to the control virus (Fig. 3A, lanes 1 and 2), and was comparable to that induced by Prx1a- Δ OAR (lane 3), suggesting that deletion of the C-terminus domain

of Prx1 has no effect in HSCs. In additional experiments, expression of other collagen mRNAs and α SMA mRNA was analyzed, as well (Fig. 3B). The level of collagen $\alpha 2$ (I) mRNA, collagen $\alpha 1$ (III) mRNA and α SMA mRNA was unchanged by overexpression of Prx1a, suggesting that collagen $\alpha 1$ (I) is a specific target gene of Prx1a *in vitro*.

To study *in vivo* effects of Prx1, adenovirus expressing Prx1a was injected into circulation of mice. Seven days after the viral injection the liver was harvested. Figure 4A shows that expression of collagen $\alpha 1$ (I) mRNA was significantly higher in the Prx1a expressing liver compared to a normal liver. The levels of collagen $\alpha 2$ (I) mRNA, collagen $\alpha 1$ (III) mRNA and α SMA mRNA were also upregulated (compare lane 2 to lane 1). Since in the liver all these mRNAs are expressed by HSCs, we analyzed expression of desmin, which is a specific marker for HSCs and equally expressed in quiescent and activated HSCs (33). The expression of desmin mRNA showed no significant change, suggesting that Prx1a probably did not change the number of HSCs; rather it promoted their activation.

To provide more evidence for the activity of Prx1 in the liver and exclude nonspecific viral effects, additional animals were injected with Prx1 and control adenoviruses. The livers were harvested 5 days after the adenovirus injection. To verify equal delivery of Prx1a and control viruses into the liver, PCR for the presence of adenoviral genome using GFP specific primers was performed. It showed a comparable signal in control and Prx1 adenovirus injected mice (Fig. 4B, bottom panel). Collagen $\alpha 1$ (I)

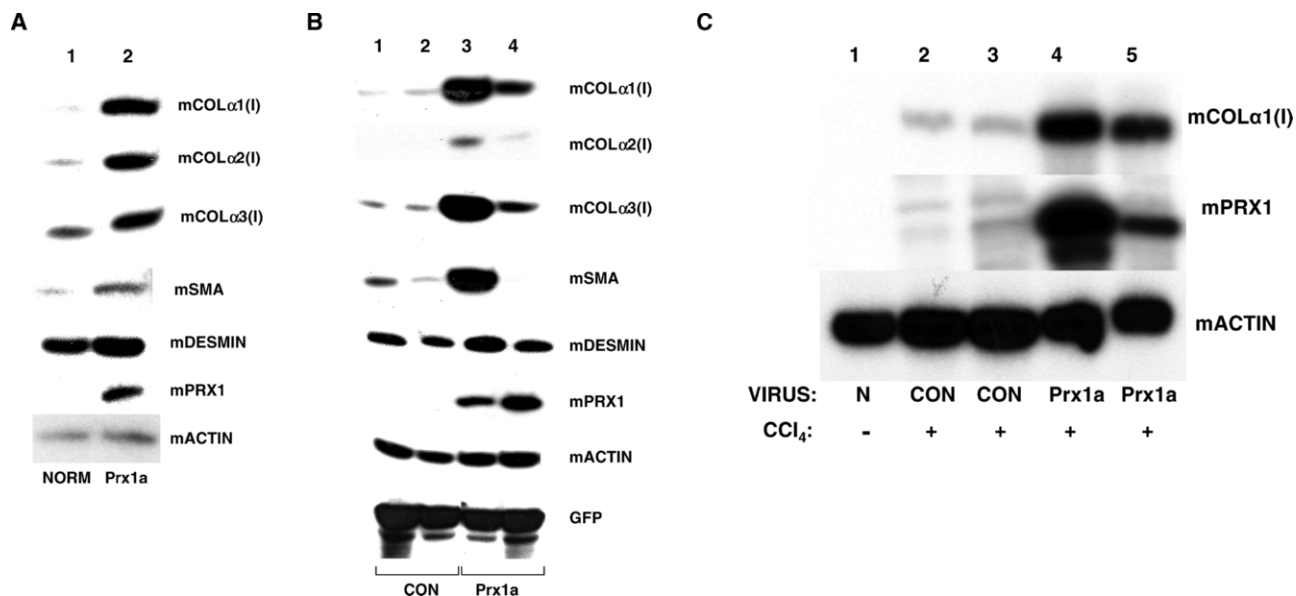


Figure 4. Effect of Prx1a on expression of collagen $\alpha 1(I)$ mRNA in mouse livers. (A) Comparison of gene expression between normal liver and Prx1a expressing liver. Prx1a adenovirus was injected at 10^{10} pfu/kg through the tail vein. Livers were harvested at day 7 after the adenovirus injections. Poly(A)⁺ RNA was extracted and analyzed by RT-PCR. Expression of collagen $\alpha 1(I)$ (mCOL $\alpha 1(I)$), collagen $\alpha 2(I)$ (mCOL $\alpha 2(I)$), collagen $\alpha 1(III)$ (mCOL $\alpha 1(III)$), smooth muscle actin (mSMA) and desmin (mDESMIN) are shown. Expression of β -actin served as an internal control, and expression of Prx1 is from the virus. (B) Comparison of gene expression between control virus and Prx1 virus injected livers. Two mice were injected with 10^{10} pfu/kg of control virus, and two mice were injected with Prx1 virus. Livers were harvested 5 days after the viral injections and analyzed as in A. Signal from viral genome marker GFP is shown as a control for equal delivery of control and Prx1 adenovirus. (C) Prx1 augments collagen expression after acute liver injury. Two mice were injected with Prx1a virus (Prx1a, lanes 4 and 5), and two mice were injected with control virus (CON, lanes 2 and 3). Twenty-four hours after viral injections, CCl₄ was injected IP, and the livers were harvested 24h after the CCl₄ injection. N is normal untreated liver without virus (lane 1). Expression of collagen $\alpha 1(I)$ mRNA (mCOL $\alpha 1(I)$) was analyzed by RT-PCR, and actin expression (mACTIN) is shown as a loading control. Prx1 expression (mPRX1) is from the endogenous gene in lanes 1–3 and from endogenous + adenovirus delivered gene in lanes 4 and 5. Note dramatic upregulation of collagen mRNA after CCl₄ treatment in livers overexpressing Prx1.

mRNA, collagen $\alpha 2(I)$ mRNA and collagen $\alpha 1(III)$ mRNA were significantly higher in the two Prx1a expressing livers, compared to the two livers injected with the control adenovirus (Fig. 4B, compare lanes 3 and 4 to lanes 1 and 2). The level of α SMA mRNA was upregulated in one of these livers, while the level of desmin mRNA showed no significant difference.

To assess if overexpression of Prx1 results in augmentation of collagen expression after liver injury, we injected the mice with Prx1 or control adenoviruses, and 24 h after the viral injections we induced acute liver injury by a single CCl₄ injection. Three days after the CCl₄ injection, expression of collagen $\alpha 1(I)$ mRNA was analyzed (Fig. 4C). Due to transient expression of the adenovirus delivered genes and immune response to the virus (34, 40), chronic liver injury models are not feasible. Figure 4C shows that Prx1 can augment collagen expression after an acute liver injury. Livers with high Prx1 expression prior to injury induced by CCl₄ showed a much higher increase in collagen $\alpha 1(I)$ mRNA level than control livers after the identical treatment (Fig. 4C, compare lanes 4 and 5 to lanes 2 and 3). Together, these data indicate that Prx1a can induce collagen expression *in vivo*.

TGF $\beta 1$, TGF $\beta 2$ and TGF $\beta 3$ are closely related members of the TGF β superfamily, which stimulate

activation of HSCs (24). We measured expression of TGF $\beta 1$, TGF $\beta 2$ and TGF $\beta 3$ mRNAs in quiescent and culture activated HSCs (Fig. 5A). Expression of TGF $\beta 1$ was constitutive, while TGF $\beta 2$ and TGF $\beta 3$ showed marked upregulation in activated HSCs. Therefore, we analyzed if Prx1 can stimulate TGF $\beta 1$, TGF $\beta 2$ and TGF $\beta 3$ expression *in vitro*. Figure 5B shows that TGF $\beta 3$ mRNA was significantly upregulated when Prx1a was overexpressed in quiescent HSCs, while no significant changes were detected in expression of TGF $\beta 1$ and TGF $\beta 2$.

To assess if Prx1 transactivates TGF $\beta 3$ promoter, we constructed a luciferase reporter gene driven by 3000 nt of the mouse TGF $\beta 3$ promoter (Fig. 6A). This reporter was cotransfected with the Prx1 expression plasmid or with the empty vector into HEK293 cells. Primary rat HSCs could not be used for this analysis, because they could not be transfected with the efficiency necessary for meaningful results. Figure 6B shows that Prx1 stimulated TGF $\beta 3$ -3-0-basic reporter 12 fold compared to the empty vector. Prx1 also stimulated the promoterless reporter (basic) 4 fold compared to the empty vector, suggesting that the specific, TGF $\beta 3$ promoter dependent effect was 3 fold.

A similar approach was made to assess the transactivation potential of Prx1 on mouse collagen $\alpha 1(I)$ promoter. Two reporter genes were initially constructed

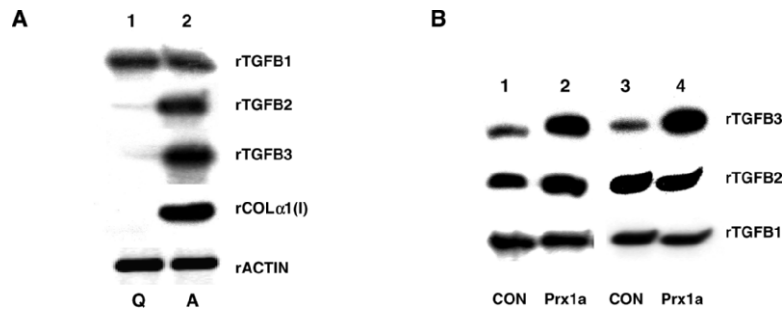


Figure 5. Effect of Prx1a on expression of TGFβ3 mRNA. (A) Expression of TGFβ1, TGFβ2 and TGFβ3 mRNA in quiescent and activated rat HSCs. Total RNA was extracted at day 2 (Q, lane 1) and day 8 (A, lane 2) of culture activated HSCs and analyzed by RT-PCR. Expression of collagen α1(I) (rCOLα1(I)) mRNA was shown as a marker of activation. β-actin served as a loading control. (B) Effect of Prx1a on expression of TGFβ1 (rTGFβ1), TGFβ2 (rTGFβ2) and TGFβ3 (rTGFβ3) mRNAs in rat HSCs. RT-PCR assay was performed with the samples as described in Figure 3B.

(Fig. 7A). A region of -6000 to -3200 of mouse collagen α1(I) promoter was cloned 5' of the SV40 driven luciferase reporter. Prx1 stimulated expression of this reporter 13 fold compared to the empty vector (Fig. 7B), while the reporter containing only the SV40 sequence was stimulated 4 fold, suggesting that the effect specific for the collagen sequence is about 3 fold. Therefore, we concluded that there is a weak far upstream Prx1 responsive element in the mouse collagen α1(I) gene.

Another reporter was constructed by ligating -3200 to -1 nucleotides of mouse collagen α1(I) promoter to the luciferase reading frame (Fig. 7A). This reporter was

stimulated more than 50 fold by Prx1. The control promoterless reporter in these experiments showed only a minimal response, suggesting a strong specific transactivation conferred by the collagen sequence (Fig. 7B). Because of this strong effect, we proceeded to map the Prx1 responsive element within these 3200 nt. Progressive deletions were made, and each deletion was tested for transactivation by Prx1 in three independent experiments. Figure 8 shows that three regions within the 3200 nt of the promoter have a significant effect on transactivation by Prx1. Deletion between nt -2400 and -1300 resulted in a reduction of the transactivation effect from 55 fold to 30 fold, suggesting that a weak positive Prx1 responsive element resides between these end points. When the promoter was deleted from -400 to -230 , the effect further decreased to 17 fold. When the promoter was truncated to only 30 nt, deleting the TATA box, Prx1 stimulation was only 4 fold. This was marginally higher than the effect on the promoterless reporter (see Figs. 6B and 7B), so we concluded that this construct has lost its ability to be transactivated by Prx1.

From the above analysis we concluded that the far upstream region of mouse collagen promoter contains one Prx1 responsive element and that the more proximal region contains three elements, one between -2400 and -1300 , one between -400 and -230 and one located within the first 230 nt of the promoter.

Discussion

Prx1 is involved in the formation of mesenchymal tissue during development (8, 38, 41, 44, 60). No data are available on expression or the effects of Prx1 in HSCs or liver. Our study is the first to demonstrate upregulation of Prx1 in fibrotic livers and in *in vivo* and *in vitro* activated HSCs. Expression of Prx2 was also increased in culture activated HSCs (Fig. 1D) and *in vivo* activated HSCs (13); however, its expression in fibrotic livers was too low to be detected. Ectopic expression of Prx1 in quiescent HSCs increased the steady-state level of collagen α1(I) mRNA, while in the normal liver it increased expression of three

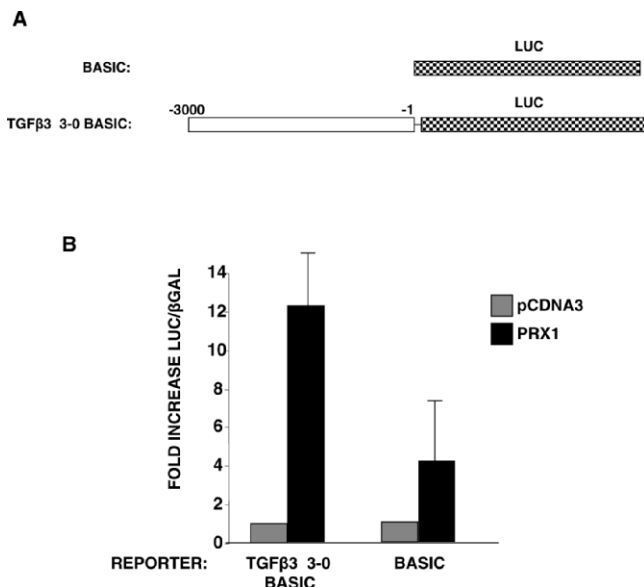


Figure 6. Transactivation of TGFβ3 promoter by Prx1. (A) Schematic representation of the reporter genes. Basic is a promoterless pGL3 vector (Promega). Three thousand nt of mouse TGFβ3 promoter were cloned into the pGL3 vector to create TGFβ3-3-0 basic reporter. (B) Transactivation of the reporters by Prx1. Prx1 expression plasmid or empty vector (pCDNA3) were cotransfected with the reporters into HEK293 cells. Fold increase in luciferase expression with Prx1 (black bars) over that with the empty vector (gray bars), which was set as 1, is shown after normalization to the expression of the internal control gene βGal. Error bars are from three independent experiments and represent ± 1 SD.

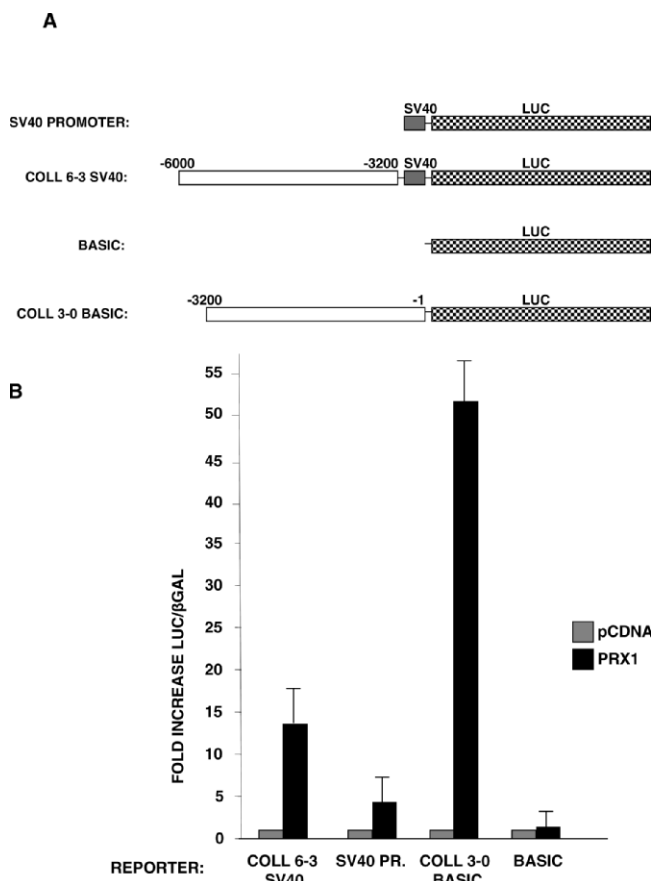


Figure 7. Transactivation of collagen $\alpha 1(I)$ promoter by Prx1. (A) Schematic representation of the reporter genes. SV40 promoter reporter is pGL3 promoter vector (Promega). The region between -6000 and -3200 nt of mouse collagen $\alpha 1(I)$ promoter was cloned into SV40 promoter reporter to create COLL 6-3 SV40 reporter. Basic is the promoterless pGL3 vector (Promega). Thirty-two hundred nt of mouse collagen $\alpha 1(I)$ promoter was cloned into the basic reporter to create COLL 3-0 basic reporter. (B) Transactivation of the reporters by Prx1. Transient transfections into HEK293 cells were done as in Figure 6B, and fold increase with Prx1 over the empty vector is shown. Error bars are from three independent experiments and represent $\pm 1SD$.

collagen mRNAs, $\alpha 1(I)$, $\alpha 2(I)$ and $\alpha 1(III)$ (Figs. 3 and 4). The more pronounced effects in the whole liver may be due to stimulation of the other liver cells or to a different response of HSCs in their natural environment. These collagens are produced in the liver only by activated HSCs and periportal fibroblasts (36), so their upregulation must reflect the effects of Prx1 in these cells. Upregulation of the specific marker of HSC activation, αSMA (which is not expressed in fibroblasts) (36), was seen in two out of three livers, while overexpression of Prx1a did not change expression of desmin (Fig. 4). Desmin is expressed to a similar level in quiescent and activated HSCs (33), indicating that the number of HSCs had remained unchanged. This suggests that Prx1 may have the potential to drive differentiation of HSCs into the activated phenotype, rather than to promote their proliferation. Collagen mRNA expression in the liver can be induced by

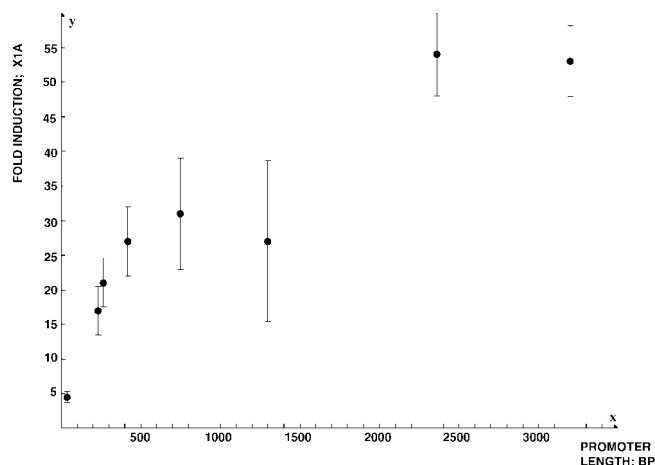


Figure 8. Several regions of collagen $\alpha 1(I)$ promoter are needed for transactivation by Prx1. Starting with COLL 3-0 basic reporter, progressive deletions of the promoter were made and tested for transactivation by Prx1. Fold increase with Prx1 over the empty vector was plotted versus promoter length. Error bars are from three independent experiments and represent $\pm 1SD$.

a single CCl_4 injection; however, it is greatly augmented if Prx1 is expressed at a high level in the liver (Fig. 4C). These effects are consistent with the previously described role of Prx1 in the regulation of differentiation of mesenchymal cells (22, 33). Expression of genes delivered by adenovirus is transient, because an immune response to adenovirus develops after several days (34, 40). This precludes the use of this system to study the effects of Prx1 in chronic liver injury and fibrosis. Prx1 knock-out mice are not viable, so to study the effect of Prx1 on development of liver fibrosis, liver specific gain or loss of function mice have to be developed.

Regulation of collagen type I expression in HSCs involves transcriptional and posttranscriptional mechanisms (57-59). For transcriptional activation of collagen $\alpha 1(I)$ promoter by Prx1, multiple sequence elements are required (Figs. 7 and 8). They are dispersed within distal and proximal regions of the promoter, spanning 6000 nt of the promoter. Each of these elements individually contributes relatively little to the transactivation, but their cumulative effect is considerable. The first 230 nt of the collagen $\alpha 1(I)$ promoter bind Sp1 and NF1 transcription factors, which are necessary for transcription (46, 51, 52). It is possible that some effects of Prx1 are due to interactions with these factors at the proximal promoter.

Our study also found that the TGF $\beta 3$ gene can be stimulated by Prx1 in HSCs (Figs. 5 and 6). Among the three members of TGF β family, TGF $\beta 3$ was the only one upregulated by Prx1a. Since TGF $\beta 2$ and TGF $\beta 3$ are upregulated in activation of HSCs (Fig. 5), it is possible that Prx1 is responsible for activation of the TGF $\beta 3$ gene. TGF $\beta 3$ was found to be mainly expressed by HSCs in the fibrotic area in chronic rejection of human liver allografts, while TGF $\beta 1$ was mainly expressed in macrophages (14).

In conclusion, our study demonstrates the role of Prx1a

in HSCs and liver as a transcriptional regulator, which promotes expression of fibrillar collagen genes and the TGF β 3 gene. Such activity may contribute to development of liver fibrosis. The expression and activity of Prx1 in activation of HSCs is a novel finding in the biology of these cells. Elucidation of the all target genes of Prx1 in HSCs and the mechanism of its transcriptional regulation of collagen α 1(I) promoter is an important future goal.

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