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RNA binding protein RBMS3 is expressed in activated hepatic stellate cells and liver fibrosis and increases expression of transcription factor Prx1

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SUMMARY

Hepatic stellate cells (HSCs) are mesenchymal cells of the liver, activation of which is responsible for excessive synthesis of extracellular matrix, including type I collagen, and development of liver fibrosis. Activation of HSCs is driven by transcription factors and pair-related homeobox transcription factor Prx1 was identified as one of the transcription factors involved in this process, because transcription of collagen $\alpha 1(I)$ gene is stimulated by Prx1 in HSCs and in the liver. Here we show that expression of the RNA binding protein RBMS3 is upregulated in activation of HSCs and fibrotic livers. Immunoprecipitation followed by differential display identified Prx1 mRNA as one of the mRNAs interacting with RBMS3. The RBMS3 sequence specific binding site was mapped to 60 nt located 1946 nt 3' of the stop codon of Prx1 mRNA. Ectopic expression of RBMS3 in quiescent HSCs, which express tracing amount of type I collagen, increased expression of Prx1 mRNA and collagen $\alpha 1(I)$ mRNA. Expression of reporter Prx1 mRNA containing the RBMS3 binding site was higher than the mRNA lacking this site. Overexpression of RBMS3 further increased the steady state level of the reporter mRNA containing RBMS3 binding site, but had no effect on the mRNA lacking this site. Binding of RBMS3 to the Prx1 3' UTR increased the half-life of this mRNA, resulting in increased protein synthesis. These results suggest that RBMS3, by binding Prx1 mRNA in a sequence specific manner, controls Prx1 expression and indirectly collagen synthesis. This is the first description of the function of RBMS3, as a key regulator of profibrotic potential of HSCs, representing a novel mechanism by which activated HSCs contribute to liver fibrosis.

Keywords

RNA binding protein; collagen expression; liver fibrosis; gene expression; mRNA stabilization

INTRODUCTION

Liver fibrosis is characterized by excessive synthesis of type I collagen by hepatic stellate cells (HSCs) ^{1; 2; 3}. HSCs are cells of mesenchymal origin in the liver, which reside in the space of Disse of liver sinusoids and store vitamin A. In their normal, quiescent state, HSCs synthesize trace amounts of type I collagen. When activated by a profibrotic stimulus, HSCs lose vitamin A droplets and differentiate into myofibroblasts ^{2; 4; 5; 6}. The most common

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profibrotic stimuli are infections with hepatitis B or C viruses, alcohol abuse and nonalcoholic steatohepatitis (NASH) ⁷. Activation and differentiation of HSCs is accompanied by changes in expression of thousand of genes ^{6; 8}. Collagen type I is a heterotrimeric protein composed of two $\alpha 1(I)$ and one $\alpha 2(I)$ polypeptides ⁹, and the genes encoding these polypeptides are among the most highly upregulated in HSCs activation, increasing expression 50–100 fold ^{1; 10; 11; 12; 13}. Activation of HSCs can be reproduced *in vitro*, when cells isolated from a normal liver are cultured on plastic ^{11; 13; 14}. Although this culture activation is not identical to activation of HSCs in fibrotic liver, the expression of many genes, including collagens, follows the pattern seen *in vivo* ^{6; 8}.

Two homeobox transcriptional factors, Prx1 (also known as PRRX1, mhox, k2, Pmx, rHOX) and Prx2 (also known as PRRX2 and S8) are upregulated 10–50 fold in fibrotic livers and in culture activated HSCs ⁸. Prx1 transactivates the collagen $\alpha 1(I)$ promoter and its ectopic expression in quiescent HSCs increased collagen $\alpha 1(I)$ gene expression ¹⁵. Overexpression of Prx1 in the normal liver resulted in upregulation of expression of three collagen genes, $\alpha 1(I)$, $\alpha 2(I)$ and $\alpha 1(III)$, the latter encodes for another fibrillar collagen, type III ¹⁵. Therefore, Prx1 is one of the factors promoting fibrogenic transformation of HSCs. Regulation of Prx1 expression has been studied in transgenic mice and it was shown that 2.4 kb of the mouse Prx1 promoter directed expression of the LacZ reporter gene in limb buds and in a subset of craniofacial mesenchyme. A 530-bp core element was identified, which contains a Prx1 recognition element. However, binding of Prx1 to this element and whether Prx1 can regulate its own expression has not been demonstrated and no other regulatory factors have been identified ¹⁶.

Posttranscriptional regulation of gene expression often involves stabilization of mRNAs. Increased half-life of an mRNA is usually achieved by binding of sequence specific RNA binding proteins to the 3' UTR ^{17; 18}. In activated HSCs, stabilization of collagen $\alpha 1(I)$ mRNA is facilitated by binding of αCP to the 3' UTR and 5' stem-loop binding proteins to the 5' UTR, dramatically contributing to the increased expression in HSCs ^{13; 19; 20; 21; 22; 23; 24}. Posttranscriptional regulation of Prx1 expression has not been studied. Prx1 mRNA has an unusually long 3' UTR of 3300 nt, but it does not contain any of the previously recognized binding motifs for RNA binding proteins.

RBMS3 was initially cloned by screening of a fibroblast expression library with the labeled DNA fragment derived from the promoter of mouse collagen $\alpha 2(I)$ gene ²⁵. The protein lacked transcriptional activity, however, it has two RNA binding domains and is localized predominantly in the cytoplasm. It also bound poly-U, suggesting that it is an RNA binding protein ²⁵. RBMS3 belongs to the family of c-myc gene single-strand binding proteins (MSSPs), which has two additional members, RBMS1 and RBMS2 ^{26; 27; 28}. RBMS1 is involved in binding of c-Myc protein ²⁸, but no such function has been described for RBMS3.

In this article we show that RBMS3 is highly upregulated in *in vivo* and *in vitro* activation of HSCs, and that it binds a 60 nt sequence element in the 3' UTR of Prx1 mRNA. Overexpression of RBMS3 increases Prx1 expression in HSCs and stabilizes reporter mRNA containing the 60 nt element. Since Prx1 upregulates collagen expression, we hypothesize that stabilization of Prx1 mRNA by RBMS3 is part of the mechanism by which HSCs initiate liver fibrosis.

RESULTS

RBMS3 is expressed at high levels in activated HSCs and fibrotic livers.

To identify genes involved in activation of HSCs we performed two microarray experiments comparing gene expression between quiescent and culture activated rat HSCs ⁸. RBMS3 was identified in both microarray experiments as a gene which was upregulated in activated HSCs

18 fold and 10 fold, respectively. To verify the microarray results we assessed expression of RBMS3 by RT-PCR in quiescent and activated rat HSCs, as well as in normal and fibrotic livers (Fig 1). Quiescent HSCs were HSCs cultured for two days after isolation. This allowed selection of viable cells with the phenotype which was still that of quiescent HSCs, as judged by undetectable expression of collagen $\alpha 1(I)$ and α -smooth muscle actin (α SMA) mRNAs, both markers of HSCs activation (not shown). Cell purity was >95% as judged by desmin staining (not shown)⁸. Upon culturing on plastic, HSCs spontaneously activate into myofibroblasts within eight days^{11; 14}. Our activated HSCs were cells cultured for eight days after isolation, when collagen $\alpha 1(I)$ and α -SMA mRNAs are expressed at their maximal levels. Fig 1A shows that RBMS3 mRNA was barely detectable in quiescent HSCs (lane 1), while its expression was high in activated HSCs (lane 2). Expression of neurotrimin⁸ and actin was unchanged, suggesting equal quality of the RNA preparations. We also analyzed RBMS3 mRNA at various time points during culture activation of HSCs to assess the temporal profile of RBMS3 expression (Fig 1B). There was a steady increase in RBMS3 expression, starting from day 3 and increasing to day 8. Such temporal expression is similar to that of Prx1 and collagen $\alpha 1(I)$ mRNAs¹⁵ (not shown).

To assess if RBMS3 is upregulated in *in vivo* model of liver fibrosis, five rats were treated with CCl₄ for four weeks¹⁰. This caused bridging fibrosis and increased expression of collagen $\alpha 1(I)$ and Prx1 mRNAs¹⁵. Expression of RBMS3 was compared between these five fibrotic livers and two normal livers. Fig 1C shows that all five fibrotic livers showed increased RBMS3 mRNA expression, with some variability between the animals. Expression in normal livers was low, consistent with low expression in quiescent HSCs. From these experiments we concluded that RBMS3 is upregulated in activated HSCs and in liver fibrosis.

RBMS3 binds 3' UTR of Prx1 mRNA.

Since RBMS3 is an RNA binding protein we wanted to find out which target mRNAs it binds. To this goal, immunoprecipitation was performed using cytosolic extract of human fibroblasts transduced with adenovirus expressing HA-tagged RBMS3. The immunoprecipitated mRNAs were analyzed by differential display after reverse transcription and PCR amplification using degenerate primers^{29; 30}. One mRNA, which was differentially retained by RBMS3, was identified as Prx1 (not shown). To verify if RBMS3 binds Prx1 mRNA, we derived a set of RNA probes from the 3' UTR of mouse Prx1 mRNA and performed gel mobility shift using cytosolic extracts of cells expressing HA-tagged RBMS3 and cytosolic extracts of control cells. Since in these initial experiments the RNA probes were long, RNA/protein complexes were digested with RNase T1 prior to loading on the gel¹³. Fig 2A shows a diagram of the probes used. In Fig 2B we used the 566 nt probe and observed strong RNA/protein complex, which was resistant to RNase T1 digestion. The complex was formed only in cells expressing RBMS3 protein (lane 2). All the other probes derived from the Prx1 3' UTR were negative in similar experiments, therefore, we concluded that this 566 nt fragment contains the binding site for RBMS3 protein. Fig 2C shows a gel mobility shift assay with the 240 nt probe. With these shorter probes RNase T1 digestion was omitted. Again, we observed an RNA/protein complex in RBMS3 containing extract (lane 2), which was retained in the slot of this gel. To verify if this complex contains RBMS3 we blotted the gel on nitrocellulose and probed the membrane with anti-HA antibody. RBMS3 protein was found colocalized with the RNA/protein complex (lane 5).

In subsequent experiments, to further narrow down the binding site and determine if RBMS3 alone is sufficient for binding, we used purified recombinant protein. Fig 3A shows the purity of the recombinant protein. Fig 3B shows a gel mobility shift using recombinant RBMS3 protein and the same probe as in Fig 2C. RNA/RBMS3 complex was formed and retained in the slot of the gel (lane 2). No such binding was seen with GST alone (lane 3). Also, no binding

of the recombinant RBMS3 protein was seen with probes derived from GAPDH mRNA (lanes 4–6) and from collagen $\alpha 1(I)$ mRNA (lanes 7–9). From these experiments we concluded that RBMS3 alone is able to bind Prx1 probe and can discriminate between Prx1 and other RNA sequences. Next, we designed two probes, one spanning the 5' part of 240 nt probe (150 A probe) and the other spanning the 3' part (150 B probe). These two probes overlapped by 60 nt. Recombinant RBMS3 bound each of the 150 nt probes. (Fig 3C, lanes 2 and 5). Two RNA/RBMS3 complexes were seen, one in the slot and one as a discrete band. These complexes may have formed due to a different number of RBMS3 subunits assembled on these shorter probes. With longer probes only the complex in the slot was seen (Fig 2 and Fig 3B).

Since both 150 nt probes bound RBMS3 and had 60 nt in common, we made the probe containing only these 60 nt (Fig 3D). This probe spontaneously formed a dimer (Fig 3D, lane 1). However, when combined with recombinant RBMS3, a discrete RNA/protein complex was formed (lane 2). No complex in the slot of the gel was found, probably because this short probe does not allow assembly of very many subunits of RBMS3. Addition of 50 fold and 250 fold molar excess of a nonspecific competitor RNA had little effect on the complex formation (lanes 3 and 4). Addition of 10 μ g of total liver RNA, as competitor, reduced the complex formation (lane 5), suggesting that there are some mRNAs in the liver which can compete for binding to RBMS3. Since normal liver does not express Prx1, these competing mRNAs must be derived from other genes. Addition of 50 fold molar excess of the specific competitor RNA completely abolished the complex formation (lane 6). To further corroborate the existence of additional mRNAs in the liver, which may compete for binding of RBMS3 to Prx1 sequence, we repeated the competition experiment using poly-A⁺ RNA isolated from mouse liver (Fig 3E). 10 μ g of total RNA contain 0.5 μ g of poly-A⁺ mRNA, assuming that poly-A⁺ RNA represents 5% of total RNA. There was a little competition of RBMS3 binding when either 10 μ g of total RNA and 0.5 μ g of poly-A⁺ RNA was used (Fig 3E, compare lanes 2, 3 and 4). However with 2.5 μ g of poly-A⁺ there was a significant competition (lane 5), suggesting that there are other mRNAs which may be the targets of RBMS3. From these experiments we concluded that RBMS3 binds 60 nt within the 3' UTR of Prx1 mRNA in a sequence specific manner. These 60 nt are located 1946 nt downstream of the stop codon.

Sequence comparison of the RBMS3 binding site in Prx1 mRNAs of various species showed about 90% conservation (Fig 3F). The rest of the 3' UTR showed only 20–30% identity, suggesting an evolutionary conserved function. No other mRNAs were found that contain a similar sequence.

Ectopic expression of RBMS3 in HSCs stimulate expression of Prx1 and collagen $\alpha 1(I)$.

Quiescent HSCs express only a low level of endogenous RBMS3 (Fig 1), thus, it is possible to perform gain of function experiments. To ectopically express RBMS3 in HSCs we constructed an adenovirus which expresses HA-tagged RBMS3. HSCs can not be transfected and adenoviral delivery assures that almost 100% of quiescent HSCs will express the gene ³¹. We transduced HSCs at day 2 after isolation and collected the cells at day 5, before they had reached full activation. By this method we could study if the premature expression of RBMS3 would stimulate Prx1 expression. As a control, we used an adenovirus which expressed a noncoding mRNA. Fig 4 shows that in two independent experiments ectopic expression of RBMS3 increased the steady-state level of Prx1 mRNA (lanes 2 and 3), compared to the control virus (lane 1). This effect seems to be specific for Prx1, because actin expression was not affected. Since Prx1 stimulates collagen transcription ¹⁵, we also tested if RBMS3 mediated Prx1 upregulation is associated with an increased level of collagen $\alpha 1(I)$ mRNA. In both experiments there was an increase in collagen $\alpha 1(I)$ mRNA level (lanes 2 and 3).

RBMS3 increases expression of the Prx1 reporter gene containing RBMS3 binding site.

To provide further evidence for the functional significance of RBMS3 binding to the Prx1 3' UTR we constructed two reporter genes (Fig 5A). Both reporter genes were driven by the CMV promoter and had the Prx1 open reading frame with an HA tag added at the N-terminus. The genes also had the 3' UTR of mouse Prx1 mRNA following the reading frame. The 5' UTR was derived from the vector. The WT gene had the 60 nt of the RBMS3 binding site intact, while in the mutant gene (MUT) these nt were deleted. The reporter genes were cotransfected into HEK293 cells with either the RBMS3 expression plasmid or a control expression plasmid and with the β -galactosidase gene, as an internal control. HSCs can not be transfected with an efficiency that would permit meaningful results²⁴. Expression of β -galactosidase verified similar transfection efficiency of all plasmids, which was within 20% between samples. The expression of HA-tagged Prx1 protein was measured by western blot in two independent experiments (Fig 5B). Expression of RBMS3 is shown in the upper panel of these blots, since it was detected by the same antibody. Expression of the control protein is not shown because it had no tag, however, similar transfection efficiencies were verified by measuring β -galactosidase. Expression of the Prx1 reporter protein revealed two facts. First, the WT reporter yielded more protein than the MUT reporter in the absence of cotransfected RBMS3 (compare lanes 2 and 4 and lanes 6 and 8). Second, overexpression of RBMS3 further increased Prx1 protein expression from the WT reporter compared to the control (compare lanes 1 and 2 and lanes 5 and 6). RBMS3 had no effect on expression of the MUT reporter, which was the same result as that with control protein (lanes 3 and 4 and lanes 7 and 8). From these experiments we concluded that inclusion of the RBMS3 binding site increases Prx1 protein expression in HEK293 cells, even in the absence of exogenous RBMS3. This effect may be due to binding of endogenous RBMS3. Overexpression of RBMS3 further stimulated Prx1 expression, with presence of its cognate binding site in Prx1 mRNA being necessary and sufficient for this stimulation.

RBMS3 increases steady state level of Prx1 mRNA.

To determine if RBMS3 stimulated Prx1 protein expression was due to increased steady state level of the mRNA or to increased translation, we estimated the steady state level of the WT and MUT reporter mRNAs. The RNA loading in these experiments was adjusted to the expression of the internal control gene β -galactosidase, which routinely was within 20%. Normalization to the endogenous mRNA would not be appropriate since the test mRNAs are coming from the transfected genes, however, the RNA preparations were treated with DNaseI to eliminate contaminating plasmids. As shown in Fig 6A, the expression of the reporter mRNAs very closely paralleled that of the protein (Fig 5B). In the absence of transfected RBMS3, WT reporter mRNA was present at higher levels than the MUT reporter mRNA (lanes 3 and 5), and overexpression of RBMS3 increased only the level of WT reporter mRNA (compare lanes 2 and 3 to lanes 4 and 5). Therefore, we concluded that binding of RBMS3 to its cognate binding site in Prx1 mRNA increases its steady state level, which in turn results in increased protein synthesis.

RBMS3 increases the half life of Prx1 mRNA.

Since RBMS3 increased the steady state level of WT Prx1 reporter mRNA, we wanted to investigate if this is due to stabilization of the mRNA. To this goal we cotransfected the reporter genes described in Fig 5A with the RBMS3 expression plasmid and treated the cells with transcriptional inhibitor actinomycin D for the time points indicated in Fig 6B. The level of the remaining mRNA was then estimated by RT-PCR and compared to the initial level at time 0. Since at time 0 MUT reporter mRNA was present at a lower level than WT reporter mRNA (Fig 5A), we adjusted the amount of RNA in the analysis to give a similar signal for WT and MUT mRNA at time 0 in order to better compare the decay rates. We then used these amounts

in the subsequent time points. Fig 6B clearly shows that WT reporter mRNA was more stable than MUT mRNA, with the apparent half life between 8–12h, compared to the half life of MUT mRNA, which was less than 2h. From this result we concluded that RBMS3 stabilizes Prx1 mRNA, which leads to its increased steady state level and enhanced protein synthesis.

DISCUSSION

Excessive synthesis of type I collagen by activated HSCs causes liver fibrosis^{3; 32}. Molecular mechanisms of activation of HSCs are poorly understood and their elucidation is necessary to devise antifibrotic therapy. We have described that transcription factor Prx1 transactivates the collagen $\alpha 1(I)$ promoter and increases the level of collagen $\alpha 1(I)$ mRNA in HSCs¹⁵. Expression of Prx1 is upregulated in activated HSCs, but how its expression is controlled in the liver is unknown. We have identified several RNA binding proteins whose expression is increased in activated HSCs⁸. Here we describe the posttranscriptional regulation of Prx1 expression by the RNA binding protein RBMS3. We showed that: 1. Expression of RBMS3 increases upon activation of HSCs with a similar temporal profile as that of Prx1 and type I collagen. RBMS3 expression is also increased in fibrotic livers. 2. Expression of RBMS3 in quiescent HSCs increases expression of Prx1 mRNA and collagen $\alpha 1(I)$ mRNA. 3. RBMS3 binds the 60 nt element in the 3' UTR of Prx1 mRNA in a sequence specific manner. This element is evolutionary conserved in Prx1 mRNAs of vertebrates, but is not found in other mRNAs. 4. Binding of RBMS3 increases expression of Prx1 protein by stabilizing its mRNA. Based on these results we hypothesize the following mechanism; expression of RBMS3 is upregulated in HSCs and this results in stabilization of the mRNA encoding the transcription factor Prx1. Increased expression of Prx1 stimulates transcription of the collagen $\alpha 1(I)$ gene. This, together with the posttranscriptional regulation of collagen $\alpha 1(I)$ expression in HSCs, leads to liver fibrosis. In culture activation of HSCs these events are probably concomitant, since we were unable to clearly show that upregulation of RBMS3 precedes upregulation of Prx1.

RBMS3 was first described as a DNA binding protein which bound the promoter sequence of collagen $\alpha 2(I)$ gene *in vitro*²⁵. The protein belongs to a family of c-myc gene single-strand binding proteins (MSSPs) which has three members. MSSPs are believed to regulate DNA replication, transcription, apoptosis and cell cycle progression by interacting with the C-MYC protein^{26; 27; 28}. However, RBMS3 has two pairs of RNA binding motives and is cytoplasmic²⁵, suggesting that it is involved in the metabolism of mRNAs. The protein was reported to be ubiquitously expressed²⁵, however, its expression in normal livers and quiescent HSCs is very low. How the expression of RBMS3 is upregulated upon the activation of HSCs remains to be elucidated.

This is the first report of a target mRNA which RBMS3 binds. A 60 nt sequence within the 3300 nt 3' UTR of Prx1 mRNA was able to bind recombinant RBMS3 protein in a sequence specific manner. In this work we did not precisely map the critical nt, however, the whole element is evolutionary conserved, suggesting an important function. A highly homologous sequence to this element is not found in any other gene. This does not exclude that RBMS3 regulates other mRNAs, but we were unable to identify other mRNAs using affinity selection and differential display. A weak competition of total liver RNA for binding of recombinant RBMS3 to the 60 nt Prx1 target site suggests that there are some other mRNAs in the liver which may sequester RBMS3. The RBMS3 binding element does not resemble any other motif that RNA binding proteins recognize^{33; 34; 35; 36; 37}, other than it is relatively AT rich, nor does it have an extensive secondary structure.

Binding of proteins in cytosolic extracts to the larger probes encompassing the RBMS3 binding site often resulted in a large RNA/protein complex which was retained in the slot of the gels

(Figs 2B and 2C). Even pure recombinant protein yielded these large complexes when 240 nt and 150 nt probes were used (Figs 3a and 3B), however, this was not seen with the 60 nt probe. While in the whole extract other RNA binding or RBMS3 interacting proteins may be involved, and formation of large complexes with purified RBMS3 suggests that RBMS3 may form multimers. The binding of RBMS3 multimers to the RNA may become more stable if additional sequences around the core binding site are present. Other RNA proteins have been described which bind RNA as homo-multimers^{38; 39; 40}. The sequence specific binding was clearly demonstrated to the core 60 nt sequence (Fig 3D), which may nucleate formation of these complexes. When the RBMS3 binding site was included in the reporter gene, the steady state level of the mRNA and protein was increased and was further augmented by overexpression of RBMS3 (Fig 5). The increased expression was a consequence of stabilization of the mRNA. Since our reporter mRNAs closely resembled that of endogenous Prx1 mRNA, we surmise that RBMS3 mediated stabilization of Prx1 mRNA operates in activation of HSCs. Although we did not measure stability of Prx1 mRNA in HSCs, ectopic expression of RBMS3 in quiescent HSCs resulted in an increase in Prx1 mRNA steady state level (Fig 4). How RBMS3 may stabilize Prx1 mRNA is not known at present. Most RNA binding proteins interact either with poly-A binding protein or cap binding proteins to stabilize the circular form of the RNA^{17; 18; 41}.

In summary, we have identified RBMS3 as an RNA binding protein which interacts with the conserved element in the 3' UTR of Prx1 mRNA. This results in stabilization of the mRNA and increased protein synthesis. Since Prx1 transactivates the collagen $\alpha 1(I)$ promoter, this mechanism may promote profibrotic phenotype of HSCs. The findings described here may help understand activation of HSCs and development of liver fibrosis.

MATERIAL AND METHODS

Isolation and culture of HSCs.

Rat HSCs were isolated from rat livers by perfusion of collagenase and pronase, followed by centrifugation over Nycodenz gradient, as described⁴². After isolation the cells were cultured for one day, trypsinized and cultured for an additional day before being harvested as quiescent HSCs. The purity was assessed by desmin staining, as described⁸, and was found to be >95%. For activated HSCs, HSCs were cultured in uncoated plastic dishes in DMEM supplemented with 10% FBS in a 5% CO₂-humidified atmosphere for a total of 8–14 days. For expression studies, two days after isolation the cells were infected at a multiplicity of infection of 500 with adenoviruses expressing RBMS3 or control virus and cultured for additional 3 days.

Plasmid constructs and adenovirus preparation.

Full-size human RBMS3 was isolated by RT-PCR using total RNA from a human lung fibroblast cell line and amplified with the primers containing a BamHI site within the 5' primer and a EcoRI site within the 3' primer. Full size 1.25 kb RBMS3 cDNA was cloned into the BamHI and EcoRI sites of a modified pCDNA3 vector (Invitrogen), which was modified to contain an HA-tag at the N-terminus. The full length Prx1 ORF with complete 3' UTR (WT) and Prx1 with the deleted 60 nt RBMS3 binding site in the 3' UTR (MUT) were cloned from mouse Prx1 cDNA (obtained from Dr. M. Kern, University of SC, Charleston) into the NotI site of the modified pCDNA3 vector (both Prx1 constructs received an HA tag added on their N-terminus). The adenovirus expressing HA-tagged RBMS3 was generated using recombination in *E. Coli*, as described⁴³.

RT-PCR analysis of gene expression.

Total cellular RNA was isolated using an RNA isolation kit (Eppendorf, Westbury, NY). RT-PCRs were done with 100 ng of total RNA using rTth reverse transcriptase (Boca Scientific,

Boca Raton, FL) and ^{32}P -dCTP was included in the PCR step to label the products ⁸; ¹³; ²⁴; ⁴⁴. PCR products were subsequently resolved on sequencing gels and visualized by autoradiography. The number of cycles was adjusted to be in the linear range of the reaction. The following primers were used for RT-PCR: h-RBMS3, 5' primer: CGCGGATCCTACCCCCAGTACACTTA, 3' primer: CCGGAATTCTTACTTAGACTGTTGGTA, m-PRX1, 5' primer: GCGGAGAAACAGGACAACAT, 3' primer: ACTTGGCTCTTCGGTTCTGA, r-PRX1 5' primer: CTTCTCCGTCAGTCACCTGC, 3' primer: CGTGCAAGATCTTCCCGTAC, r- β -actin, 5' primer: CGTGCGTGACATTAAAGAGAAGC, 3' primer: TGCATGCCACAGGATTCCATACC, r-neurotrimin, 5' primer: CTGGAATGACAAGTGGTGC, 3' primer: CTATGCAAGTGAGGCTGATG.

Immunoprecipitation.

Expression vector (PCDNA3) encoding HA-tagged human RBMS3 or a truncated form of HA-tagged human lysyl oxidase, as control, was transfected into human fibroblasts and stable transfected cell lines were developed. Expression was verified by Western blotting. Cell lysate was prepared by homogenizing the cells in phosphate-buffered saline containing 0.5% NP-40 and, after removal of nuclei by centrifugation, 1 mg of clear lysate was incubated with either 10 μl of anti-HA monoclonal antibody or 10 μl of anti-GFP monoclonal antibody, as a control, for 1 h at 4°C. A 50 μl volume of prewashed protein A/G plus-agarose (Santa Cruz Biotechnology) was added to each tube, and incubation continued for 3 h. After washing the beads three times in RNA binding buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl_2 , 150 mM KCl) with 0.5% Triton X-100, 3 μl of protein A/G plus-agarose was used for western blotting, while the rest of the A/G plus-agarose was used for extraction of total RNA. Identical immunoprecipitation experiments were performed with human HSCs that were infected with adenovirus expressing HA-tagged RBMS3.

Differential display PCR.

Total RNA isolated from RBMS3 or control immunoprecipitation experiments was used in a reverse transcription reaction with one of three different 3' primers containing oligo dT, followed by an A, C, or G nucleotide ²⁹; ⁴⁵; ⁴⁶. The first strand is then used in PCR amplification reaction with the same 3' primer and one of 15 random 5' primers. The radioactively labeled PCR products were resolved on sequencing gels and visualized by autoradiography. The bands representing RNAs differentially precipitated with RBMS3 were excised from the gel, eluted, reamplified with the same set of primers, cloned into PGEM3 vector (Promega), and sequenced.

Recombinant protein expression.

The recombinant GST-RBMS3 was expressed from the pGEX-2T plasmid (Amersham) in BL21 *E. coli* by induction with 0.1 M isopropyl-1-thio- β -D-galactopyranoside for 3 h prior to purification. Recombinant protein was purified after cell lysis with the addition of 500 μl of a 50% slurry of glutathione-sepharose beads. The beads were washed three times with ice-cold phosphate-buffered saline and recombinant protein was eluted using 50 mM reduced glutathione in 100 mM Tris, pH 7.6, 100 mM NaCl_2 . Purity of the recombinant protein was assessed by Coomassie staining and the protein was devoid of any visible contaminants.

Gel mobility shift experiments.

Uniformly labeled RNA probes were prepared by standard *in vitro* transcription protocols using [α - ^{32}P]UTP (3,000 Ci/mmol; ICN) after linearization of plasmids with appropriate restriction enzymes ¹³; ²²; ²³; ⁴⁷. Gel mobility shift experiments were performed by incubating 50 μg of cytoplasmic lysates in a binding buffer (12.5 mM HEPES, pH 7.9, 15 mM KCl, 0.25 mM

EDTA, 5 mM MgCl₂, 1 mM dithiothreitol, 0.2 mg/ml tRNA, 50 units/ml RNasin, 10% glycerol) with 10,000 cpm of RNA probe for 30 min on ice. For the 566 nt probe, the reactions were digested with U of RNase T1 for 30 min at room temperature (RT) prior to loading on the gel. Binding reactions were electrophoresed in 6% native gels at 200 V, gels were then dried and signals visualized by autoradiography. Competitor RNAs were added in some experiments as indicated. These RNAs were made by *in vitro* transcription and their integrity was assessed by agarose gel electrophoresis. In gel mobility experiments in which purified recombinant proteins were used, 250 ng of protein was incubated with the RNA probe.

Reporter gene analysis.

0.5 µg of HA-tagged PCDNA3-PRX1 WT or PCDNA3-PRX1 MUT plasmids were cotransfected into HEK293 cells with either 0.5 µg of PCDNA3-RBMS3 or with 0.5 µg of plasmid encoding procollagen C-endopeptidase enhancer protein (PCOLCE), as control. The latter clone did not have an HA tag. 0.2 µg of plasmid containing a β-galactocidase gene driven by a CMV promoter was included in all cotransfection experiments as an internal control. Transfection was done using the HEK293 cell specific transfection reagent (Mirus *transit*-293, Mirus Corp.). Cells were harvested 2 days after transfections and total RNA or protein was extracted. Transfection efficiency was determined by a β-galactocidase assay and the readings were considered significant if the A₄₂₀ was less than 1 and greater than 0.05. Protein and RNA concentrations used for Western blots and RT-PCR, respectively, were adjusted based upon the transfection efficiency.

mRNA stability determinations.

Cell transfections were performed as outlined under reporter gene analysis section. HEK293 cells were treated with Actinomycin D (10 µg/ml of medium) for 2h, 4h, 8h, 12h, or 24h. After the designated Actinomycin D incubation period was reached, the cells were scraped and total RNA was extracted. RT-PCR was performed using 5' and 3' primers specific for mPrx1 mRNA. RNA extracted from cells at time point 0 (no addition of Actinomycin D) was used as initial level of PRX1 mRNA.

Western blot analysis.

Total and cytosolic proteins were prepared by standard procedure. Protein concentration was estimated by Bradford assay with BSA as standard. Western blots were done using 10 µg of protein and anti-HA antibody (Sigma).

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Fig 1.

Expression of RBMS3 in HSCs and fibrotic livers. A. Expression of RBMS3 in quiescent (Q) and culture activated rat HSCs (A). HSCs were harvested two days after isolation (lane 1, Q) or eight days after isolation (lane 2, A) and the level of RBMS3 mRNA was estimated by RT-PCR. Expression of neurotrimin (NTRM) and β -actin (ACTIN) is shown as control. B. Time course of RBMS3 expression during culture activation of rat HSCs. HSCs were cultured for the indicated time points and expression of RBMS3 was estimated as in A. C. Expression of RBMS3 in animal model of liver fibrosis. Five rats were treated with CCl_4 for four weeks to induce liver fibrosis and expression of RBMS3 mRNA in fibrotic livers (lanes 3–7) was compared to that in normal livers (lanes 1 and 2) by RT-PCR. Actin is shown as loading control.

Fig 2.

RBMS3 interacts with a sequence within the 3' UTR of Prx1 mRNA. A. schematic representation of RNA probes used to map the RBMS3 binding site. Relationship between the full size 3' UTR and various probes is shown (not to scale). 60 nt of the RBMS3 binding site is shown as black box. The length of probes is given in nt. B. Binding of RBMS3 to the 566 nt probe. HA-tagged RBMS3 was overexpressed in human fibroblasts by adenoviral delivery. Cytosolic extract of RBMS3 expressing cells was used in gel mobility shift with 566 nt probe (RBMS3, lane 2) and compared to shift with extracts of cells infected with control virus (CON, lane 3). Lane 1 is probe alone. Prior to loading on the gel the samples were digested with RNase T1. Migration of RNase resistant RNA/protein complex is indicated, as well as that of undigested probe. C. Experiment as in B, except the 240 nt probe was used and RNase digestion was omitted (lanes 1–3). Migration of RNA/protein complex is indicated. Lanes 4–6 is western blot of the gel shown in lanes 1–3, probed with anti-HA antibody. Arrow indicates position of RBMS3 protein in the slot of the gel.

C

F

MOUSE :	AAATAGGTTTATATTTTCAATATCTCAACAATAACATCATTTGACCAAAGAATAAT
HUMAN :	AAATAGTTTATATTTTTCAGATCTCTCAAAAATCACATCATTTGACCAAAGAATAAT
RABBIT :	TTTATATTTTCAAATATCTCAACAATAACATCATTTGACCAAAGAATAAT
DOG :	AAATAGTTTATATTAACAGACTTCTCAAAAATCACATCACTTGACCAAAGAATAAT
CAT :	AAATAGGTTTACTTCCAGACCTCTCAAAAATCACATCACTTGACCAAAGATAA
ELEPHANT :	AAATAGTTTATATTTCCAGATCTCTCAACAATCACTTTCATTTGACCAAAGAGTAAT
BAT :	AAATAGTTTATATTTATAGACCTCTCAAAAATCACATCATTTGACCAAAGGATAAT

Fig 3.

Recombinant RBMS3 interacts with 60 nt within the 3' UTR of Prx1 mRNA. A. Purity of the recombinant RBMS3. RBMS3 was purified as GST fusion protein after expression in *E. coli*. The purified protein was analyzed by SDS-PAGE and Coomassie staining (lane 3). Lane 2 is GST alone and lane 1 is size marker (M). Sizes in kD is shown to the left. B. Binding of the recombinant protein to 240 nt probe. Gel mobility shift with purified GST- RBMS3 fusion protein (RBMS3, lane 2) and GST alone (CON, lane 3). Lane 1 is probe alone. Migration of RNA/RBMS3 complex and free probe is indicated. In lanes 4–5 a probe derived from mouse GAPDH sequence was used. This probe shows spontaneous formation of a dimer (lane 4). In lanes 7–8 a probe encompassing 5' stem-loop of mouse collagen $\alpha 1(I)$ was used (5'SL). C.

Binding of recombinant RBMS3 to 150 nt probes. Two probes from the 5' end (150A) and 3' end (150B) of 240 nt probe sequence, respectively, were derived. These probes overlap in 60 nt. Gel mobility shift experiment was done as in A. Migration of RNA/RBMS3 complexes and free probe is indicated. D. Binding of recombinant RBMS3 to the 60 nt overlapping sequence shared by probes 150A and 150B. Lane 1 is 60 nt probe alone. This probe spontaneously forms a dimer, what is indicated. Lane 2 is binding of recombinant RBMS3 to the probe, In lanes 3 and 4 two different nonspecific competitor RNAs were added at the molar excess indicated, while in lane 5 10 µg of total liver RNA was added. In lane 6 specific competitor RNA was added at the molar excess indicated. Migration of RBMS3/RNA complex is indicated. E. Poly-A+ RNA from the liver competes binding of RBMS3 to the Prx1 sequence. Experiment as in D, but 0.5 µg of poly-A+ RNA from mouse liver (lane 4) or 2.5 µg of poly-A+ RNA from mouse liver (lane 5) was used as competitor. Lane 3 is competition with 10 µg of total liver RNA and lane 2 is binding without competitor. Lane 1 is probe alone and the migration of RNA and RBMS3/RNA complex is shown. F. Sequence comparison of the RBMS3 binding site in vertebrate Prx1 mRNAs.

Fig 4.

Ectopic expression of RBMS3 upregulates Prx1 and collagen $\alpha 1(I)$ mRNA. Quiescent HSCs at day 2 after isolation were transduced with control adenovirus (CON, lane 1) or adenovirus expressing RBMS3, which was done in duplicate (RBMS3, lanes 2 and 3). At day 5, expression of Prx1 and collagen $\alpha 1(I)$ (COL) was analyzed by RT-PCR. Arrows indicate migration of the specific PCR products. B-actin was used as loading control.

Fig 5.

RBMS3 increases expression of Prx1 protein when encoded by the mRNA containing the RBMS3 binding site. **A.** Schematic representation of reporter constructs. The genes were driven by CMV promoter (white box), the 5' UTR was derived from the vector, followed by Prx1 open reading frame (gray box) with HA tag at the N-terminus (black box). The reading frame was followed by the complete Prx1 3' UTR (WT) or by the 3' UTR in which RBMS3 binding site (stippled box) was deleted (MUT). All Prx1 sequences were derived from the mouse gene. **B.** Prx1 protein expression from the reporter constructs. WT reporter together with expression plasmid for RBMS3 (RBMS, lane 1) or control protein expression plasmid (CON, lane 2) were transiently transfected into HEK293 cells. Lanes 3 and 4 are identical, except the mutant reporter (MUT) was transfected. All transfections were normalized to expression of β -gal internal standard gene. Western blot was performed with anti-HA antibody. Expression of RBMS3 was visualized (indicated by arrow), because it contained HA tag. Control protein can not be seen, because it did not have a tag. Expression of Prx1 is indicated in the bottom part by the arrow. Lanes 5 to 9 represent an identical independent experiment and correspond to lanes 1 to 4.

A **B** **Fig 6.**

Steady state level and stability of reporter mRNAs is affected by RBMS3. A. Steady state mRNA level. RNA was extracted from samples shown in A and expression of reporter mRNAs (REP) was analyzed by RT-PCR. Amounts of RNA used in analysis were normalized to the transfection efficiencies of individual samples, which were within 20%. Lane 1 are nontransfected cells. Expression of reporter mRNAs (REP) is indicated. B. Stability of mRNAs. WT and MUT reporter genes were cotransfected with RBMS3 expression plasmid and 48h after transfections the cells were treated with actinomycin D for the indicated time periods. RNA was extracted and analyzed as in A. For better comparison, the amount of total

RNA in MUT samples was increased to give the Prx1 signal at time 0 comparable to that of WT samples.