



# MMP-13 deletion attenuates N-nitrosodimethylamine induced hepatic fibrosis in mice

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## Abstract

The pathogenesis of hepatic fibrosis is a dynamic process involving several cell types and molecular events. Connective tissue growth factor (CTGF) is a major profibrogenic molecule and plays a significant role in the pathogenesis of hepatic fibrosis. Since matrix metalloproteinase-1 (MMP-1) is absent in mice, MMP-13 is responsible for cleavage and activation of CTGF. Here, we elucidated the role of MMP-13 and CTGF in the pathogenesis of hepatic fibrosis using MMP-13 knockout mouse. Hepatic fibrosis was induced in wild-type and MMP-13 knockout mice through intraperitoneal injections of N-nitrosodimethylamine (NDMA) in doses of 1 mg/100 g body weight on 3 consecutive days of every week over a period of 4 weeks. NDMA administrations resulted in marked elevation of serum AST, ALT, hyaluronic acid (HA), TGF- $\beta$ 1 and procollagen-III peptide in wild-type mice. There was marked activation of hepatic stellate cells, deposition of collagen and HA in the liver. However, all these changes were attenuated in NDMA administered MMP-13 knockout mice. Immunohistochemical staining demonstrated marked upregulation of CTGF in the necrotic and fibrotic areas of NDMA-treated wild-type mice but not in the knockout. Semiquantitative and qRT-PCR for collagen I,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), CTGF and TGF- $\beta$ 1 mRNA demonstrated marked upregulation in NDMA treated wild-type mice, but not in similarly treated MMP-13 knockout mice. Western blotting showed increased levels of collagen I,  $\alpha$ -SMA, CTGF, and TGF- $\beta$ 1 in wild-type fibrotic mice, but not in knockout. Our results demonstrated that MMP-13 plays a significant role in the pathogenesis of hepatic fibrosis through cleavage and activation of CTGF. Furthermore, our study indicates that blocking of CTGF using effective molecules has potential therapeutic application to prevent hepatic fibrosis.

## Introduction

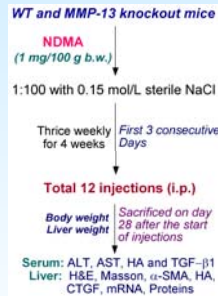
Hepatic fibrosis initiates with a chronic liver injury, either alcohol, drugs, virus, metabolic disorders or unknown reasons. The pathogenesis of hepatic fibrosis is always associated with oxidative stress and release of reactive oxygen species (ROS), which trigger a chain of molecular events that culminates in hepatic fibrosis. If the causative agent is not controlled or prevented hepatic fibrosis could lead to cirrhosis and ultimate death. The molecular pathogenesis of hepatic fibrosis is a very complex and dynamic process that involves several cell types and recruit of hepatic progenitor and inflammatory cells. The activation and transformation of resting hepatic stellate cells into myofibroblasts with the expression of several growth factors and connective tissue proteins play the key role in the progression of hepatic fibrosis.

Connective tissue growth factor (CTGF or CCN2) is a multifunctional cysteine-rich protein, acts through integrins and heparan sulfate-containing proteoglycans (HSPGs), involved in the regulation of cell growth and tissue remodeling. CTGF is expressed in mesenchymal cells during development and wound healing and is over expressed in hepatic fibrosis. CTGF plays a significant role in the transformation of resting hepatic stellate cells into myofibroblasts, which leads to the production of more CTGF. The upregulation of transforming growth factor beta-1 (TGF- $\beta$ 1) during pathogenesis of hepatic fibrosis also stimulates excessive production of CTGF. Since matrix metalloproteinase-1 (MMP-1) is absent in mice, MMP-13 is responsible for cleavage and maturation of CTGF. The active subunit of CTGF stimulates the synthesis of collagens and other connective tissue proteins that accumulate in the extracellular matrix of the liver. This process leads to scarring, loss of normal hepatic architecture, and cirrhosis. Appropriate strategy to inhibit the cleavage and maturation of CTGF would be promising to prevent the progression of hepatic fibrosis into cirrhosis.

## Materials and Methods

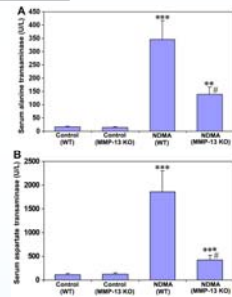
MMP-13 knockout and wild type male littermates in a C57BL/6J and 129/Sv hybrid background were generated from the intercross between MMP-13<sup>-/-</sup> mice. Hepatic fibrosis was induced by serial intraperitoneal injections of N-nitrosodimethylamine (NDMA) in doses of 1 mg (diluted 1:100 with 0.15 mol/L sterile NaCl)/100 g body weight. The injections were given on three consecutive days of each week over a period of 4 weeks. Control animals also received an equal volume of 0.15 M NaCl without NDMA. All the injected animals were sacrificed on day 28th of the experiment. Hepatic injury was assessed through hematoxylin and eosin as well as Masson's trichrome staining. The activation of hepatic stellate cells, evaluated through immunohistochemical staining for the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), was also considered as a marker for the extent of fibrosis. Alanine transaminases (ALT), aspartate transaminases (AST), hyaluronic acid (HA), and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) levels were measured in the serum. The expression of CTGF, TGF- $\beta$ 1,  $\alpha$ -SMA and collagen type I were also determined both at mRNA and protein levels in the hepatic tissue.

**Figure 1**



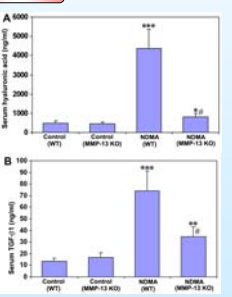
**Figure 1.** NDMA induced model of chronic liver injury and fibrosis. WT and MMP-13 knockout mice were injected intraperitoneally with NDMA thrice a week (1 mg/100 g body weight) on three consecutive days for 4 weeks. The animals were sacrificed on day 28 after the start of injections. Sera were collected and analyzed for ALT, AST, HA and TGF- $\beta$ 1 to determine the extent of liver injury and fibrosis. Livers were removed for histopathology, immunohistochemistry, Western blotting and PCR analysis.

**Figure 2**



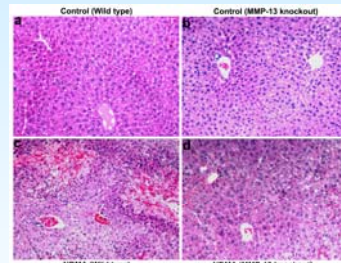
**Figure 2.** (A) Serum alanine transaminase (ALT), and (B) aspartate transaminase (AST) activities in WT and MMP-13 knockout mice. Both ALT and AST activities were significantly decreased in MMP-13 knockout mice compared to WT mice. (\*\*\*) $P < 0.001$  compared to the respective untreated control mice and # $P < 0.001$  compared to NDMA treated WT mice, N=6.

**Figure 3**



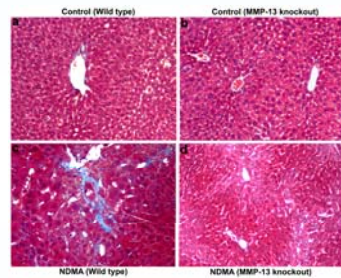
**Figure 3.** (A) Serum hyaluronic acid (HA) and (B) transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) levels in WT and MMP-13 knockout mice. Both HA and TGF- $\beta$ 1 levels were markedly reduced in MMP-13 knockout mice compared to WT mice. (\*\*\*) $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$  compared to the respective untreated control mice and # $P < 0.001$  compared to NDMA treated WT mice, N=6.

**Figure 4**



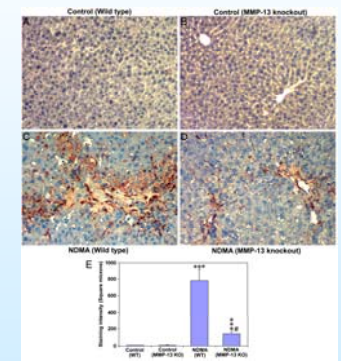
**Figure 4.** H&E staining. (A) WT mice (x100). (B) MMP-13 knockout mice (x100). No visible pathological alterations. (C) NDMA treated WT mice (x100). Massive hepatic necrosis and loss of normal architecture. Severe congestion of hepatic arteries and extensive hemorrhage. Infiltration of mononuclear and inflammatory cells. (D) NDMA treated MMP-13 knockout mice (x100). Slight hemorrhagic and mild centrilobular necrosis.

**Figure 5**



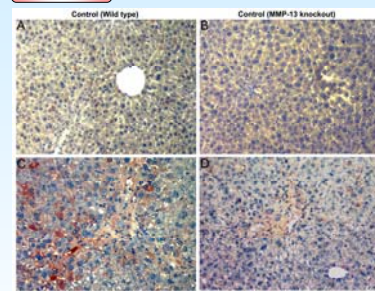
**Figure 5.** Masson's trichrome staining. (A) WT mice (x100). (B) MMP-13 knockout mice (x100). Absence of collagen staining. (C) NDMA treated WT mice (x100). Marked hepatic fibrosis with deposition of thick collagen fibers stained in blue. (D) NDMA treated MMP-13 knockout mice (x100). Absence of collagen staining and fibrosis.

**Figure 6**



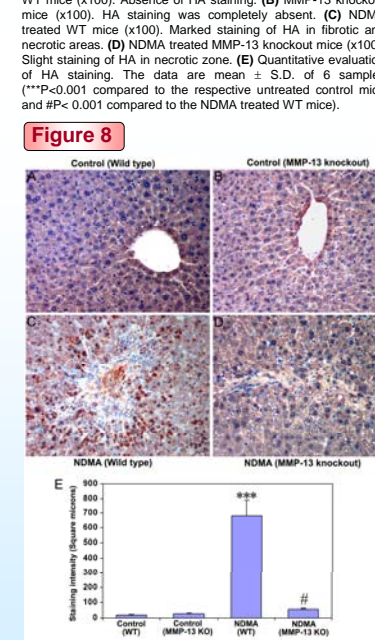
**Figure 6.** Immunohistochemical staining for  $\alpha$ -SMA demonstrating activated hepatic stellate cells. (A) WT mice (x100). Absence of  $\alpha$ -SMA staining. (B) MMP-13 knockout mice (x100). Absence of activated hepatic stellate cells (C) NDMA treated WT mice (x100). Extensive staining of  $\alpha$ -SMA demonstrating large number of activated stellate cells. (D) NDMA treated MMP-13 knockout mice (x100). Staining of  $\alpha$ -SMA in the necrotic areas. (E) Quantitative evaluation of activated stellate cells. The data are mean  $\pm$  S.D. of 6 samples (\*\*\*) $P < 0.001$  compared to the respective untreated control mice and # $P < 0.001$  compared to the NDMA treated WT mice.

**Figure 7**



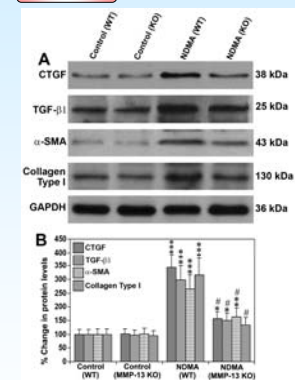
**Figure 7.** Histochemical staining for hyaluronic acid (HA). (A) WT mice (x100). Absence of HA staining. (B) MMP-13 knockout mice (x100). HA staining was completely absent. (C) NDMA treated WT mice (x100). Marked staining of HA in fibrotic and necrotic areas. (D) NDMA treated MMP-13 knockout mice (x100). Slight staining of HA in necrotic zone. (E) Quantitative evaluation of HA staining. The data are mean  $\pm$  S.D. of 6 samples (\*\*\*) $P < 0.001$  compared to the respective untreated control mice and # $P < 0.001$  compared to the NDMA treated WT mice.

**Figure 8**



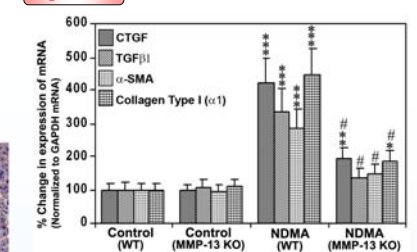
**Figure 8.** Immunohistochemical staining for connective tissue growth factor (CTGF). (A) WT mice (x100). Absence of CTGF staining in the liver parenchyma. (B) MMP-13 knockout mice (x100). Absence of CTGF staining in the liver parenchyma. (C) NDMA treated WT mice (x100). Moderate staining for CTGF surrounding hepatic vein. (D) NDMA treated MMP-13 knockout mice (x100). Slight staining of CTGF in the necrotic zone. (E) Quantitative evaluation of CTGF staining. The data are mean  $\pm$  S.D. of 6 samples (\*\*\*) $P < 0.001$  compared to the respective untreated control mice and # $P < 0.001$  compared to the NDMA treated WT mice.

**Figure 9**



**Figure 9.** (A) Western blotting for CTGF, TGF- $\beta$ 1,  $\alpha$ -SMA and collagen type I in the liver tissue of WT and MMP-13 knockout mice (B) Quantitative evaluation of Western blotting. The Western blot images were quantified using Gel-Pro analyzer software. The data are mean  $\pm$  S.D. of 6 samples (\*\*\*) $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$  compared to the respective untreated control mice and # $P < 0.001$  compared to the NDMA treated WT mice.

**Figure 10**



**Figure 10.** Quantitative real-time RT-PCR (qRT-PCR) for the expression of CTGF, TGF- $\beta$ 1,  $\alpha$ -SMA and collagen type I ( $\alpha$ 1) mRNA in the livers of WT and MMP-13 knockout mice. The data are mean  $\pm$  S.D. of 6 samples (\*\*\*) $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$  compared to the respective untreated control mice and # $P < 0.001$  compared to the NDMA treated WT mice.

## Conclusions

- \* Serial administrations of NDMA produced centrilobular necrosis and well developed fibrosis in mouse liver within 28 days.
- \* Treatment with NDMA resulted in increased levels of serum AST, ALT, HA, and TGF- $\beta$ 1, activation of hepatic stellate cells, upregulation of CTGF, TGF- $\beta$ 1,  $\alpha$ -SMA and collagen type I in wild type mice.
- \* Treatment with NDMA in MMP-13 knockout mice showed inhibition of fibrosis, marked reduction in the activation of hepatic stellate cells, downregulation of CTGF, TGF- $\beta$ 1,  $\alpha$ -SMA and collagen type I compared to similarly treated wild type mice.
- \* MMP-13 plays a crucial role in the pathogenesis of hepatic fibrosis through activation of CTGF.
- \* Effective blocking of CTGF has potential therapeutic implication to prevent hepatic fibrosis.