

Activator protein-1 is a novel regulator of mesencephalic astrocyte-derived neurotrophic factor transcription

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Received February 14, 2018; Accepted October 3, 2018

DOI: 10.3892/mmr.2018.9601

Abstract. Mesencephalic astrocyte-derived neurotrophic factor (MANF) is an endoplasmic reticulum stress-inducible protein, which has been suggested to be upregulated in inflammatory diseases; however, how inflammation regulates its transcription remains unclear. Activator protein-1 (AP-1), which is a transcription factor complex composed of c-Fos and c-Jun, is activated during the inflammatory process. The present study aimed to investigate whether the AP-1 complex regulates MANF transcription. The results of a luciferase reporter assay revealed that one of three putative AP-1 binding sites in the MANF promoter region is essential for enhancement of MANF transcription. Mechanistically, AP-1 was revealed to directly bind to the promoter region of the MANF gene by chromatin immunoprecipitation assay. Furthermore, MANF was strongly expressed in the liver tissues of patients with hepatitis B virus (HBV) infection, compared with in normal liver tissues from patients with hepatic hemangioma. Furthermore, c-Fos and c-Jun were also upregulated in the nuclei of hepatocytes from patients with HBV infection. In mice treated with carbon tetrachloride, the expression patterns of MANF, c-Fos and c-Jun were similar to those in patients with HBV. These results suggested that the AP-1 complex may be a novel regulator of MANF transcription, which may be involved in liver inflammation and fibrosis.

Introduction

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a recently discovered neurotrophic factor, which was initially isolated from astrocyte culture medium and was

revealed to selectively exert protective effects on dopaminergic neurons (1,2). Subsequent studies indicated that MANF is induced and secreted in response to experimental endoplasmic reticulum (ER) stress *in vitro* and *in vivo* (3-6). MANF has a protective role under physiological and pathophysiological conditions, including in neurodegenerative diseases, ischemic heart disease, diabetes mellitus, chondrodysplasia and polycystic ovary syndrome (4,6-15). Further studies have suggested that MANF protects against various forms of ER stress-induced cell damage via its C-terminal domain. Our previous studies also confirmed that MANF protects neurons in rats with middle cerebral artery occlusion-induced focal cerebral ischemia via alleviating ER stress (5,7,16,17). A recent study identified MANF as an immune modulator that serves a critical role in mediating tissue repair in the retina (18). Our previous study also indicated that MANF is involved in regulation of inflammation. Notably, it is upregulated in patients with rheumatoid arthritis and systemic lupus erythematosus, and in rabbits with antigen-induced arthritis (19). Further studies have demonstrated that MANF is a novel negative regulator of inflammation, functioning as an inhibitor of the nuclear factor (NF)- κ B signaling pathway by blocking the binding of p65 to the promoter of its target genes (20,21). In addition, it has been reported that MANF inhibits oxygen-glucose deprivation-induced cell damage and inflammatory cytokine secretion via suppressing ER stress in rat astrocytes in primary culture (22). Furthermore, MANF may decrease lipopolysaccharide (LPS)-induced proinflammatory cytokines, interleukin (IL)-1 β , tumor necrosis factor (TNF)- α and interferon- γ , through regulating the NF- κ B signaling pathway and phosphorylation of p38-mitogen-activated protein kinases in neural stem cells (23). However, how inflammation regulates MANF expression in inflammatory diseases remains unknown.

Activator protein-1 (AP-1) is a transcription factor complex composed of heterodimers or homodimers of members of the Fos, Jun, activating transcription factor and MAF protein families (24). The c-Fos/c-Jun heterodimer is the prototypic form of the AP-1 complex, which binds to its target DNA motifs to transcriptionally activate target genes (24,25). AP-1 and NF- κ B are the principal components of two critical inflammatory signaling pathways involved in the response

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Key words: mesencephalic astrocyte-derived neurotrophic factor, activator protein-1, c-Fos, c-Jun, inflammation, liver fibrosis

to inflammatory stimuli (26,27). Proinflammatory factors, such as TNF- α and IL-1, can activate the AP-1 signaling pathway (28,29). Using the Database of Transcriptional Start Sites (DBTSS) and Transcription Element Search Software (TESS), three putative binding sites of AP-1 were detected in the 5'-flanking region (-1,239 to +176 bp) of the human MANF gene in the present study.

Since the effects of AP-1 on MANF expression in inflammatory diseases remain to be elucidated, it was hypothesized that MANF may be a potential target gene of transcriptional activation by AP-1. The present study investigated the association between AP-1 and MANF expression in human liver tissues and a mouse liver injury model. In addition, the binding of AP-1 to the MANF promoter region was analyzed using chromatin immunoprecipitation (ChIP) assays.

Materials and methods

Collection of human liver tissues. Human liver tissues were obtained from 10 patients with hepatocellular carcinoma (HCC) and HBV infection and four control patients with hepatic hemangioma (HHG); these patients were admitted to the First Affiliated Hospital of Anhui Medical University (Hefei, China) and the Chinese People's Liberation Army 123 Hospital (Bengbu, China) between January and December 2014. General patient information is presented in Table I. All patients received partial hepatectomy. Final diagnosis of the hepatic nodules was confirmed by histological examination of biopsy specimens. All tissue samples were fixed in 4% neutral buffered formaldehyde at 4°C for 24 h and were embedded in paraffin. The use of human liver tissues was in accordance with the ethical standards of the Declaration of Helsinki. Written informed consent was obtained from all patients and the present study was approved by the Human Research Ethics Committee of Anhui Medical University (license number: 20131359).

Preparation of a murine model of liver fibrosis. A total of 12 C57BL/6J mice (age, 6 weeks; weight, ~20 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were individually housed in ventilated cages at the Anhui Medical University animal facility. The mice were kept in a controlled temperature (21 \pm 1°C) and humidity (50 \pm 5%) environment, under a 12 h light/dark cycle, with *ad libitum* access to food and water. To induce liver fibrosis, six male mice received an intraperitoneal injection of 2 μ l/g carbon tetrachloride (CCl₄) in sterile olive oil three times per week for 4 weeks. The six control mice were injected with same volume of sterile olive oil three times per week for 4 weeks. The body weight of the mice was monitored each week. The mice were sacrificed after 4 weeks, and livers were removed and were immediately fixed in 4% paraformaldehyde at 4°C for 24 h. All mouse studies were conducted according to protocols approved by the Animal Ethics Committee of Anhui Medical University (license number: 20160329).

Histological analysis of liver fibrosis. Mouse liver sections were stained with hematoxylin at 25°C for 8 min and eosin at 25°C for 3 sec (H&E), and Sirius red at 25°C for 1 h (SR), in order to evaluate the degree of inflammation and collagen

deposition, respectively. The degree of mouse hepatic fibrosis was determined by Masson trichrome staining according to the manufacturer's protocol (cat. no. MST-8003; Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, Fujian, China).

Immunohistochemistry (IHC). Paraffin-embedded mouse and human tissues sections (3–5 μ m) were deparaffinized and rehydrated prior to IHC for the detection of MANF, c-Fos and c-Jun proteins, as previously described (30). Briefly, antigen retrieval was performed by pressure cooking slides for 5 min in 0.01 M citrate buffer. Slides were incubated for 30 min at 37°C in 20% (vol/vol) hydrogen peroxide to block endogenous peroxidase activity and then washed in phosphate-buffered saline (PBS). Following blocking with 10% normal goat serum, the sections were incubated with primary antibodies, including rabbit anti-MANF (dilution 1:1,000; cat. no. ab126321; Abcam, Cambridge, MA, USA), rabbit anti-c-Fos (dilution 1:100; cat. no. 2250; Cell Signaling Technology, Inc., Danvers, MA, USA) and rabbit anti-c-Jun (dilution 1:250; cat. no. ab32137; Abcam), at 4°C overnight, followed by horseradish peroxidase-conjugated secondary antibody at 37°C (dilution 1:2,000; cat. no. TA140003, OriGene Technologies, Inc., Beijing, China) for 30 min. Normal IgG (dilution 1:100; cat. no. 3900; Cell Signaling Technology, Inc.) was used as a control. DAB was used for visualization and dark-brown staining was considered positive. Immunohistochemical staining was visualized and images were captured using an Olympus BX35 microscope (Olympus Corporation, Tokyo, Japan); staining was analyzed by a blinded independent pathologist. For analysis, the total cells were counted in >5 views from one slide and the positive intensity was measured by ImageJ (version 1.8.0, National Institutes of Health, Bethesda, MD, USA). Briefly, positive staining for each antibody was determined based on the percentage of positive cells (0, negative; 1+, weak; 2+, moderate; 3+, strong). Representative images were collected using the Olympus BX35 microscope.

Cell culture and transfection. The HepG2 human liver cancer cell line and 293 cells, selected for ease of transfection, were obtained from CHI Scientific, Ltd. (Jiangyin, Jiangsu, China). All cells were maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Tianjin Ankangyuan Technology Development Co., Ltd., Tianjin, China) at 37°C in a humidified atmosphere containing 5% CO₂. The HepG2 human liver cancer cells (0.8 \times 10⁵ cells per well in 24-well plates in serum-free medium) were transiently transfected with the pGL3-hMANF plasmids and pcDNA3.1/c-Fos or pcDNA3.1/c-Jun plasmids with 800 ng/ml at 37°C for 24 h using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Prediction of transcription factor binding sites. The putative binding sites for AP-1 in the 5'-flanking region (-1,239 to +176 bp) of the human MANF gene were predicted using the DBTSS (<http://dbtss.hgc.jp/index.html>, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo,

Table I. Clinical characteristics of the selected subjects.

| Characteristic | HBV (n=10) | HHG (n=4) |
|---|-----------------|----------------|
| Sex, n (%) | | |
| Male | 9 (90) | 3 (75) |
| Female | 1 (10) | 1 (25) |
| Age, years (means \pm standard deviation) | 51.2 \pm 3.9 | 53.0 \pm 6.7 |
| Etiology, n (%) | | |
| HBV | 10 (100%) | - |
| HCV | 0 | - |
| Others | 0 | - |
| Liver injury, U/l (means \pm standard deviation) | | |
| Serum ALT | 74.2 \pm 69.2 | 13.8 \pm 4.9 |
| Serum AST | 52.8 \pm 42.6 | 18.5 \pm 1.7 |

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; HHG, hepatic hemangioma.

Japan) and TESS version 10.0 beta (<http://www.cbil.upenn.edu/teess/>, Computational Biology and Informatics Laboratory, University of Pennsylvania, Philadelphia, PA, USA).

Cloning and mutagenesis. The 1,415-bp human MANF promoter region spanning -1,239 to +176 bp was amplified by polymerase chain reaction (PCR) from human genomic DNA extracted from 293 cells using the primer sets 5'-CCATTG TCCCAAGAGGTATTTT-3' (forward) and 5'-CTATCCCGC ACCTTCGCAG-3' (reverse). PCR products were then ligated into a pMD-18-T vector (cat. no. 6011; Takara Biomedical Technology Co., Ltd, Beijing, China) using *KpnI* and *HindIII*, followed by subcloning into the dual luciferase expression vector pGL3-Basic (Promega Corporation, Madison, WI, USA), in order to prepare the recombinant plasmid pGL3-hMANF (-1,293/+176) expressing the entire promoter region of human MANF. The sequencing results were compared with the human MANF cDNA sequence reported in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>). Three truncates, pGL3-hMANF (-880/+83), pGL3-hMANF (-423/+83), and pGL3-hMANF (-265/+83), expressing 963-, 506- and 348-bp fragments of the human MANF promoter region, respectively, were generated using pGL3-hMANF (-1,293/+176) as a template and the following primer sets: pGL3-hMANF (-880/+83), forward 5'-CGGGGTACCCAGTGCTTCTCTGGTGAT TCCC-3', reverse 5'-GGGAAGCTTCATCCTCCTCATCCT CCTCATC-3'; pGL3-hMANF (-423/+83), forward 5'-CGG GGTACCGTCTTGCTGACCCAGAACTC-3', reverse 5'-GGGAAGCTTCATCCTCCTCATCCTCCTCATC-3'; and pGL3-hMANF (-265/+83), forward 5'-CGGGGTACCCCA CACCGCTTCCGTCG-3' and reverse 5'-GGGAAGCTTCAT CCTCCTCATCCTCCTCATC-3'. The deletion mutants were subcloned into the luciferase expression vector pGL3-Basic to generate pGL3-hMANF (-880/+83), pGL3-hMANF (-423/+83) and pGL3-hMANF (-265/+83) plasmids.

Mutagenesis of putative AP-1 binding sites located at position -421 and -326 of the MANF promoter was performed using a Multipoints Mutagenesis kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. All mutations/substitutions in the DNA were confirmed by Sanger sequencing (Sangon Biotech Co., Ltd., Shanghai, China).

Luciferase assay. A luciferase assay was performed using a Dual Luciferase Reporter Assay system (Promega Corporation), according to the manufacturer's protocol. Briefly, HepG2 cells were seeded in 24-well plates at a density of 0.8×10^5 cells/well and were cotransfected with the pGL3-hMANF plasmids and c-Fos or c-Jun-expressing plasmids with 800 ng/ml at 37°C for 24 h. The *Renilla* luciferase reporter plasmid pRL-TK (Promega Corporation) was used as an internal control. Cells were lysed 24 h post-transfection using lysis buffer (Promega Corporation) and luciferase activity was immediately determined using a GloMax™ 20/20 luminometer (Promega Corporation). The results were normalized to corresponding pRL-TK activity. Independent experiments were performed at least three times.

ChIP assay. ChIP was performed using a Agarose ChIP assay kit (cat. no. 26156; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The nuclei were isolated from HepG2 liver cancer cells following treatment with phorbol-12-myristate-13-acetate (10 nM) at 37°C for 2 h. Chromatin complexes were immunoprecipitated with 4 μ g anti-c-Jun (cat. no. ab32137; Abcam, Cambridge, MA, USA) or anti-c-Fos (cat. no. ab7963; Abcam), according to the manufacturer's protocol. A parallel immunoprecipitation was carried out using 2 μ l normal rabbit IgG in 500 μ l diluted lysate (cat. no. 26156, Thermo Fisher Scientific, Inc.) as a negative control and 10 μ l anti-RNA polymerase II antibody in 500 μ l diluted lysate (cat. no. 26156, Thermo Fisher Scientific, Inc.) as a positive control. PCR was performed to amplify a 236-bp fragment containing the putative AP-1 binding sites using appropriate primers (forward, TCACATTCTCACCAG CCACT; reverse, CAGGTCGATCTGCTTGTCATAC), as previously described (21). PCR products were resolved on a 1.5% agarose gel and were visualized by ethidium bromide (cat. no. 123945-8, Sangon Biotech Co., Ltd.) staining.

Statistical analysis. Data are presented as the means \pm standard deviation. All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Two-way analysis of variance with Tukey's post hoc correction was conducted for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Screening of putative AP-1 binding sites in the MANF promoter region. To screen the transcriptional regulators of MANF, the promoter region of the human MANF gene within a sequence located 1,239 bp upstream of the transcription start site was analyzed using DBTSS and TESS. Three putative AP-1 binding sites were identified as follows: At -554 bp (5'-GTCA-3'; AP-1-1), at -421 bp (5'-TGAC-3'; AP-1-2) and at -326 bp (5'-TGCA-3'; AP-1-3) (Fig. 1A).

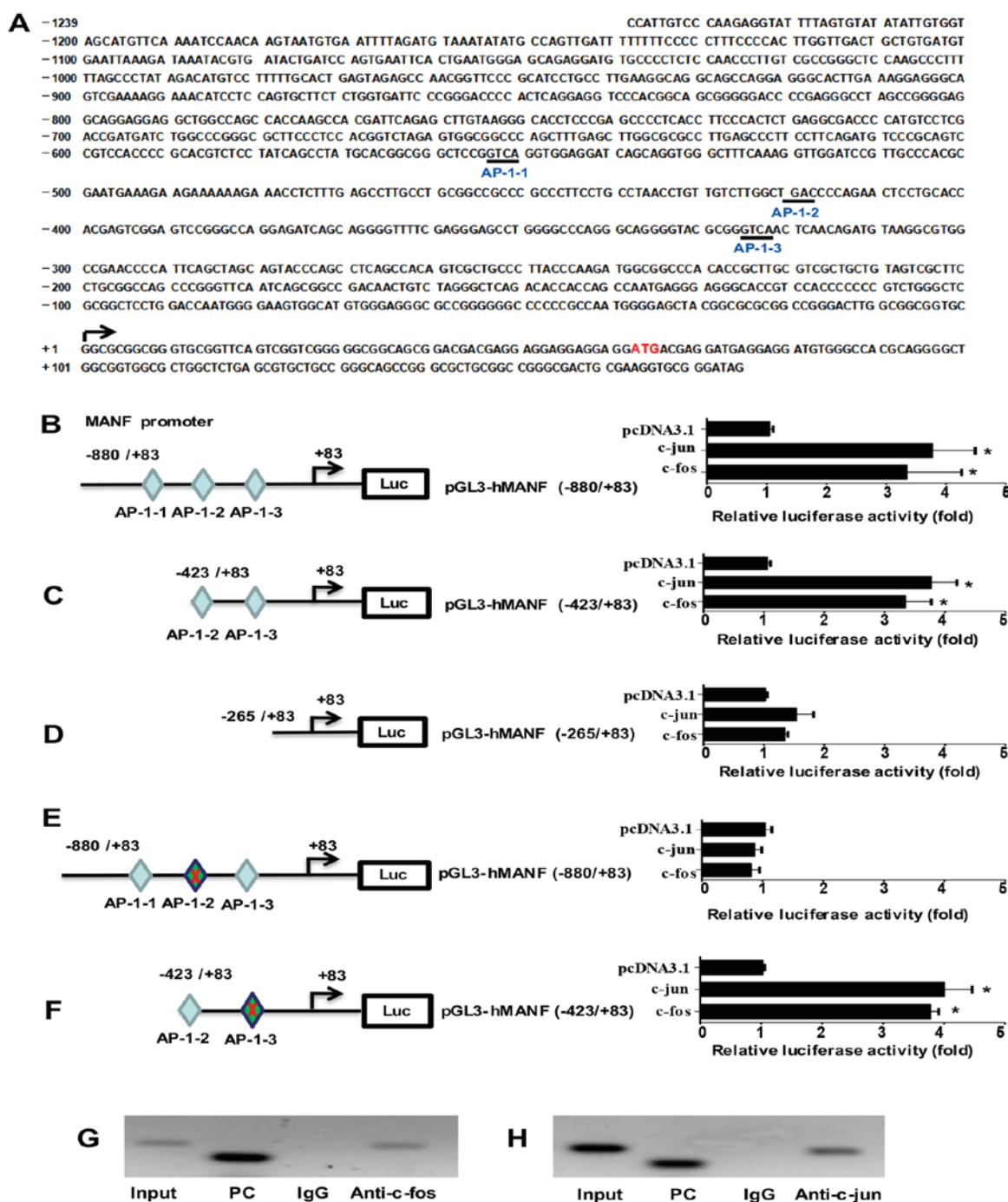


Figure 1. AP-1 binds to the MANF promoter region and regulates MANF transcription. (A) Prediction of three putative AP-1 binding sites in the promoter region of the human MANF gene. (B-D) Overexpression of c-Fos or c-Jun enhanced MANF transcription. Luciferase assay was performed in HepG2 liver cancer cells transfected with plasmids expressing c-Fos or c-Jun together with (B) MANF promoter fragment (-880/+83) containing all three putative AP-1 binding sites, (C) truncate (-423/+83) containing two of the putative AP-1 binding sites, or (D) truncate (-265/+83) without the putative AP-1 binding sites cloned in pGL3 basic. (E and F) Effects of mutation of the putative AP-1 binding sites on MANF transcriptional activity. (E) Mutation of AP-1-2 abolished the enhancement of MANF transcription by c-Fos or c-Jun. (F) AP-1-3 mutation had no effect on the enhancement of MANF transcription by c-Fos or c-Jun. (G and H) Binding of c-Fos or c-Jun to the MANF promoter region. The nuclei were isolated from HepG2 liver cancer cells following treatment with phorbol-12-myristate-13-acetate (10 nM) for 2 h, and a ChIP assay was performed with antibodies against (G) c-Fos or (H) c-Jun. Normal goat IgG and anti-RNA polymerase II antibody were used as a negative control and PC, respectively. Data are presented as the means \pm standard deviation. The experiments were repeated at least three times. * $P < 0.05$, compared with the pcDNA3.1 empty vector. AP-1, activator protein-1; IgG, immunoglobulin G; MANF, mesencephalic astrocyte-derived neurotrophic factor; PC, positive control.

Identification of the AP-1 binding site involved in MANF transcriptional regulation. To confirm the aforementioned sites as AP-1 binding sites, the MANF promoter region and the truncates were cloned into the pGL3-Basic vector to form pGL3-hMANF (-880/+83) (Fig. 1B), pGL3-hMANF

(-423/+83) (Fig. 1C) and pGL3-hMANF (-256/+83) (Fig. 1D). Subsequently, luciferase activity was detected after co-transfecting HepG2 liver cells with the pGL3-hMANF plasmids and c-Fos or c-Jun-expressing plasmids. The plasmids were successfully transfected, as verified using

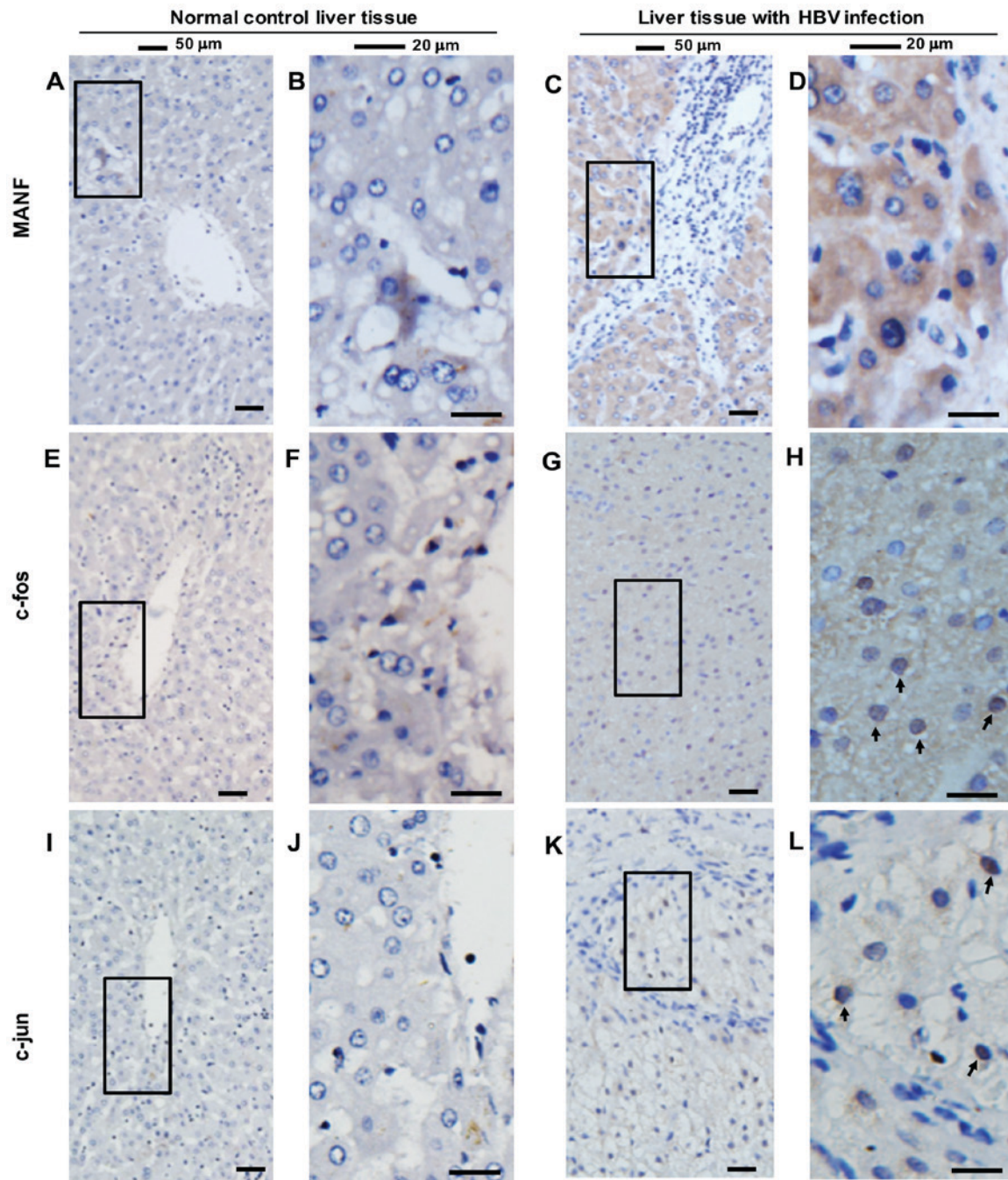


Figure 2. Expression of MANF and AP-1 complex in the liver tissues of patients with HBV infection. (A-D) MANF expression was detected in liver tissues from patients with (A and B) HHG and (C and D) HBV. (B) and (D) are higher magnification images of the areas shown in (A) and (C), respectively. (E-H) c-Fos expression was detected in liver tissue from patients with (E and F) HHG and (G and H) HBV. (F) and (H) are higher magnification images of the areas shown in (E) and (G), respectively. (I-L) c-Jun expression was detected in liver tissues from patients with (I and J) HHG and (K and L) HBV. (I) and (K) are higher magnification images of the areas shown in (J) and (L), respectively. Arrows indicate positive cells. HBV, hepatitis B virus; HHG, hepatic hemangioma; MANF, mesencephalic astrocyte-derived neurotrophic factor.

immunofluorescence and western blotting (data not shown). As shown in Fig. 1B-D, c-Jun and c-Fos were able to significantly increase the luciferase activity of cells transfected with plasmids containing the MANF promoter and the -423/+83 truncate, but not the -256/+83 truncate, which does not contain the putative AP-1 binding site. In addition, there was no significant difference in luciferase activity between pGL3-hMANF (-880/+83) and pGL3-hMANF (-423/+83) truncates was observed (Fig. 1B and C), thus suggesting that the AP-1-1 binding site may not be required for regulating

MANF transcription. These findings indicated that the two putative binding sites AP-1-2 and AP-1-3 are required for the enhancement of MANF promoter activity by AP-1.

To further confirm the essential binding sites for AP-1 in the MANF promoter region, mutations were introduced into the putative binding sites AP-1-2 (TGAC→CAGC) (Fig. 1E) and AP-1-3 (GTCA→CAGC) (Fig. 1F). HepG2 liver cells were co-transfected with the mutated plasmids and c-Fos or c-Jun, and the reporter activity was detected. The results revealed that the AP-1-2 mutation abolished the enhancement of MANF

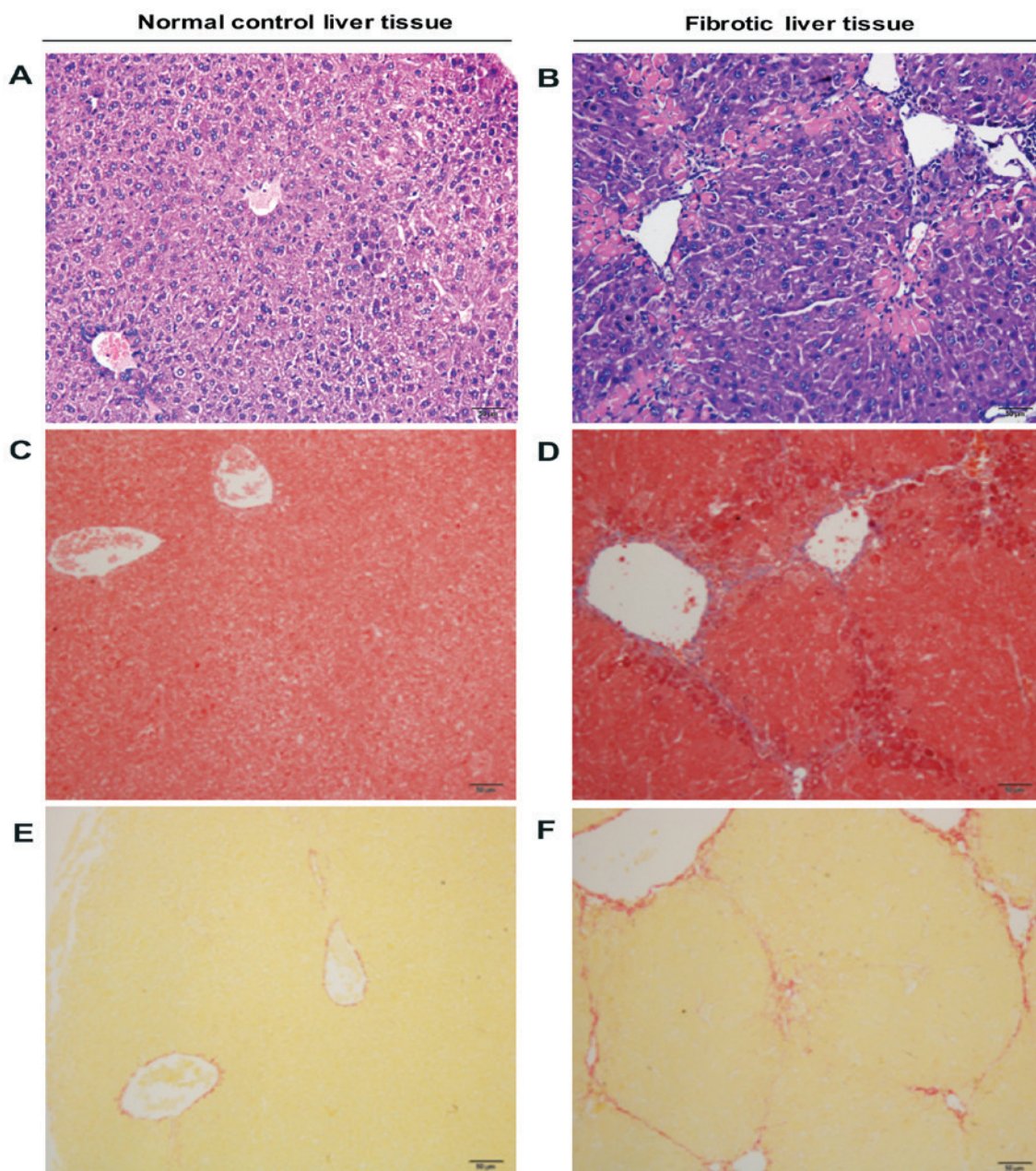


Figure 3. Histological analysis of liver fibrosis in mice. (A and B) Hematoxylin and eosin staining; (C and D) Masson trichrome staining; (E and F) Sirius red staining. (A, C and E) Tissues from normal control mice; (B, D and F) tissues from model mice treated with carbon tetrachloride. Scale bar, 50 μ m. MANF, mesencephalic astrocyte-derived neurotrophic factor.

promoter activity by c-Fos or c-Jun (Fig. 1E). However, the AP-1-3 mutation did not alter the effects of c-Fos and c-Jun on MANF promoter activity (Fig. 1F), thus suggesting that AP-1-2 (5'-TGAC-3') is the essential binding site of AP-1 required for MANF transcriptional regulation.

AP-1 directly binds to the promoter region of the MANF gene. The present study performed ChIP analysis to validate the direct binding of AP-1 to the MANF promoter region. For this experiment, ChIP with anti-c-Fos and c-Jun antibodies was performed. The pulled down DNA was subjected to PCR (Fig. 1G, lane 4 and Fig. 1H, lane 4) to amplify the MANF promoter region containing the AP-1 binding site. As shown in Fig. 1G and H, c-Fos and c-Jun were revealed to bind to the MANF promoter, thus suggesting that upregulation of MANF

may be caused by the direct binding of AP-1 to the promoter region of the MANF gene.

Expression of MANF and AP-1 in human and murine liver samples. Previous studies have demonstrated that MANF is an ER stress-inducible protein (3), which is upregulated in inflammatory diseases (19,21). To determine the expression of MANF in human liver samples, liver tissues were collected from 10 patients with hepatocellular carcinoma (HCC) and HBV infection, and from four patients with HHG (Fig. 2). It was revealed that MANF was strongly expressed in the paracancerous liver tissue of five patients with HCC and HBV infection (Fig. 2C and D), compared with in normal liver tissues from patients with HHG (Fig. 2A and B). Additionally, it was revealed that c-Fos and c-Jun were upregulated in the nuclei

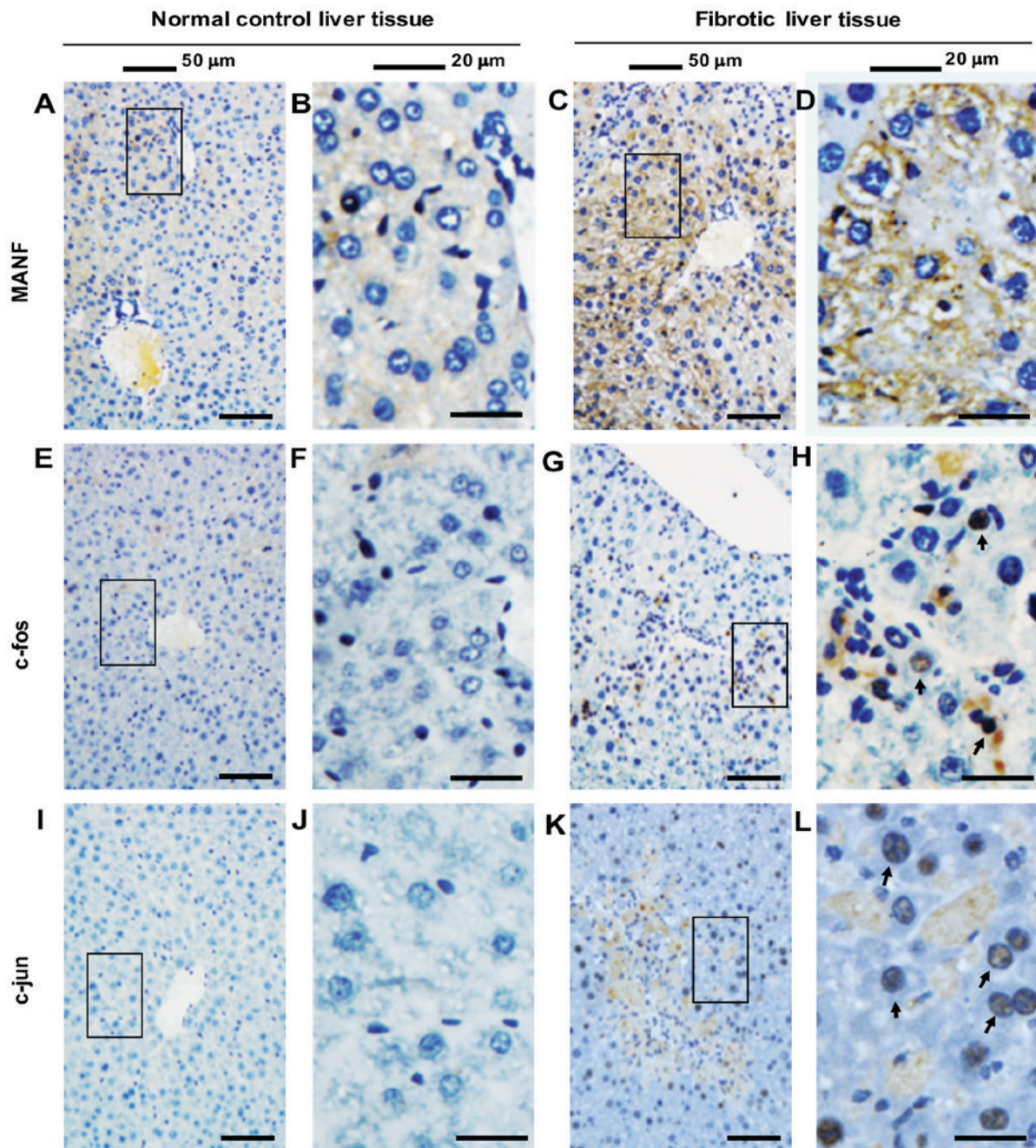


Figure 4. Expression of MANF and AP-1 complex in the liver tissues of mice treated with carbon tetrachloride. (A-D) MANF expression was detected in the (A and B) normal control liver tissues and (C and D) fibrotic liver tissues. (B) and (D) are higher magnification images of the areas shown in (A) and (C), respectively. (E-H) c-Fos expression was detected in (E and F) normal control liver tissues and (G and H) fibrotic liver tissues. (F) and (H) are higher magnification images of the areas shown in (E) and (G), respectively. (I-L) c-Jun expression was detected in (I and J) normal control liver tissues and (K and L) fibrotic liver tissues. (I) and (K) are higher magnification images of the areas shown in (J) and (L), respectively. Arrows indicate positive cells. MANF, mesencephalic astrocyte-derived neurotrophic factor.

of hepatocytes of patients with HBV infection (Fig. 2E-L, indicated by arrows).

MANF expression was also detected in liver tissues from mice treated with CCl_4 . In the mouse liver injury model, CCl_4 -induced liver fibrosis was initially evaluated via H&E, SR and Masson trichrome staining. As shown in Fig. 3, CCl_4 treatment for 4 weeks resulted in liver inflammation and fibrosis. Furthermore, it was revealed that few MANF-positive cells were detected in the liver samples obtained from the control group. Conversely, in CCl_4 -treated mice, the majority of hepatocytes were MANF-positive (Fig. 4A-D). Coincidentally,

c-Fos and c-Jun were also strongly expressed in the nuclei of hepatocytes in CCl_4 -treated mice, compared with in the control mice (Fig. 4E-L). These results suggested that MANF and AP-1 may be involved in liver inflammation, and that MANF expression may be associated with AP-1 under such a condition.

Discussion

Our previous studies demonstrated that MANF is upregulated under various conditions, including ischemia and hypoxia,

and in response to inflammatory stimuli, all of which are associated with ER stress (5,7,19,21). However, how MANF is upregulated in response to ER stress, particularly in an inflammatory state, remains unclear. A previous study demonstrated that the unfolded protein response is able to induce mouse MANF expression via ER stress-responsive element (ERSE) II (31). Recently, it was reported that ERSE I serves a dominant role in mediating X-box binding protein 1-induced MANF expression, whereas ERSE II exerts little impact on MANF transcription (32). The present study demonstrated that the AP-1 complex, comprised of c-Fos and c-Jun subunits, may enhance MANF transcription through binding to the sequence TGAC (-421/-418 bp) within the MANF promoter region. Collectively these findings suggested that MANF transcription may be regulated by numerous mechanisms. The current findings may aid to understand how MANF is regulated under inflammatory conditions.

AP-1 is part of an important inflammatory signaling pathway and is activated by LPS and TNF- α via the signaling pathways of TNF receptor-associated factor 6 and transforming growth factor- β -activated kinase 1 (33,34). Activation of the AP-1 signaling pathway promotes the expression of inflammatory factors, including IL-1 β , IL-6 and inducible nitric oxide synthase (35). The present study revealed that MANF was a downstream target, which was upregulated by c-Fos and c-Jun as part of the AP-1 complex, via a direct binding interaction with the MANF gene promoter. In addition, only one binding site was effective in enhancing MANF transcription among the three putative AP-1 binding sites identified. Unlike other AP-1 targets, MANF reportedly exerts anti-inflammatory activity by negatively regulating the NF- κ B signaling pathway (21), thus suggesting that the AP-1 signaling pathway may be involved in dual-directional regulation of inflammation.

Previous studies have reported that HBV replication and expression of hepatoviral proteins, such as HBV protein X, are associated with potent activation of AP-1 (36-39), and that AP-1 expression is increased in HBV (40,41). Upregulation of AP-1 has also been reported in mouse liver fibrosis induced by CCl₄ treatment and common bile duct ligation (42,43), which is consistent with the present findings. Although c-Fos and c-Jun were initially considered as oncogenes, it has been revealed that c-Fos can suppress the growth of murine hepatocytes by inducing cell cycle inhibition and cell death (44). Regarding the mechanisms underlying the effects of AP-1 activation on liver fibrosis, this likely occurs via the ER stress response/extracellular signal-regulated kinase (ERK)/AP-1 signaling pathway, as it has been reported that ER stress inducers activate AP-1-associated genes, including c-Fos and c-Jun, in an ERK-dependent manner in hepatic cells and murine livers (45). In the present study, MANF expression was increased in the liver tissues of patients with HBV infection and of mice with CCl₄-induced liver fibrosis. Therefore, it may be hypothesized that the increase in MANF expression in the liver may partially be stimulated by AP-1. However, the impact of MANF on liver inflammation requires further investigation. We have prepared hepatocyte-specific MANF knockout mice and mono-macrophage-specific MANF knockout mice, and aim to compare liver inflammation and fibrosis in wild-type mice and MANF knockout mice in

the future; in addition, the underlying mechanisms will be investigated.

In conclusion, the present study demonstrated that the AP-1 complex may be a novel regulator of MANF transcriptional enhancement, and that MANF is a novel downstream target of AP-1, which may indicate a novel role of AP-1 in regulating inflammatory pathways. Therefore, targeting the AP-1/MANF signaling pathway may be a potential therapeutic strategy for the treatment of inflammation and liver injury.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 91129729, 81372576 and 81673438 awarded to YXS) and the Youth Fund Project of the First Affiliated Hospital of Anhui Medical University (grant no. 2009kj19 awarded to CHW).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CHW performed the experiments and wrote the manuscript; TCJ, WMQ and LZ performed some of the experiments; YJS and LJF helped to analyze the data and revised the manuscript; YXS designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of human liver tissues was in accordance with the ethical standards of the Declaration of Helsinki. Written informed consent was obtained from all patients and the present study was approved by the Human Research Ethics Committee of Anhui Medical University (license number: 20131359). All mouse studies were conducted according to protocols approved by the Animal Ethics Committee of Anhui Medical University (license number: 20160329).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have competing interests.

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