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Zinc finger protein 267 is up-regulated during the activation process of human hepatic stellate cells and functions as a negative transcriptional regulator of MMP-10 ☆

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Abstract

Activation of hepatic stellate cells (HSCs) is the central event in the development of liver fibrosis and cirrhosis. The transdifferentiation process of quiescent into activated HSCs requires a complete reprogramming in gene expression, which is governed by modulation of transcriptional activators or repressors. Using microarray analysis to identify genes differentially expressed during the activation process of human HSCs, zinc finger protein 267 (ZNF267) mRNA was up-regulated in activated HSCs and in cirrhotic human liver. ZNF267 belongs to the family of Kruppel-like zinc fingers and contains a conserved KRAB (Kruppel associated box) A and B domain in the N-terminal part outside the C-terminal region of zinc fingers. ZNF267 constructs containing enhanced cyan fluorescence protein were constitutively localized in the nucleus. When fused to GAL4 DNA binding domain, full-length ZNF267 and all constructs encompassing KRAB A domain showed transcriptional repressor activity. Microarray analysis and RNase protection assays showed that ZNF267 represses MMP-10 gene expression, which was confirmed by reporter gene assays. Furthermore, ZNF267 binds to the MMP-10 promoter region as demonstrated by chromatin immunoprecipitation assays. In conclusion, our results suggest that ZNF267 as a negative transcriptional regulator of MMP-10 might promote liver fibrogenesis through alteration of matrix degradation in vivo.

Keywords: Kruppel-like zinc finger proteins; Kruppel associated box; Transcriptional repressor; Liver fibrosis; Collagen

Liver cirrhosis is characterized by an excessive deposition of extracellular matrix (ECM) proteins, including collagens type I and type III. Activated hepatic stellate cells (HSCs) play a central role as the main ECM protein producing cells during hepatic fibrogenesis [1]. Hepatic injury from various causes including toxic, viral and immune, induces HSCs to undergo a transdifferentiation or activation process, which is characterized by a loss of their intracellular vitamin A stores, an increase in proliferation, a change in cellular morphology to a more myofibroblast-like cell type, and an up-regulation of ECM proteins [1]. Many of these phenotypic and

☆ *Abbreviations:* HSCs, hepatic stellate cells; ZNF267, zinc finger protein 267; KRAB, Kruppel associated box; ECM, extracellular matrix; KLF, Kruppel-like factor; ECFP, enhanced cyan fluorescent protein; MMP, matrix metalloproteinase; HEK, human embryonic kidney; TSA, trichostatin A; IL, interleukin; SNAP, *S*-Nitroso-*N*-acetylpenicillamine; CRBP-I, cellular retinol binding protein I; RACE, rapid amplification of cDNA ends; ChIP, chromatin immunoprecipitation assay; HDAC, histone deacetylase; NO, nitric oxide; TIMP, tissue inhibitor of metalloproteinases; NF-Y, nuclear factor Y.

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metabolic changes during the activation process of HSCs are also observed when primary cultures of HSCs are grown on plastic or type I collagen [2].

The molecular mechanisms of activation and perpetuation of the activated phenotype in HSCs include an extensive reprogramming of gene expression. Modulation in gene expression requires at least in part a change in the expression and activity of transcription factors acting as transcriptional activators or repressors. Several transcription factors have been identified and found to be involved in the activation process of HSCs including AP-1 [3,4], NFκB [5,6], C/EBP [7,8], PPARγ [9,10] and members of the Kruppel-like zinc finger family, like Kruppel-like factor (KLF)6 [11], KLF9 [12], and SP1 [13].

Kruppel-like factors contain multiple zinc fingers, which represent one of the most common DNA binding domains. A zinc finger contains two cysteine and two histidine residues (Cys_2His_2 zinc finger) that coordinate a single zinc ion and fold the domain into a finger-like projection [14]. A subfamily of Kruppel-like factors contains a conserved Kruppel associated box (KRAB) domain in the amino terminal part outside the conserved carboxy terminal region of zinc fingers [15]. The KRAB domain mediates transcriptional repression [16,17] and is divided into a KRAB A and KRAB B domain. The KRAB A domain plays the central role in transcriptional repression [14]. One member of the Kruppel-like zinc finger family is zinc finger protein 267 (ZNF267; also named human zinc finger 2 or HZF2), whose amino terminal KRAB domain is separated through a linker region consisting of 193 amino acids from a clustered zinc finger domain [18]. The first zinc finger is followed by three degenerated fingers, and then continues with 13 zinc fingers at the carboxy terminus [18]. The function of ZNF267 is unknown; its mRNA is up-regulated upon nitric oxide treatment in venous endothelial cells [19]. In this study, we report the nuclear localization, transcriptional regulation, and functional properties of ZNF267.

Materials and methods

Plasmid constructs. Full size human ZNF267 was generated by reverse transcription of total RNA from activated human HSCs and amplified by PCR with primers 5′-CGCGCGGATCCGTATGGGACTGTTGACATTCAGGGA-3′ and 5′-CCGCTCGAGTTAAAGTTTTTCTCTAGTATGACTTCT-3′. The two oligonucleotides introduced a *Bam*HI and *Xho*I site at the 5′- and 3′-end, respectively. ZNF267 cDNA was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Sequencing of the full insert confirmed the identity to the published sequence for ZNF267 (GenBank Accession No. XM_008061). From this clone pCR2.1-TOPO/ZNF267, the *Bam*HI–*Xho*I fragment was cloned in-frame into pcDNA3-HA vector [20] for mammalian expression (pcDNA-ZNF267).

For fluorescent studies pCR2.1-TOPO/ZNF267 and pECFP-C1 (BD Biosciences, Palo Alto, CA) were cut with *Spe*I and *Bam*HI, respectively, and blunt ends were generated using Klenow polymerase. After phenol/chloroform extraction and ethanol precipitation, both vectors were cut with *Apa*I, and the released full-length ZNF267 was recloned in-frame generating a fusion of ZNF267 to the C-terminus of ECFP (pECFP-ZNF267).

The Gal4 luciferase (pGAL4-Luc) constructs contain four GAL4 DNA consensus binding sites derived from the *Saccharomyces cerevisiae* GAL4 gene upstream of luciferase [21]. GAL4-ZNF267 constructs were obtained by subcloning various regions from pCR2.1-TOPO/ZNF267 in-frame into the effector plasmid pFA-CMV (Stratagene, La Jolla, CA) containing the GAL4 DNA binding domain. For pGAL4-ZNF267(1–184), a *Bam*HI–*Sty*I (both blunt) fragment of pCR2.1-TOPO/ZNF267 was subcloned into *Bam*HI (blunt) site of pFA-CMV; for pGAL4-ZNF267(1–2332) a *Bam*HI (blunt)–*Xba*I fragment of pCR2.1-TOPO/ZNF267 was subcloned into *Bam*HI (blunt)–*Spe*I site of pFA-CMV to generate a fusion protein between GAL4 DNA binding domain and full-length ZNF267. To obtain pGAL4-ZNF267(1–248) and pGAL4-ZNF267(1–717), pGAL4-ZNF267(1–2332) was cut with *Xba*I/*Sac*I and *Ppu*MI/*Sac*I, respectively, and the blunt ends were ligated. For pGAL4-ZNF267(184–2332) a *Sty*I (blunt)–*Spe*I fragment of pCR2.1-TOPO/ZNF267 was subcloned into *Sma*I–*Xba*I sites of pFA-CMV. The in frame location of all GAL4 fusion proteins was verified by sequencing.

To obtain a matrix metalloproteinase (MMP)-10 reporter gene plasmid, a 2.6-kb fragment (−2579 to +37; +1 corresponding to the transcription start site) consisting of the 5′-flanking sequence of the MMP-10 gene was PCR amplified from genomic DNA, which was isolated from human HSCs using the DNeasy kit (Qiagen, Valencia, CA). The PCR primers used to obtain the MMP-10 promoter were 5′-TCTCACTGCCCTTACCTTCTTTGTCTACTGGGCTTCTAGC-3′ and 5′-GTGAGTCAGTGAGTGAGTGGTGAGTGAATCTGTACAC-3′. The fragment was inserted into pCR2.1-TOPO. The MMP-10 reporter gene plasmid pGL3-MMP-10(−2579/+37) was constructed by subcloning a *Xho*I–*Sac*I fragment into pGL3-Enhancer Vector (Promega, Madison, WI). The insert was sequenced to confirm the identity to published sequence of genomic DNA (GenBank Accession No. AP000647). We also prepared a 5′-deleted MMP-10 luciferase construct, encompassing the MMP-10 promoter region −533/+37. pGL3-MMP-10(−533/+37) was PCR generated using pGL3-MMP-10(−2579/+37) as template, a forward primer 5′-GGGGTACCCCTTCTCACAATACCCCTACTCCACG-3′ and a reverse primer 5′-GCAGATCTCGAGCGG-3′. The forward primer introduced a *Kpn*I site at the 5′-end. PCR products were gel purified, digested with *Kpn*I and *Xho*I and the fragments were cloned into pGL3-Enhancer Vector.

Cell culture. Isolation and culture of human HSCs were previously described [22,23]. Human HSCs, HeLa cells, and human embryonic kidney (HEK) 293 cells were cultured on uncoated plastic tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum and standard antibiotics in 95% air–5% CO_2 humidified atmosphere at 37 °C.

Transfections and luciferase reporter assays. Gal4-based transactivation assays were performed using 1×10^6 HEK 293 cells in 60-mm dishes transfected with 0.5 μg of GAL4-Luc and 0.5 μg of the various effector plasmids GAL4-ZNF267 using LipofectAMINE Plus (Invitrogen). Cell lysates were collected 48 h after transfection and assayed for luciferase activity using Luciferase Assay System (Promega, Madison, WI). Luciferase activity was normalized to total cellular protein. For trichostatin A (TSA; Sigma, St. Louis, MI) experiments, TSA was added 2 h after transfection and incubated for another 22 h before harvesting. For agonist studies, 24 h after transfection cells were serum starved for 8 h and subsequently stimulated with interleukin (IL)1β (2.5 ng/ml; R&D Systems, Minneapolis, MN) or *S*-nitroso-*N*-acetylpenicillamine (SNAP, 500 μM; Merck, Germany) in medium without serum for additional 24 h before harvesting. For MMP-10 reporter genes with or without 0.25 μg of the effector plasmids per 60-mm dish, and cell lysates were collected 48 h later. For microarray, immunofluorescence and immunoprecipitation studies, cells were

either transfected in 60-mm dishes (2.5 μg of plasmid DNA) or in 100-mm dishes (10 μg of plasmid DNA) by the calcium phosphate technique [24]. Cells were analyzed 48 h after transfection.

RNA isolation and RT-PCR. Total RNA from normal and cirrhotic human livers was extracted using TRIZOL (Invitrogen) according to the manufacturer's protocol. Total RNA from cultured cells was isolated by use of a RNA extraction kit (Qiagen, Valencia, CA). RNA for RT-PCR was digested with DNAse I for 10 min at 37 °C to get rid of genomic DNA. RT-PCR was performed as described previously [4,22,25]. The synthesized cDNA was amplified using specific primer sets for β-actin or cellular retinol binding protein I (CRBP-I), as described previously [26,27]. The sequence of the primer set for ZNF267 was 5′-CCTTTCGCTGTAGTTCATAC-3′ and 5′-CCGATGCACAG AAAGACCT-3′, the expected length of the amplified product was 538 bp. The cycle number for each amplicon was determined to be in the linear range of amplification.

RNase protection assays. RNase protection assays were performed as described previously [4,22,25]. For in vitro transcription of MMP-10 antisense RNA, a fragment of MMP-10 was amplified by PCR with primers described previously [28], and cloned into pCR2.1-TOPO. This plasmid was linearized with *Hin*dIII and transcribed with T7 Polymerase to generate a 404 nt antisense riboprobe, which protects 335 nt of MMP-10 mRNA. Riboprobes for the housekeeping gene GAPDH were derived from the plasmid pTRI-GAPDH-human (Ambion, Austin, Texas).

Gene chip microarrays. Hybridization of Affymetrix human genome U133A (HG-U133A) high density oligonucleotide arrays (Affymetrix, Santa Clara, CA) was performed as described previously [23,29].

Immunoprecipitation and Western blot analysis. For immunoprecipitation experiments, whole cell extracts were prepared 2 days after transfection using RIPA buffer. Clear lysate (2 mg) was incubated with 10 μl of anti-HA polyclonal antibody (Sigma, St. Louis, MI) or anti-GAL4 monoclonal antibody (Santa Cruz, Santa Cruz, CA) for 1 h at 4 °C in a final volume of 1 ml PBS. Twenty microliters of prewashed Protein A/G PLUS–Agarose (Santa Cruz, Santa Cruz, CA) or Protein G–Sepharose 4 Fast Flow (Amersham, Piscataway, NJ) were added and incubation continued for 12 h. After washing three times in PBS, samples were analyzed by Western blotting as described previously [4,25].

Rapid amplification of cDNA ends. The 5′-terminus of MMP-10 mRNA was determined by rapid amplification of cDNA ends (5′RACE) using First Choice 5′ RLM RACE (Ambion, Austin, Texas) according to the manufacturer's protocol and as described [30]. The sequence for the gene specific outer primer was 5′-GCCCCACTCA GAGGATAGGCAGAGCAGAC-3′ and for the gene specific inner primer 5′-GGCAGACACAACAGCACAAGGAATGCAAGATGC-3′. The PCR amplified products were subcloned into pCR2.1-TOPO and three clones were sequenced.

Chromatin immunoprecipitation assay. HEK 293 cells (1×10^6) were seeded in 100-mm dishes, transfected with 10 μg of pcDNA or pcDNA-ZNF267 using LipofectAMINE Plus (Invitrogen), and cultured for 48 h in regular medium containing 10% FCS. Chromatin immunoprecipitation assays were performed using a chromatin immunoprecipitation assay (ChIP) Assay Kit (Upstate, Lake Placid, NY) according to the manufacturer's protocol. Sonication was performed in 600 μl lysis buffer per 100-mm dish. After centrifugation 200 μl of the supernatant was used as ChIP input control, 200 μl for immunoprecipitation using 4 μg of anti-HA antibody (Roche, Indianapolis, IN), and 200 μl for immunoprecipitation using 4 μg of isotype control antibody (anti-IgG; Sigma). The precipitated DNA fragments were analyzed by PCR using a primer set 5′-GTGAGTCAGTGAG TGAGTGGTGAGTGAATCTGTACAC-3′ and 5′-ACCTAAGTGT TATTACAAAAGAGTC-3′. The length of the amplified product was 200 bases, and the amplicon corresponds to the fragment from −2579 to −2379 of the 5′-flanking sequence of the MMP-10 gene. PCRs were cycled as follows: initial denaturation at 95 °C for 5 min; then 26 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 45 s. The final extension was carried out at 72 °C for 10 min. Negative controls without DNA were performed with each PCR. PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining.

Statistical analysis. The results were analyzed for statistical significance according to the Mann–Whitney *U* statistic test.

Results

Induction of ZNF267 mRNA expression during culture activation of human HSCs and in cirrhotic human liver

To identify genes differentially expressed in HSCs, we performed gene chip microarrays to compare gene expression profiles of activated HSCs and normal liver containing quiescent HSCs [31]. ZNF267 was up-regulated 3.1-fold in activated HSCs. To confirm this result, expression of ZNF267 mRNA was assessed in culture-activated human HSCs by semi-quantitative RT-PCR analysis. Activated HSCs on culture day 2 showed an induction of ZNF267 mRNA as compared to quiescent human HSCs after the isolation from normal human liver (day 0; Fig. 1A). CRBP-I serves as housekeeping gene, since CRBP-I is similarly expressed in quiescent and activated human HSCs [32]. However, ZNF267 expression minimally increased when HSCs are cultured for a period up to 17 days (Fig. 1B).

To further investigate changes in gene expression in vivo, gene chip microarray analysis was employed to identify differentially expressed genes in normal liver and cirrhotic liver secondary to chronic hepatitis C infection. In cirrhotic liver, mRNA expression of ZNF267 was 3.1-fold higher as compared to normal liver. Semi-quantitative RT-PCR confirmed the up-regulation of ZNF267 expression in cirrhotic liver (Fig. 1C). Thus, ZNF267 mRNA is induced during the activation process of human HSCs in culture as well as in cirrhotic human liver in vivo.

ZNF267 shows a nuclear localization

To investigate the subcellular localization of ZNF267, full-length ZNF267 cDNA was cloned and a fusion protein between ZNF267 and the C-terminus of ECFP was generated. Using fluorescence microscopy of HeLa cells transfected with the ECFP reporter vector alone showed both a cytoplasmic and nuclear localization (Figs. 2A and B). However, in cells transfected with pECFP-ZNF267 the fusion protein ECFP-ZNF267 was exclusively localized in the nucleus (Figs. 2C and D).

ZNF267 displays transcriptional repressor activity

To test whether ZNF267 contains a transcriptional regulatory domain, a GAL4 based reporter gene system was used. Full-length ZNF267 and various constructs containing deletions in the N-terminal or C-terminal

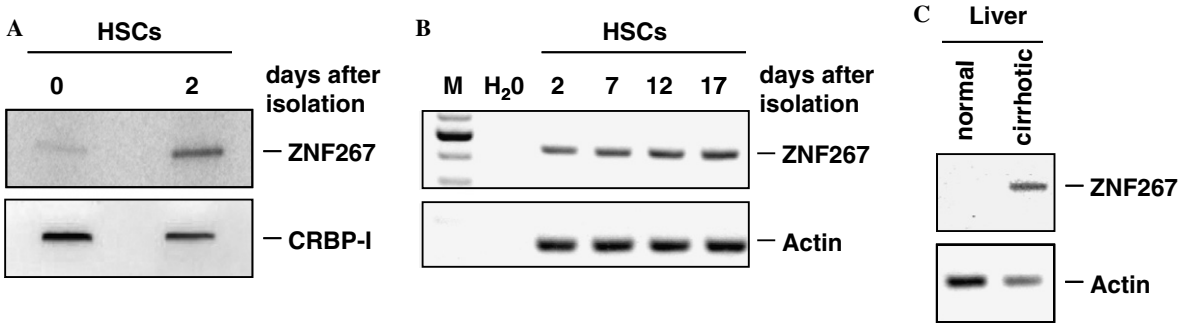

Fig. 1. Expression of ZNF267 mRNA is up-regulated in culture activated human HSCs and in cirrhotic human liver. The expression of ZNF267 was analyzed by semi-quantitative RT-PCR using 1 μg of total RNA. With primers specific for ZNF267, cDNA was analyzed from human HSCs freshly isolated (day 0) or cultured for 2, 7, 12 or 17 days as indicated (A,B), and in normal liver or cirrhotic liver secondary to chronic hepatitis C infection, respectively (C). CRBP-I (A) and actin (B,C) were amplified to confirm equal amount of mRNA was present in each sample. Negative controls without cDNA (H_2O) were performed with each PCR. Lane M contains the molecular weight marker.

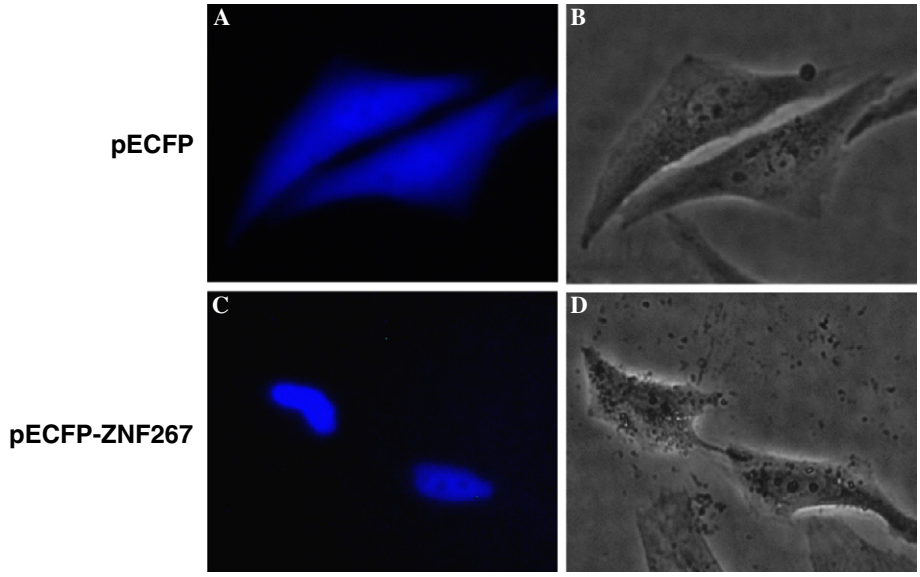

Fig. 2. ZNF267 is localized in the nucleus. Photomicrographs are of HeLa cells transfected with pECFP (A,B) or pECFP-ZNF267 (C,D) and analyzed 48 h later with light (B,D) or fluorescent microscopy (A,C).

region of ZNF267 were fused with a GAL4 DNA binding domain to transactivate a GAL4 responsive promoter driving luciferase expression (Fig. 3A).

Western blotting verified the expression of the various constructs with the predicted molecular weights (Fig. 3B). However, the expression of fusion proteins 6 and 7 was only detectable using immunoprecipitation indicative of a short half-life with a rapid turnover of full length (construct 6) or nearly full-length ZNF267 (construct 7). Two bands representing full-length ZNF267 or nearly full-length ZNF267 are probably due to differences in posttranslational modifications.

In transient transfections all three constructs encompassing KRAB A domain alone showed transcriptional repressor activity (Fig. 3C; constructs 3–6) as compared to GAL4 DNA binding domain alone (Fig. 3C; construct 2). The KRAB A domain acts as the strongest transcriptional repressor (Fig. 3C; construct 3). In contrast, a construct containing KRAB B, linker and zinc fingers (without KRAB A) did not modulate transcriptional activity in this assay (Fig. 3C; construct 7).

Taken together, these results along with sequence analysis of the DNA binding domain of Kruppel-like factors and nuclear localization support a role for ZNF267 as a transcription factor.

Transcriptional repression activity of ZNF267 is independent from histone deacetylase activity

Since transcriptional repression of genes by zinc finger proteins containing the KRAB domain is associated with their deacetylation [33], we investigated whether histone deacetylase (HDAC) mediates transcriptional repression by ZNF267. Shortly after transfection, cells were treated with various concentrations of TSA, an inhibitor of HDAC activity. TSA treatment increased

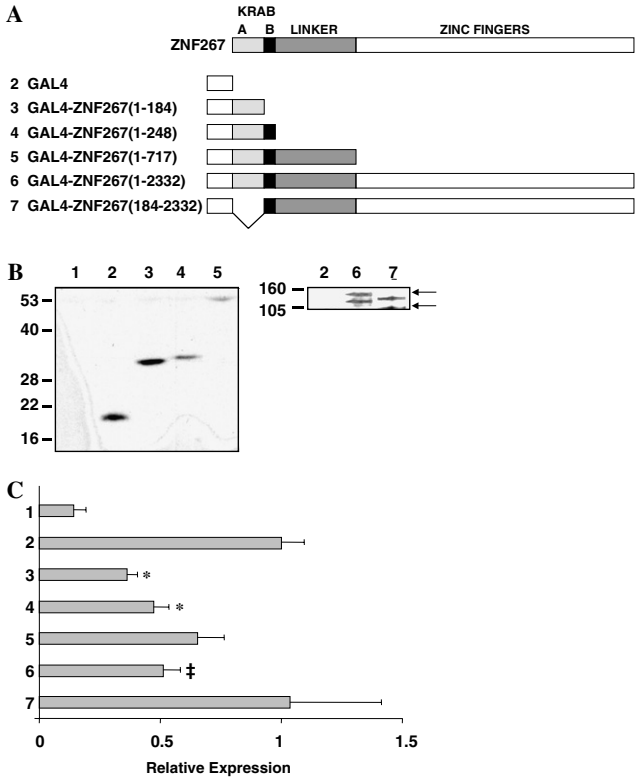

Fig. 3. Transcriptional repressor activity of ZNF267. (A) A schematic diagram of ZNF267 with KRAB, linker and zinc fingers [18] (top panel) in addition to various GAL4-ZNF267 constructs. All constructs (constructs 3–7) were generated in the expression vector pFA-CMV containing the GAL4 DNA binding domain (construct 2) resulting in a fusion protein between the GAL4 DNA binding domain and parts of ZNF267. (B) HEK 293 cells were transiently transfected with the various pGAL4-ZNF267 constructs (2.5 μg per 60-mm dish) using the calcium phosphate method. Western blotting of the GAL4-ZNF267 constructs (construct 1–5; left panel), or immunoprecipitation and Western blotting (construct 2, 6 and 7; right panel) were performed using an anti-Gal4 antibody. Molecular weights (kDa) are indicated on the left side of each immunoblot. The numbers on top correspond to the numbers of the various expression constructs in (A). Constructs 6 and 7 are indicated by arrows. Lane 1 represents cells transfected with the luciferase reporter plasmid pGAL4-Luc alone. (C) HEK 293 cells were transiently transfected with the various pGAL4-ZNF267 constructs (0.5 μg per 60-mm dish) along with luciferase reporter plasmid pGAL4-Luc (0.5 μg per 60-mm dish) containing four GAL4 DNA consensus binding sites (lanes 3–7). Background measurements were performed in cells transfected with luciferase reporter plasmid pGAL4-Luc alone (lane 1). Basal transcriptional activity was measured using the reporter gene pGAL4-Luc with a plasmid containing the GAL4 DNA binding domain alone (lane 2). Luciferase activity was normalized to total cellular protein. Data represent means ± standard error of the mean (SEM) of three experiments performed in duplicate and are expressed relative to the luciferase activity of the reporter gene pGAL4-Luc with a plasmid containing the GAL4 DNA binding domain (lane 2). $^{*}p \leqslant 0.005$, $^{\ddagger}p \leqslant 0.05$ when compared to the basal transcription activity of cells (lane 2).

the luciferase expression in pGAL4 and pGAL4-ZNF267 full-length transfected cells in a dose-dependent manner (Fig. 4A). However, TSA did not block repressor activity of ZNF267 on the GAL4-luciferase reporter gene (Fig. 4A). Note that due to cytotoxicity

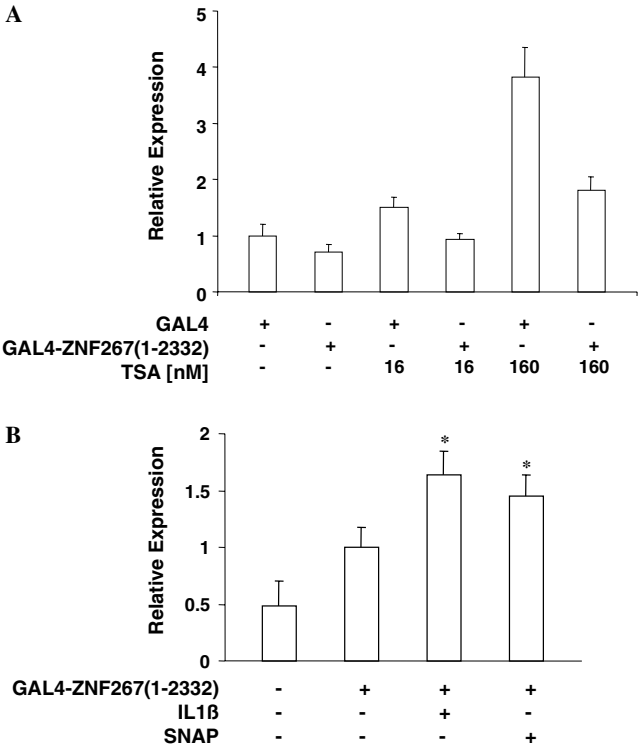

Fig. 4. Transcriptional repressor activity of ZNF267 does not involve HDAC. (A) To examine whether HDAC mediates the repression activity of ZNF267, HEK 293 cells were co-transfected with a luciferase reporter plasmid (0.5 μg per 60-mm dish) containing four GAL4 DNA consensus binding sites, and the empty vector pGAL4 (0.5 μg per 60-mm dish) or pGAL4-ZNF267 (0.5 μg per 60-mm dish) expressing full-length ZNF267. Two hours after transfection, cells were treated with 16 or 160 nM TSA for 22 h. Luciferase activity was normalized to total cellular protein. Data represent means ± standard error of the mean (SEM) of four experiments performed in duplicate and are expressed relative to the luciferase activity of untreated cells transfected with pGAL4 alone. (B) A luciferase reporter plasmid (0.5 μg per 60-mm dish) containing four GAL4 DNA consensus binding sites was transiently transfected into HEK 293 cells alone (first bar) or with 0.5 μg pGAL4-ZNF267(1–2332) per 60-mm dish expressing full-length ZNF267. Twenty-four hours after transfection, cells were serum starved for 8 h and stimulated with IL-1β (2.5 ng/ml) or SNAP (500 μM) in medium without serum for additional 24 h. Luciferase activity was normalized to total cellular protein. Luciferase activity was compared to untreated cells co-transfected with the luciferase reporter gene and pGAL4-ZNF267(1–2332). Data represent means ± standard error of the mean (SEM) of four experiments performed in duplicate. $^{*}p \leqslant 0.05$, when compared to untreated cells transfected with the reporter gene and pGAL4-ZNF267(1–2332).

of TSA, cells were harvested 24 h after transfection. Compared to the cells analyzed 48 h after transfection (Fig. 3C), this shorter time does not result in maximal repressor activity of ZNF267 (50% 48 h after transfection versus 30% 24 h after transfection).

Thus, it is unlikely that HDAC mediates transcriptional repression of ZNF267.

To further characterize the transactivation of ZNF267, the potential of various mediators and cytokines was screened to modulate transactivation of ZNF267. IL-1β and SNAP, a nitric oxide (NO) donor,

both significantly increased the transactivation of ZNF267 (Fig. 4B). In a control experiment, IL-1β or SNAP showed no effect on pGAL4 luciferase activity in cells co-transfected with the empty expression vector pFA (data not shown). Other factors including transforming growth factor (TGF)β, interferon γ, tumor necrosis factor (TNF)α, or lipopolysaccharide (LPS) did not change the transactivation potential of ZNF267 (data not shown).

Gene expression pattern of cells ectopically expressing ZNF267

To further characterize the functional properties of ZNF267, the HA-tagged plasmid pcDNA-ZNF267 with a CMV promoter driving the expression of full-length ZNF267 was transiently transfected into HEK 293 cells.

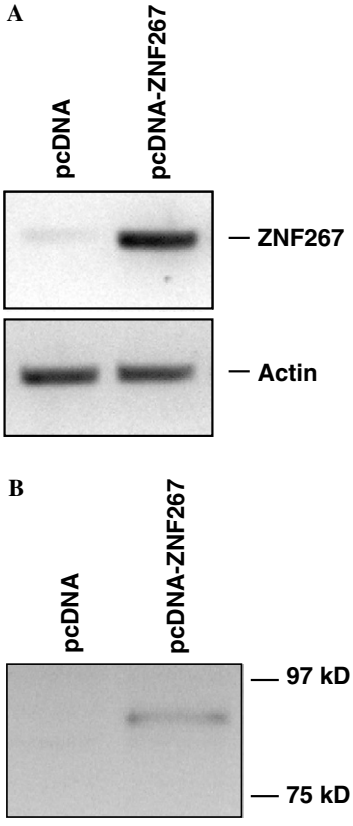

Fig. 5. Over-expression of ZNF267. HEK 293 cells were transiently transfected with pcDNA-ZNF267 expressing HA-tagged full-length ZNF267 or the empty vector pcDNA for 48 h. (A) The expression of ZNF267 was analyzed by semi-quantitative RT-PCR using 1 μg of total RNA. cDNA was analyzed from cells transfected with control vector pcDNA or pcDNA-ZNF267. Actin (below) was amplified to confirm equal amount of mRNA present in each sample. Shown is a representative experiment. (B) Total cell lysates were prepared from transfected cells and immunoprecipitated with an anti-HA antibody. After SDS–PAGE, proteins were transferred to nitrocellulose and immunoblotted with an anti-HA antibody. Molecular weights (kDa) are indicated on the right side of the immunoblot. Shown is a representative experiment.

RT-PCR analysis confirmed ectopic expression of ZNF267 mRNA in cells transfected with pcDNA-ZNF267 compared to cells transfected with the control vector pcDNA (Fig. 5A). Using immunoprecipitation and Western blotting an HA-antibody detected the expression of ZNF267 with an approximate molecular weight of 90 kDa protein in cells transfected with pcDNA-ZNF267 (Fig. 5B). Structural prediction revealed that ZNF267 has a calculated molecular weight of 87 kDa. Having confirmed that ZNF267 can be transiently over-expressed on the mRNA and protein level, gene chip microarray analysis was employed to compare changes in gene expression patterns of cells over-expressing ZNF267. Genes that differed in intensity by at least 3-fold were considered differentially regulated. Focusing on genes involved in fibrogenesis, several were down-regulated in response to ZNF267 overexpression including members of the MMP and ADAM family of proteases, like MMP-10, MMP-1, ADAM-12, ADAM-22, and ADAM-28. Compared with cells over-expressing ZNF267, control transfected cells showed higher mRNA levels for extracellular matrix proteins, including proteoglycans, keratin 9, and collagens, although collagen type IV, α6 showed a decreased expression in control cells (data not shown). Thus, ZNF267 induces changes in gene expression that would decrease matrix degradation.

ZNF267 inhibits MMP-10 gene expression and promoter activity

To confirm microarray data, we focused on the most suppressed gene, MMP-10. Control transfected cells showed detectable MMP-10 mRNA expression as determined by RNase protection assay. The level of MMP-10 mRNA was decreased in cells overexpressing ZNF267 (Fig. 6A). After normalization to GAPDH mRNA levels, overexpression of ZNF267 resulted in a decrease of MMP-10 mRNA by 67% (Fig. 6B).

To confirm the negative transcriptional regulation of MMP-10 by ZNF267, MMP-10 promoter luciferase reporter constructs were generated. Therefore, we first determined the 5′ untranslated region of MMP-10 gene. Using 5′RACE, a prominent DNA fragment with an approximate size of 100 bp was detected (Fig. 7A). After subcloning, the product was sequenced, and the transcription initiation site was determined to be 37 nt upstream of the start codon ATG. The difference between the fragment length detected on the agarose gel and the 37 nt, corresponds to and includes the RNA adaptor oligonucleotide, which was ligated to the 5′-end of MMP-10 mRNA, and to the gene specific primer, which hybridizes downstream of the ATG in the first exon (corresponding to nucleotide +43 to +76, being +1 the transcription initiation site). Next, we constructed plasmids containing approximately 2.6 and

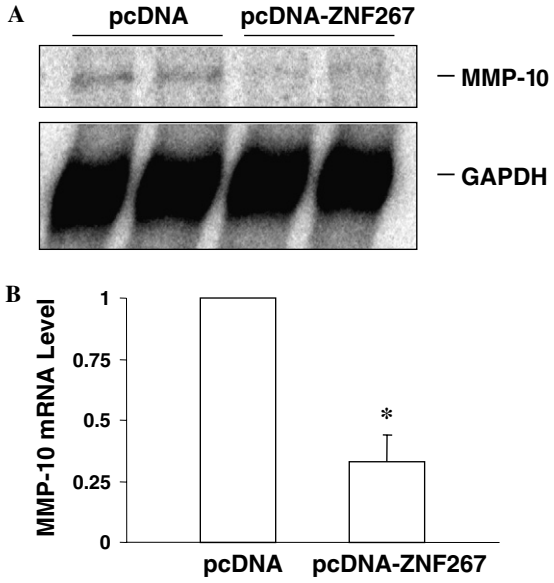

Fig. 6. ZNF267 inhibits endogenous MMP-10 gene expression. (A) HEK 293 cells were transfected with pcDNA or pcDNA-ZNF267 and harvested 48 h later. Total RNA (50 μg) was subjected to RNase protection assay with riboprobes specific for MMP-10 and GAPDH mRNA. Radiolabeled markers were run in parallel to determine the size of the protected bands (not shown). Shown is a representative result of two transfection experiments. (B) Expression of MMP-10 mRNA was normalized to GAPDH mRNA and summarized as result of four experiments. Data represent means ± standard error of the mean (SEM). Results are expressed relative to cells transfected with pcDNA; $*p \leqslant 0.05$.

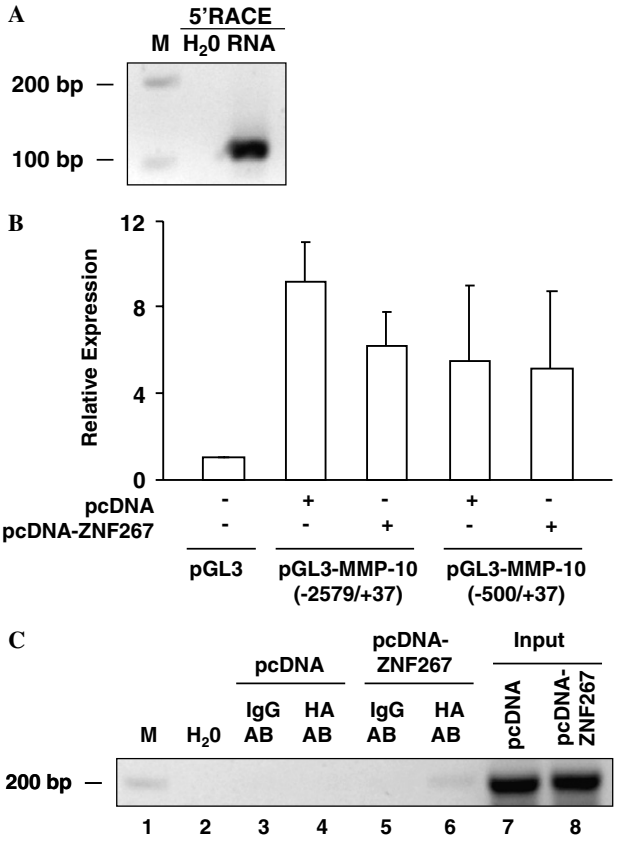

Fig. 7. ZNF267 decreases MMP-10 promoter activity and binds to the MMP-10 promoter region. (A) The transcriptional initiation site of the human MMP-10 gene was determined by 5′RACE and a DNA fragment was amplified. A negative control (H_2O) was included with PCRs. Lane M contains the molecular weight marker. (B) HEK 293 cells were co-transfected with the reporter plasmid pGL3-MMP-10(−2579/+37) (0.5 μg per 60-mm dish) or pGL3-MMP-10(−533/+37) (0.5 μg per 60-mm dish), which contains a 2.6 or 0.6 kb promoter fragment upstream from the start codon, respectively, and pcDNA-ZNF267 (0.25 μg per 60-mm dish) expressing full-length ZNF267, or the empty vector pcDNA (0.25 μg per 60-mm dish). Background measurements were performed in cells transfected with the empty luciferase reporter plasmid pGL3 alone. Luciferase activity was measured 48 h after transfection and was normalized to total cellular protein. Data represent means ± standard error of the mean (SEM) of three experiments performed in duplicate and are expressed relative to the luciferase activity of the empty luciferase reporter plasmid pGL3. (C) ChIP assay of genomic DNA extracted from HEK 293 cells, which were transfected with pcDNA-ZNF267 (lanes 5 and 6) or pcDNA as control (lanes 3 and 4), using anti-HA antibody (lanes 4 and 6) or anti-IgG as isotype control antibody (lanes 3 and 5) for immunoprecipitation. Precipitated DNA fragments were analyzed using specific primers amplifying a fragment from −2579 to −2379 of the 5′-flanking sequence of the MMP-10 gene. As positive ChIP input control for the PCR, an aliquot of genomic DNA of cells transfected with pcDNA-ZNF267 or pcDNA was used (lanes 7 and 8). Water served as negative control for the PCR (lane 2). Lane 1 contains the molecular weight marker.

0.6 kb of the 5′ region of the MMP-10 gene inserted upstream of a luciferase reporter gene. Both constructs had a common 3′-end. Luciferase activities of the constructs pGL3-MMP-10(−2579/+37) and pGL3-MMP-10(−533/+37) were measured by transient co-transfections with plasmids expressing ZNF267 (pcDNA-ZNF267) or the empty vector (pcDNA) in HEK 293 cells. When pcDNA-ZNF267 was co-transfected with the reporter construct pGL3-MMP-10(−2579/+37), MMP-10 promoter activity was reduced as compared to the control transfected cells (Fig. 7B). In contrast, co-transfection of pGL3-MMP-10(−533/+37) with pcDNA-ZNF267 did not decrease promoter activity as compared to cells transfected with pcDNA as control (Fig. 7B). These results indicate that ZNF267 acts within a region from −2579 to −533 of the MMP-10 promoter. The repression by ZNF267 was not due to a general suppressive effect on transcriptional regulation, since ZNF267 showed no effects on the luciferase activity of the collagen α1(I) promoter in co-transfection experiments in HEK 293 cells (data not shown).

To investigate whether ZNF267 directly binds to the MMP-10 promoter, we then performed ChIP assays. HEK 293 cells were transfected with pcDNA-ZNF267 or pcDNA as control, and the cell lysate prepared after fixation was immunoprecipitated with anti-HA antibody or anti-IgG isotype control antibody, followed by PCR with primers amplifying a fragment from −2579 to −2379 of the 5′-flanking sequence of the MMP-10 gene. In cells transfected with pcDNA-ZNF267, anti-HA antibody enriched the chromatin-containing DNA of the

MMP-10 promoter region (Fig. 7C, lane 6) as compared to incubation with isotype control antibody (Fig. 7C, lane 5). In HEK 293 cells transfected with the empty vector pcDNA alone, no enrichment was detected using anti-HA antibody or isotype control antibody for the immunoprecipitation (Fig. 7C, lanes 3 and 4). As a control, primers designed for a promoter region located further downstream did not reveal a difference in pcDNA-ZNF267 or pcDNA transfected cells using anti-HA antibody or isotype control antibody (data not shown). From these experiments, we conclude that ZNF267 binds to the MMP-10 promoter.

Discussion

The activation process of HSCs is the central event leading to hepatic fibrosis. Activated HSCs are the major source of the extracellular matrix proteins that are deposited in liver fibrosis and cirrhosis. In this report we demonstrate that ZNF267 mRNA is up-regulated during the activation process of human HSCs and in cirrhotic human liver in vivo. We show that ZNF267 protein is constitutively localized in the nucleus, and has an approximate molecular weight of 90 kDa. Using the GAL4 based transcriptional system identified the KRAB A domain in ZNF267 as a potent transcriptional repression domain. ZNF267 binds to the MMP-10 promoter region, and inhibits MMP-10 gene expression and promoter activity. Thus, inhibition of MMP-10 expression in activated HSCs might result in an increased accumulation of ECM and might promote liver fibrogenesis in vivo.

Transcription factors play a central role in gene regulation. While much attention has been focused on understanding transcriptional activation, it has become evident that transcriptional repression also plays a crucial role in gene regulation. Gene expression is fine tuned through transcription factors acting as activators or repressors. Among the DNA binding domains mediating repression is the KRAB domain [15–17]. The KRAB domain represses transcription through interaction with a common co-repressor known as KAP-1, TIF1β or KRIP-1 [34–36]. KAP-1 bound to KRAB associates with heterochromatin protein 1 isoforms, Setdb1 and histone deacetylases, which results in gene silencing [33,37]. Consistent with this, in our study the KRAB domain in ZNF267 acts as potent repressor of activated transcription when linked to the GLA4 DNA binding domain, whereas a construct lacking KRAB A domain does not modulate transcriptional activity. However, the repressor function is not abrogated using TSA, an inhibitor of class I and II HDAC activity. Class III HDACs are not inhibited by TSA [38], and might be involved in mediating the gene silencing effect of ZNF267.

Following liver injury, stimuli including oxidative stress, cytokines and early changes in ECM composition, initiate the activation process of HSCs. The initiation of this transdifferentiation process is associated with transcriptional events and is governed by transcription factors, which exert either positive or negative modulation of gene expression [1]. The activity of transcriptional regulators is modified at multiple levels, including expression, subcellular localization, and post-translational modifications. During the activation process of HSCs several transcription factors are either induced, like KLF6 [11], or show an increase in their DNA binding activity, like SP1 and nuclear factor-Y (NF-Y) [13]. We have identified another transcription factor ZNF267, a member of the family of Kruppel-like zinc fingers, as being up-regulated during the activation process in vitro. Analyzing the promoter of ZNF267, we have previously demonstrated that NF-Y is critical for ZNF267 gene expression [30]. Thus, increased NF-Y DNA binding activity might be responsible for the induction of ZNF267 mRNA expression during the activation process of HSCs. However, the upstream stimulus for increased NF-Y DNA binding activity in HSCs is currently unknown.

It has been reported that NO induces ZNF267 mRNA in a time- and dose-dependent manner [19], but is unlikely to be the stimulus for the induction of ZNF267, since NO levels decrease in acute liver injury [39]. Thus, the initial mediator to increase gene expression of ZNF267 in HSCs and during fibrosis in vivo remains to be identified. Although IL-1β and SNAP slightly increased the transactivation potential of ZNF267, no change in transcription rate or transactivation of ZNF267 was observed in response to several other stimuli known as potent initiators of fibrogenesis and HSC activation, including TGFβ1 and LPS. In addition, no change in the nuclear localization of ZNF267 was observed. Taken together, beside molecular mechanisms leading to the induction of ZNF267 mRNA, factors that modulate ZNF267 activity remain to be identified.

In acute liver injury, there is a balance between fibrogenesis and fibrolysis. Fibrolysis is governed by proteolytic enzymes, the most important are MMPs. In recurrent or chronic injury, fibrogenesis prevails over fibrolysis resulting in ECM accumulation and fibrosis. Fibrogenesis in chronic liver injury is characterized by the down-regulation of MMPs, an increase in expression of MMP inhibitors (TIMPs) and an up-regulation of ECM protein synthesis [40]. Similar observations have been made in cultured HSCs. The initial phase of the transition towards activation is accompanied by an increased expression of MMP-1 and MMP-3 indicative of a matrix degrading phenotype [41–43]. With increasing duration of culture

MMP expression is decreased, while the expression of TIMPs is markedly increased [43]. In addition, activated human HSCs express MMP-10 mRNA (data not shown).

Supportive of these observations is an animal model of acute liver injury, demonstrating that members of the MMP family, including MMP-10, are induced [44]. Another animal model to study reversibility of liver fibrosis showed that regression and resolution of liver fibrosis was accompanied by an increase of collagenase activity and a decrease in TIMP-1 and TIMP-2 expression [45], indicative that diminished degradation of fibrillar liver matrix is a central aspect in progression of liver fibrosis.

In this paper, we provide a novel molecular mechanism that might contribute to matrix accumulation in the extracellular space and might promote liver fibrogenesis in vivo. ZNF267, which is up-regulated in the initial phase of HSC activation, inhibits MMP-10 promoter activity in reporter gene assays. The finding of MMP-10 being a putative target gene of ZNF267 was confirmed by analyzing the endogenous MMP-10 mRNA product, which was inhibited in cells overexpressing ZNF267, and by demonstrating direct binding of ZNF267 to the MMP-10 promoter region using ChIP assays. Thus, inhibition of MMP-10 expression during the activation and perpetuation of the activated phenotype might tip the balance between fibrolysis and fibrogenesis in favor of ECM accumulation and progression of liver fibrosis. However, we are aware that MMP-10 expression was studied at the mRNA level, but most MMPs are closely regulated at the level of transcription [44,46]. MMP-10 has broad substrate specificity, being able to degrade proteoglycans, fibronectin, collagen type III and IV [46,47]. These ECM proteins accumulate in fibrotic and cirrhotic liver.

In conclusion, this is the first report of ZNF267 identifying its function as a transcriptional repressor. The inhibition of MMP-10 gene expression in activated HSCs suggests that ZNF267 might promote the development and progression of liver fibrosis by altering matrix degradation in vivo. However, further studies are required to investigate the pathophysiologic role of ZNF267 in the process of liver fibrosis and cirrhosis.

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