

# Hepatitis C Virus E2 Protein Induce Reactive Oxygen Species (ROS)-Related Fibrogenesis in the HSC-T6 Hepatic Stellate Cell Line

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## ABSTRACT

Chronic infection of hepatitis C virus (HCV) leads to hepatic fibrosis and subsequently cirrhosis, although the underlying mechanisms have not been established. Previous studies have indicated that the binding of HCV E2 protein and CD81 on the surface of hepatic stellate cells (HSCs) lead to the increased protein level and activity of matrix metalloproteinase (MMP) 2, indicating that E2 may involve in the HCV-induced fibrosis. This study was designed to investigate the involvement of HCV E2 protein in the hepatic fibrogenesis. Results showed that E2 protein may promote the expression levels of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and collagen  $\alpha$ (I). Furthermore, several pro-fibrosis or pro-inflammatory cytokines, including transforming growth factor (TGF)- $\beta$ 1, connective tissue growth factor (CTGF), interleukin (IL)-6 and IL-1 $\beta$ , were significantly increased in E2 transfected-HSC cell lines, while the expression of MMP-2 are also considerably increased. Moreover, the significant increases of CTGF and TGF- $\beta$ 1 in a stable E2-expressing Huh7 cell line were also observed the same results. Further molecular studies indicated that the impact of E2 protein on collagen production related to higher production of ROS and activated Janus kinase (JAK)1, JAK2 and also enhance the activation of ERK1/2 and p38, while catalase and inhibitors specific for JAK, ERK1/2, and p38 abolish E2-enhanced expression of collagen  $\alpha$ (I). Taken together, this study indicated that E2 protein involve in the pathogenesis of HCV-mediated fibrosis via an up-regulation of collagen  $\alpha$ (I) and oxidative stress, which is JAK pathway related. *J. Cell. Biochem.* 112: 233–243, 2011. © 2010 Wiley-Liss, Inc.

**KEY WORDS:** HEPATITIS C VIRUS E2 PROTEIN; FIBROSIS; COLLAGEN; OXIDATIVE STRESS; TGF- $\beta$ ; JAK

Liver fibrosis results from chronic damage to the liver, causing excessive accumulation of extracellular matrix (ECM). Extensive investigation over the last two decades has established that the major effector of fibrogenesis is HSCs [Iredale, 2001; Beljaars et al., 2002], which may be activated via a number of ways, including hepatic injury and several cytokines, such as TNF- $\alpha$  [Tilg and Diehl, 2000] and transforming growth factor (TGF)- $\beta$  [Breitkopf et al., 2006]. In most cell types, TGF- $\beta$ 1, a prominent profibrogenic cytokine and released from almost any cell during inflammation, tissue regeneration and fibrogenesis, could significantly promote the production and deposition of major ECM molecules [Gressner, 1992; Friedman, 2000; Bissell, 2001]. Activated HSCs produce a

variety of compounds, including growth factors, cytokines, and chemokines that have pleiotrophic effects in the local environment. Activated HSCs also produce abundant quantities of ECM proteins, together with matrix-degrading enzymes including over 15 matrix metalloproteinases (MMPs) [Benyon and Arthur, 2001] and tissue inhibitors of metalloproteinases (TIMPs) [Iredale, 1997]. An early event in hepatic injury appears to be the degradation of type IV collagen and laminin by MMP-2, which was produced by activated HSCs [Zhao et al., 2004]. Subsequently, the degradation of the normal matrix then promotes the activation of HSCs and production of interstitial collagens. With such vicious circle, an inadequate activation of MMPs result in the removal of regular ECM leading to

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subsequent unfavorable tissue remodeling and a fibrogenic response.

As one of the major long-term complications of hepatitis C virus (HCV) infection, liver cirrhosis is a progressive process with fibrosis being the initial step [Negro, 2006]. Although the mechanisms of hepatic fibrogenesis by HCV were not clear yet, a study with an in vitro co-culture system has shown that the expressions of several fibrosis-related molecules were significantly increased by the co-culture of stable HepG2-HCV core with HSC [Shin et al., 2005]. This study initially suggested that HCV core protein contribute to the hepatic fibrogenesis via up-regulation of connective tissue growth factor (CTGF) and TGF- $\beta$ 1. However, the contribution of other HCV proteins has not been elucidated. Since a recent study has indicated that HCV E2 glycoprotein bind to the cell surface of human hepatic stellate cells (HSCs) and induced a time-dependent increase of MMP-2 gelatinolytic activity [Mazzocca et al., 2005], it is possible that up-regulation of MMP-2 by HCV E2 in stellate cells could lead to enhanced penetration of inflammatory cells to sites of injury, which promote the process of fibrogenesis. Therefore, the aim of this study is to clarify the impact of HCV E2 protein on fibrogenesis by examining the expression levels of certain molecules and related pathways involved in fibrogenesis in an HCV E2-expressing HSC cell line to elucidate the role of HCV E2 protein in establishing chronic hepatic injury.

## MATERIALS AND METHODS

### CELL CULTURE AND TRANSFECTION

Mouse hepatic stellate cells (HSC-T6), obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan), were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 1 mM glutamine, 1% penicillin/streptomycin, 1.5 g/L sodium bicarbonate, and 1 mM sodium pyruvate (Sigma, St. Louis, MO). The cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. At the day prior to transfection, cells were plated on 6-cm tissue culture dishes at a density of 4 × 10<sup>5</sup> cells/dish and cultured for overnight. A mixture of 4 μg DNA of pEGFP-E2, a previously constructed E2 expression plasmid [Chiou et al., 2006], and 6 μl lipofectamine reagent (Invitrogen, Life Technologies) in 1.0 ml DMEM (serum free) was added to cells and incubated for 6 h, and then an additional 2.0 ml of fresh DMEM (serum free) was added to each plate. A mock transfection with the vector pEGFP-N1 only was simultaneously performed to act as a mock control. The transfection efficiency was monitored by observation under fluoroscope since pEGFP-N1 encodes an enhanced green fluorescent protein.

### RNA EXTRACTION, REVERSE TRANSCRIPTION-PCR (RT-PCR), AND REAL-TIME PCR

Cellular RNA was extracted using TRIzol (Sigma) and then subjected to a reverse transcription reaction using oligo (dT) primers (New England Biolabs, Beverly, MA) and Superscript II enzyme (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Resultant cDNA was initially dissolved in 20 μl DEPC-H<sub>2</sub>O used in

TABLE I. Primers Employed for RT-PCR

	Sequence	Size (bp)
mCollagen (αI)	5'-GACGCCATCAAGGTCTACTG-3' 5'-ACGGGAATCCATCGGTCA-3'	154
mα-SMA	5'-GTGCTATGTAGCTCTGGACT-3' 5'-ACATCTGCTGGAAGGTAGAC-3'	419
mIL-6	5'-GTGTTCTCAAGGTCTGAGTC-3' 5'-GTAGCCACCTTGCTTACAG-3'	423
mIL-1β	5'-ATAACCTGCTGGTGTGTGACG-3' 5'-GGTGAAGTCAACTATGTCCC-3'	458
mTGF-β1	5'-GCCCTGGATACCAACTATTGC-3' 5'-GCAGGAGCGACAATCATGTT-3'	326
mCTGF	5'-CCGCCAACCGCAAGATT-3' 5'-AGGCGGCTCTGCTTCTCCA-3'	449
mMMP-2	5'-GAGTTGGCAGTGCAATCATGTT-3' 5'-GCCGTCCTTCTCAAAGTGTG-3'	665
mGAPDH	5'-ACCACAGTCCATGCCATCAC-3' 5'-TCCACCACCTGTGTGTA-3'	451

m, Mouse.

the following PCR reactions with primers specific for α-smooth muscle actin (α-SMA), collagen α(I), interleukin (IL)-6, IL-1β, TGF-β1, CTGF MMP-2 and E2. The thermal cycling conditions comprised an initial denaturation at 94°C for 5 min, followed by 20 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. The final extension step was conducted at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel and then quantified using ChemImager 4400 software (Alpha Innotech Corporation, San Leandro, CA). Real-time PCR analysis was performed using SYBR green (Applied Biosystems) on Applied Biosystems 7000 Real-Time PCR System. Expression data were normalized with GAPDH as the internal control. Sequences of used primers are listed in Table I.

### IN SITU IMMUNOFLOURESCENCE ASSAY

Cells were grown on cover glasses overnight and then transfected with pEGFP-N1 or pEGFP-E2 plasmids, while cells treated with TGF-β (5 ng/ml) were employed as positive control. After being incubated in serum-free medium for 24 h, cells were fixed with 2% paraformaldehyde (Sigma) for 12 min and then incubated with 0.5% Triton X-100 (Sigma) for 10 min. Extensive PBS washing was conducted between each reaction to remove any residual solvent. Afterwards, fixed cells were incubated with 4% BSA at room temperature for 2 h and then with the appropriate primary antibodies at 4°C. After overnight incubation, cells were washed and then incubated with rhodamine conjugated affinipure goat anti-mouse IgG secondary antibody (Roche) with light protection. Meanwhile, another set of cells were subjected to DAPI staining for 10 min without antibody reaction. At the end of incubation, cells were covered with medium containing 70% glycerol, 20% PBS, and 5% (w/v) propylgallate (Aldrich) and then observed under fluorescence microscopy equipped with filters for UV, blue 488 nm and green 543 nm.

### HCV E2 GENE SILENCING

To construct a recombinant containing siRNA specific for HCV E2, the pcDNA3-HU6 vector (denoted by Dr. J. Tsai Chang, Institute of Toxicology, College of Medicine, Chung Shan Medical University, Taichung, Taiwan) was employed to be the vector backbone. The 25-

nucleotide siRNA-HCVE2 duplex (GenBank accession no. AJ\_238800) was designed using the BLOCK-iT RNAi Design to produce hairpin RNAs identical to the oligonucleotide siRNA duplex sequences are follows: sense: 5'-GGATCGATCGTTCGGGTGCC-TACGTATATTCAAGAGA-3' and antisense: 5'-AAGCTAAAAA-GATCGTTCGGGTGCCCTACGTATATTCAAGAGA-3'. To generate siRNA duplex, sense and antisense oligonucleotides (40  $\mu$ M) were incubated in the PCR thermocycler at 37°C for 30 min to anneal and then 65°C for 15 min. The completed siRNA duplex was then cloned into the pcDNA3-HU6 vector in frame of the *Bam*HI and *Hind*III sites to yield SiE2, an E2-specific siRNA expression vector. The insert was screened by PCR and confirmed by sequencing with HU6 primer. HSC cells were cotransfected with the EGFP-E2 plasmid and SiE2. Briefly, 4  $\times$  10<sup>5</sup> cells were suspended in 50  $\mu$ l of serum- and antibiotic-free DMEM medium and then cultured in 6 cm culture dish. A preincubated mixture of lipofectamine 2000 reagent (Invitrogen) containing 4  $\mu$ g of EGFP-E2 plasmid and 8  $\mu$ g of SiE2 was added to HSC cells. After the cells were exposed to the DNAs for 6 h, an additional 2.0 ml of fresh DMEM (without serum and antibiotic) was added to each plate and cells were harvested and then subjected to RT-PCR at indicated times. Non-functional siRNA (Ambion) was used as a negative control (SiNT).

#### WESTERN BLOT ANALYSIS

Cell lysates were separated in a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The blot was subsequently incubated with 5% non-fat milk in PBS for 1 h to block non-specific binding, and probed with a corresponding antibody against a specific protein (H1920-19 for E2 was obtained from United States Biological; 1A4 for  $\alpha$ -SMA, C-18 for collagen  $\alpha$ (I), 6B13 for CTGF and 8B4 for MMP-2 were purchased from Santa Cruz, CA; anti-pJAK1 was obtained from Abcam, Cambridge Science Park, UK; C80C3 for pJAK2 was obtained from Cell Signaling; anti-JAK1 and anti-JAK2 were obtained from BD Biosciences, USA; anti-pERK1/2 was obtained from Upstate, Charlottesville, VA; anti-p-p38, anti-p38, and anti-ERK1/2 were obtained from Biosource, Camarillo, CA) for overnight at 4°C, and then with an appropriate peroxidase conjugated secondary antibody for 1 h. All incubations were carried out at 37°C and intensive PBS washing was performed between incubations. After the final PBS washing, signal was developed by ECL detection system and relative photographic density was quantitated by a gel documentation and analysis system (Alpha Imager 2000, Alpha Innotech Corporation).

#### GELATINOLYTIC ZYMOGRAPHY

Conditioned media from the cell cultures were analyzed for gelatin degradation activity by SDS-PAGE under non-reducing conditions. The culture media was mixed with non-denatured 5 $\times$  sample buffer (1 M Tris-HCl pH 6.8, 1% bromophenol blue, 20% SDS). Media mixture was electrophoresed in 8% polyacrylamide gel containing 1 mg/ml gelatin (Sigma), and the gel was rinsed twice in distilled water with 2.5% Triton X-100 for 30 min and then incubated in the activation buffer (5 mM CaCl<sub>2</sub>, 150 mM NaCl, and 50 mM Tris) at 37°C for 16 h. The gelatinolytic activities were visualized by staining the gel with Coomassie Blue R-250.

#### DETECTION OF H<sub>2</sub>O<sub>2</sub> VIA FLOW CYTOMETRY

H<sub>2</sub>O<sub>2</sub> levels were assessed by flow cytometry as the fluorescence of 2',7'-dichlorofluorescein (DCF), which are the oxidation products of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, Madrid, Spain) with a sensitivity for H<sub>2</sub>O<sub>2</sub>/NO<sup>-</sup> based radicals and O<sub>2</sub><sup>-</sup>, respectively. Activated cells were incubated with 5  $\mu$ M DCFH-DA in 37°C for 30 min and then washed twice, resuspended in PBS, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA). The fluorescence was measured at excitation wavelength of 488 nm and emission at 525 nm in a spectrophotometer. For NAC treatment (*N*-acetyl-L-cysteine; Sigma), appropriate amounts of H<sub>2</sub>O solution of NAC (5 mM) were added into culture medium before transient transfection and then incubated with cells for 2 h, while H<sub>2</sub>O solution without NAC was used as blank reagent.

#### TREATMENT OF VARIOUS INHIBITORS

Cultured HSC-T6 cells were washed with serum-free DMEM and then incubated in medium containing indicated phosphorylation inhibitors, including AG490 (50  $\mu$ M) for JAK (Calbiochem, San Diego, CA), SB203580 (20  $\mu$ M) for p38 (Sigma), PD098059 (30  $\mu$ M) for ERK1/2 (Sigma), or catalase (1,000 U/ml; Sigma) for 1 h before being subjected to transfection [Cao et al., 2006].

#### STATISTICAL ANALYSIS

Statistical significances of difference throughout this study were calculated by Student's *t*-test (SigmaStat 2.0, Jandel Scientific) with *P* < 0.05 being regarded as statistically significant.

## RESULTS

#### THE MRNA AND PROTEIN LEVELS OF $\alpha$ -SMA AND COLLAGEN ( $\alpha$ )I IN HSC WERE UP-REGULATED OF BY A TRANSFECTION WITH HCV E2

A previously constructed E2-expressing plasmid [Chiou et al., 2006], pEGFP-E2, containing the entire E2 open-reading frame and EGFP gene, was transiently transfected into HSC by lipofectamine. The successful transfection was indicated by fluorescent signal under a microscope while the correct expression of E2 protein was confirmed by Western blot using a monoclonal antibody against E2 (data not shown). After being transfected and then cultured in serum-free medium for 12 or 24 h, E2-transfected and mock-transfected HSC cells were subjected to RNA extraction followed by semi-quantitative RT-PCR to see the impact of E2 protein on the expression levels of fibrosis-related molecules, including  $\alpha$ -SMA and collagen ( $\alpha$ )I. Results indicated that in response to the expression of E2, mRNA levels of  $\alpha$ -SMA and collagen ( $\alpha$ )I both increased gradually and such increase was time-dependent (Fig. 1A). Western blot analysis further revealed that protein levels of  $\alpha$ -SMA and collagen ( $\alpha$ )I were also enhanced in E2-transfected HSC, as compared with that of mock-transfected or un-treated HSC (Fig. 1B). The same results also observed in Figure 1C that analyzed by qRT-PCR. To confirm the abovementioned increase of  $\alpha$ -SMA was directly related to E2 expression, in situ expression of  $\alpha$ -SMA and E2 was detected by immunofluorescence assay to reveal the co-localization of these two molecules (Fig. 1D). These results

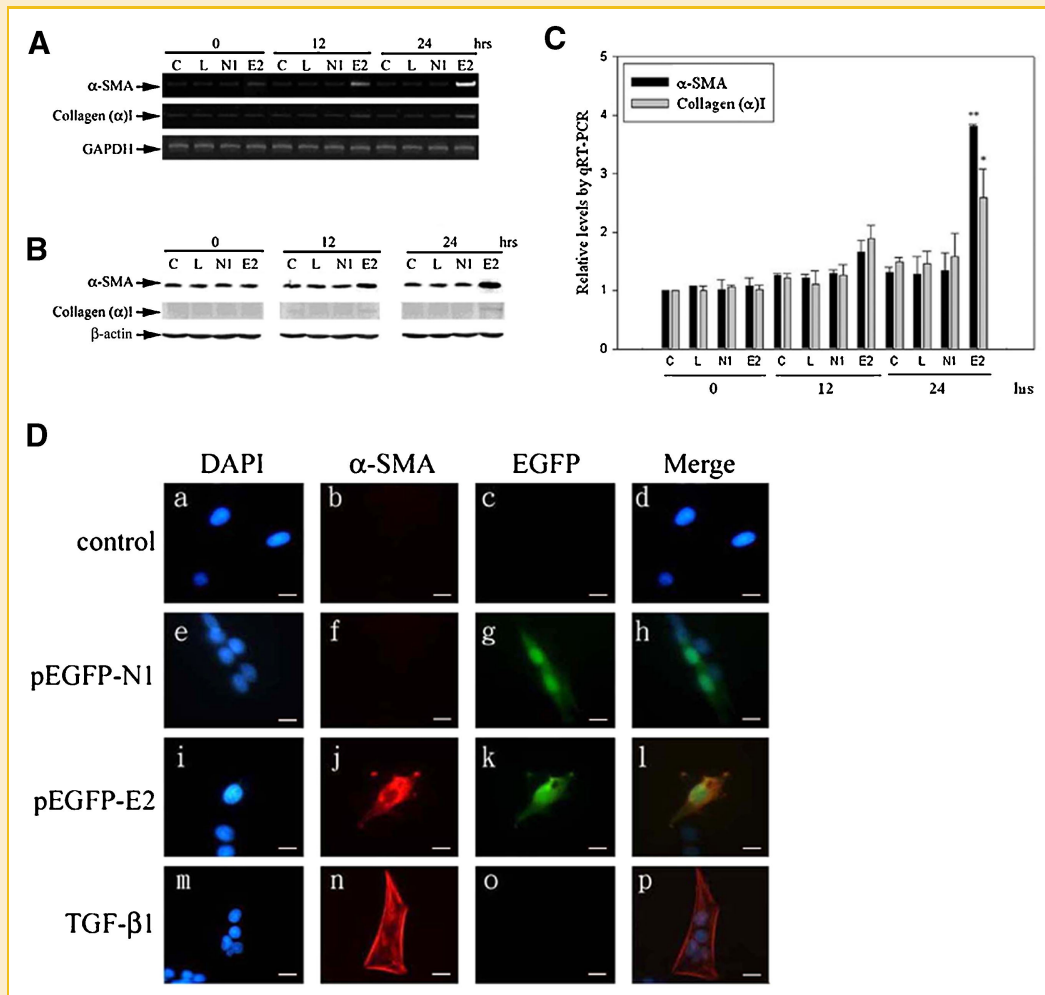


Fig. 1. Up-regulation of mRNA and protein levels of  $\alpha$ -SMA and collagen ( $\alpha$ I) in E2-transfected HSC. At the indicated time after transfection in serum-free medium, cells were harvested and then subjected to (A) mRNA extraction followed by semi-quantitative RT-PCR or (B) whole cell lysate preparation followed by Western blotting with an antibody against  $\alpha$ -SMA and collagen ( $\alpha$ I). C: The mRNA expression was quantitatively analyzed by qRT-PCR. Results are expressed as averages  $\pm$  SD ( $n = 3$ ); \* $P < 0.05$ , \*\* $P < 0.01$ . C: control, L: lipofectamine, N1: pEGFP-N1, E2: pEGFP-E2. D: Immunostainings with antibodies specific for  $\alpha$ -SMA and then a secondary antibody with rhodamine (TRITC) conjugate were performed to detect the expression of  $\alpha$ -SMA in E2-transfected HSCs (400 $\times$ ) (scale bars, 10  $\mu$ m). TGF- $\beta$ 1: HSCs treated with TGF- $\beta$ 1 (5 ng/ml) for 24 h in serum-free medium, EGFP: enhanced green fluorescent proteins. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

initially suggested that HCV E2 protein involved in the process of fibrogenesis.

#### EXPRESSIONS OF INFLAMMATION-RELATED CYTOKINES AND MMP-2 WERE INCREASED IN E2-TRANSFECTED HSC

After showing that E2 involved in hepatic fibrogenesis, whether E2 stimulate certain cytokines was investigated by measuring mRNA levels with semi-quantitative RT-PCR and qRT-PCR. As shown in Figure 2A,B, the mRNA level of TGF- $\beta$ 1 in HSC at 12 or 24 h after E2 transfection was significantly higher than in that in untransfected and vector-transfected-HSC. Furthermore, the mRNA level of CTGF, a cytokine known to be involved in fibrogenic processes, was also up-regulated by E2 protein, suggesting that HCV E2 protein may enhances TGF- $\beta$ 1 and CTGF expression which promotes liver fibrogenesis. As for other inflammation-related cytokines, the impact of E2 protein on the expression of IL-6 and IL-

1 $\beta$  was also proved since mRNA levels of IL-6 and IL-1 $\beta$  were clearly higher in a time-dependent manner (Fig. 2C,D). MMPs, especially MMP-2, were known to be involved in fibrogenesis and therefore, whole cell lysate of E2-expression HSC was subjected to RT-PCR and Western blot while the culture medium was analyzed by zymography to see the expression level and activity of MMP-2. As data shown in Figure 2, levels of mRNA (E), protein (F), and activity (G) of MMP-2 were consistently enhanced by E2 expression and such increase was time-dependent. These data suggested that HCV E2-promoted fibrogenesis involve the increased expression of MMP-2 and inflammation-related cytokines.

#### THE EXPRESSION OF FIBROSIS-RELATED MOLECULES IN E2-TRANSFECTED HSC WAS RESTORED UPON E2 SILENCING BY SIRNA

To further confirm the involvement of E2 during fibrogenesis, TGF- $\beta$ 1, a well-known fibrosis inducer, served as a positive control and

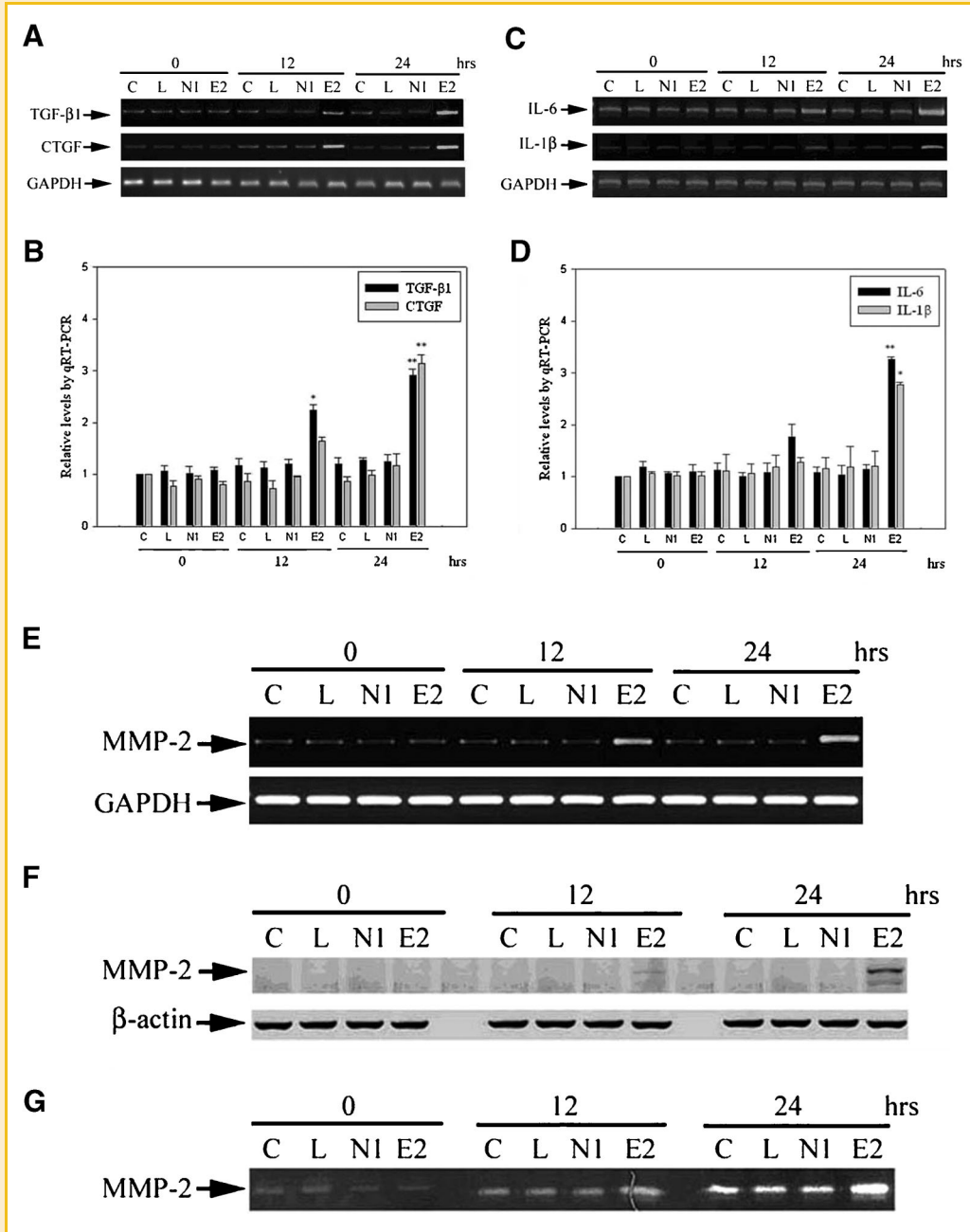


Fig. 2. Increased expressions of inflammation-related cytokines and MMP-2 in E2-transfected HSC. At the indicated time after transfection in serum-free medium, cells were harvested and then subjected to RNA extraction followed by semi-quantitative RT-PCR and quantitative RT-PCR for certain cytokines, including TGF-β1 and CTGF (A) (B), IL-6 and IL-1β (C) (D). Cells were harvested and then subjected to (E) RNA extraction followed by semi-quantitative RT-PCR, (F) whole cell lysate preparation followed by Western blotting with an antibody against MMP-2, or (G) the cultured medium was analyzed by zymography to see the expression activity of MMP-2. Results are expressed as averages ± SD (n = 3); \*P < 0.05, \*\*P < 0.01. C: control, L: lipofectamine, N1: pEGFP-N1, E2: pEGFP-E2.

siRNA silencing was performed. Levels of analyzed molecules were examined by semi-quantitative RT-PCR and Western blotting. According to data shown in Figure 3, mRNA levels of fibrosis-related molecules were enhanced by E2 expression to an extent comparable to TGF-β1, but such enhancement was abolished with a combined transfection with E2Si plasmid (Fig. 3A). Results of Western blotting gave the same observation (Fig. 3B).

#### UP-REGULATION OF TGF-β1 AND CTGF EXPRESSION IN STABLE HUH7-E2 CELLS

To investigate the effect of E2 protein on the expression of proinflammatory cytokine, TGF-β1 levels in the supernatant of cultured Huh7-E2 cells and Huh7 cells at 6, 12, and 24 h was measured by ELISA. Results indicated that TGF-β1 level in stable Huh7-E2 cells was significantly higher than that in Huh7 cells,

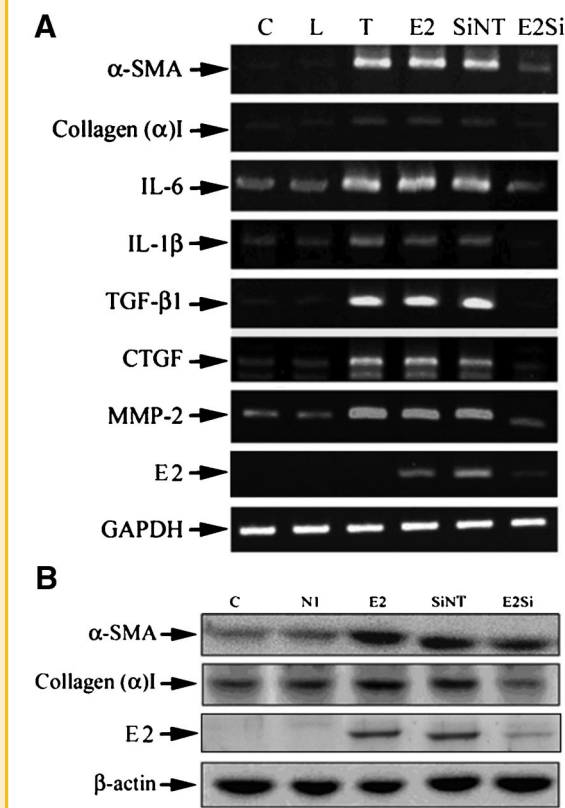


Fig. 3. The abolishment of increased expressions of fibrosis-related molecules by E2 silencing. A: After transfection or TGF- $\beta$  treatment in serum-free medium for 24 h, cells were harvested and then subjected to RNA extraction followed by semi-quantitative RT-PCR. B: After transfection for 24 h, cell lysate preparation was subjected to Western blotting with an antibody against  $\alpha$ -SMA and collagen ( $\alpha$ )I. C: control, L: lipofectamine, T: TGF- $\beta$  (5 ng/ml), N1: pEGFP-N1, E2: pEGFP-E2, SiNT: co-transfection with pEGFP-E2 plasmid and siRNA negative control plasmid, E2Si: co-transfection with pEGFP-E2 plasmid and SiE2 expressing plasmid.

suggesting that HCV E2 protein enhances TGF- $\beta$ 1 expression which promotes liver fibrogenesis (data not shown). The expression of CTGF was also evaluated by RT-PCR and Western blot analysis to show that the expression of CTGF was more prominent in Huh7-E2 cells, as compared with that in the parental Huh7 cells (data not shown).

#### HCV E2 INDUCE THE EXPRESSION OF $\alpha$ -SMA AND COLLAGEN ( $\alpha$ )I BY STIMULATING ROS GENERATION

Since oxidative stress has been shown to be associated with liver fibrosis and HSC activation in vivo [Svegliati Baroni et al., 1998], the capability of E2 to enhance ROS production was investigated. HSCs were transfected with pEGFP-E2 and then incubated for 24 h. At the end of incubation, DCFH-DA in serum-free medium was added to transfected cells and H<sub>2</sub>O<sub>2</sub> levels were detected with flow cytometry. The results showed that E2 increase H<sub>2</sub>O<sub>2</sub> levels and a co-transfection with SiE2 restore the levels to that of control. A pretreatment with NAC also has the same effect for reversing the

action of E2 (Fig. 4A). Further investigation observed that the mRNA expression levels of  $\alpha$ -SMA and collagen ( $\alpha$ )I induced by E2 were decreased by pretreatment with NAC at 2 h before E2 transfection (Fig. 4B). These data suggested that HCV E2 protein enhance H<sub>2</sub>O<sub>2</sub> expression to promote the expression of fibrosis-related factor expression and therefore involve in liver fibrogenesis.

#### HCV E2 STIMULATE THE PHOSPHORYLATION OF JAK1 AND JAK2

The JAK/STAT pathway is activated in response to cytokines and growth factors, as well as oxidative stress [Darnell, 1997]. In addition, JAK-STAT pathway has been shown to be activated in response to H<sub>2</sub>O<sub>2</sub> in fibroblasts, carcinomas cells [Simon et al., 1998] and human lymphocytes [Carballo et al., 1999]. To test whether the JAK-STAT pathway was activated by H<sub>2</sub>O<sub>2</sub> preconditioning, the levels of phosphorylated (p)-JAK1 and p-JAK2 in E2-transfected HSC-T6 cells were analyzed. Results indicated that E2 raised the levels of p-JAK1 and p-JAK2 (Fig. 5A,C), and such increase were abolished by a co-transfection of specific E2 siRNA. Further investigation with a treatment of AG490, a JAK inhibitor, showed that levels of p-JAK1 and p-JAK2 in E2-transfected HSC-T6 cell were both decreased to a level comparable to that of un-transfected cells (Fig. 5B,D), suggesting that the capability of E2 to induce phosphorylation of JAK.

#### HCV E2 INCREASE p38 MAPK PHOSPHORYLATION

It has been reported that H<sub>2</sub>O<sub>2</sub> stimulate the production of TIMP by activating the extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 of the mitogen-activated protein kinase (MAPK). Furthermore, the activation of p38 by H<sub>2</sub>O<sub>2</sub> also result in a up-regulation of procollagen ( $\alpha$ )I mRNA in HSC cells [Cao et al., 2004]. Therefore, if E2-induced H<sub>2</sub>O<sub>2</sub> subsequently affect the status of p38 was analyzed. As results shown in Figure 6, the expression of E2 in HSC-T6 cells increase the phosphorylation of p38 and a co-transfection of specific E2 siRNA reverse such increase, proving that such increase were indeed E2 related. Furthermore, a treatment of p38 inhibitor, SB203580, was not able to totally diminish such increase (Fig. 6A,B). However, this E2-enhanced p38 phosphorylation was totally inhibited by a pretreatment of catalase (Fig. 6C), demonstrating the involvement of H<sub>2</sub>O<sub>2</sub> in E2-related p38 activation.

#### HCV E2 INCREASES ERK1/2 MAPK PHOSPHORYLATION

It has been reported that H<sub>2</sub>O<sub>2</sub> enhance the transcription of MMP-1 via an increased phosphorylation of ERK1/2 in human fibrosarcoma cells [Cao et al., 2007]. In this study, the presence of E2 result in an increased p-ERK1/2 level (Fig. 7A,B) and such increase was abolished by a co-transfection of specific E2 siRNA. Again, the rise was not totally inhibited by a pretreatment of PD98059, an ERK phosphorylation inhibitor, but completely diminished by a pretreatment with catalase (Fig. 7). These results further demonstrated the vital involvement of H<sub>2</sub>O<sub>2</sub> in E2-induced phosphorylation.

#### HCV E2-INDUCED INCREASE OF COLLAGEN ( $\alpha$ )I WAS INHIBITED BY JAK INHIBITOR AG490, CATALASE, p38 INHIBITOR SB203580, AND ERK1/2 INHIBITOR PD098059

To further investigate the involvement of H<sub>2</sub>O<sub>2</sub>-related pathways, the effects of inhibitors of JAKs, H<sub>2</sub>O<sub>2</sub>, p38, and ERK1/2 on E2-

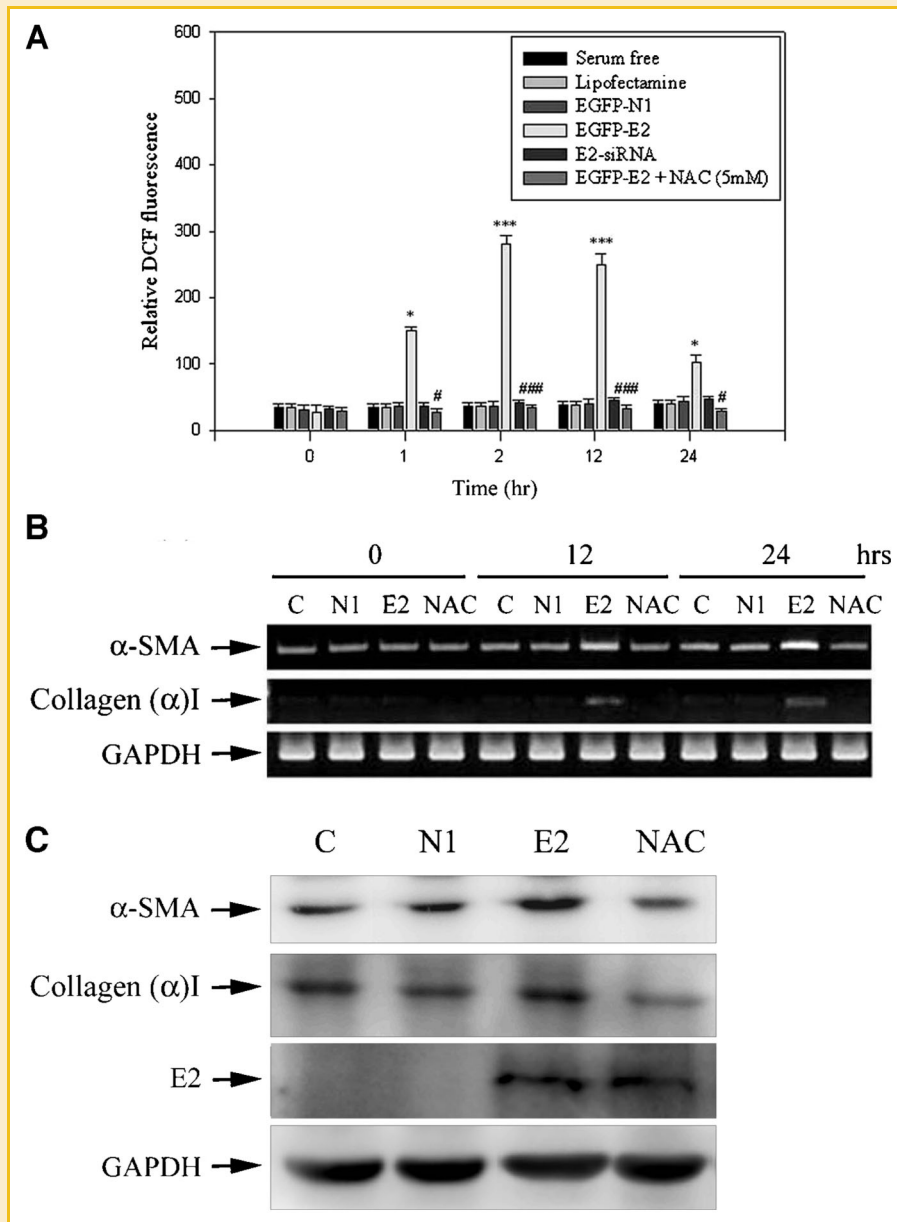


Fig. 4. Generation of intracellular ROS by HCV E2 and its prevention by *N*-acetyl cysteine. A: HSC-T6 cells transfected with EGFP-E2 or co-transfected with SiE2 for 24 h. E2-transfected cells were incubated in serum-free medium with 5  $\mu$ M DCFH-DA at 37°C, 30 min and then washed twice, resuspended in PBS, and analyzed on a FACSCalibur flow cytometer. For a treatment of NAC, an excellent antioxidant, cells were incubated with 5 mM NAC before transfection for 2 h. Values for the DCF fluorescence are expressed as percentage of the control at 0 time. \* $P$  < 0.05 and \*\*\* $P$  < 0.001 versus 0 time. # $P$  < 0.05 and ### $P$  < 0.001 versus E2-transfected alone. E2 siRNA: EGFP-E2 co-transfected with SiE2. B: After transfection, cells were incubated in serum-free medium for the indicated period, and then harvested and subjected to RNA extraction followed by semi-quantitative RT-PCR. C: After transfection, cells were incubated in serum-free medium for 24 h and then harvested and subjected to cell lysate followed by Western blotting. C: control, N1: pEGFP-N1, E2: pEGFP-E2, NAC: a pretreatment of NAC (5 mM) for 2 h and then a transfection with pEGFP-E2.

induced collagen ( $\alpha$ )I production were studied. Results in Figure 8 showed that these inhibitors all reduce E2-induced collagen ( $\alpha$ )I production to various extents. A pretreatment of catalase has the most significant effect to nearly completely alleviate the impact of E2, while the effect other inhibitors was only partly or slightly. This results further suggested that the impact of E2 collagen ( $\alpha$ )I protein was H<sub>2</sub>O<sub>2</sub> dependent.

## DISCUSSION

Patients chronically infected with HCV are prone to liver diseases of various severities, including symptomatic or asymptomatic hepatitis, liver cirrhosis, and even hepatic carcinoma [Rodis, 2007]. While most studies on the pathogenesis of HCV have focused on carcinogenesis, the detailed mechanism for cirrhosis has not been

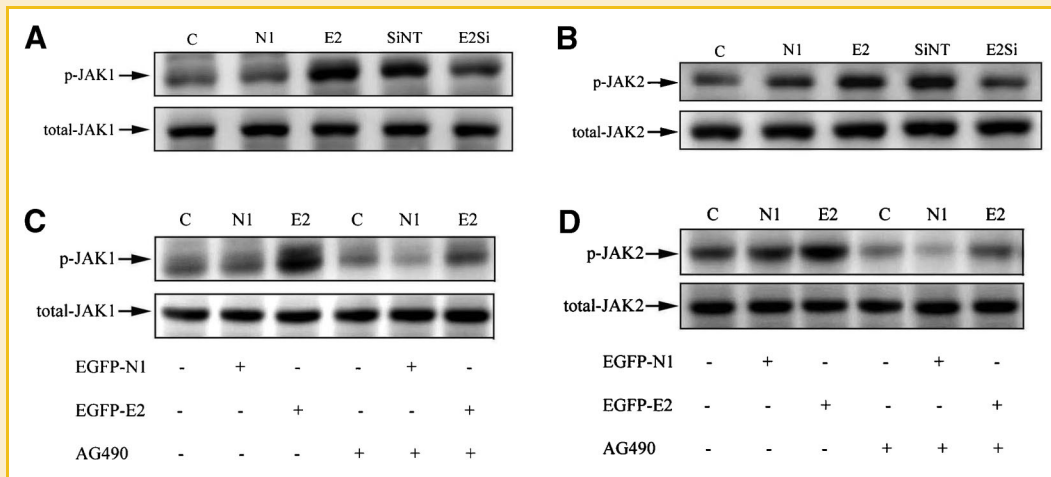


Fig. 5. Increased phosphorylation of JAK1 and JAK2 by HCV E2. HSC-T6 cells were transfected with EGFP-E2 alone or co-transfected with SiE2 for 24 h. Cells were harvested and the whole cell lysate were prepared for Western blotting with an antibody against p-JAK1 (A) and p-JAK2 (B) proteins. For inhibitor treatment, cells were incubated with AG490 (50  $\mu$ M) in the serum-free medium before transfection for 2 h and then transfected with EGFP-E2 alone or co-transfected with SiE2 for 24 h in the presence or absence of AG490, followed by Western blotting for p-JAK1 (C) and p-JAK2 (D). C: control, N1: pEGFP-N1, E2: pEGFP-E2, SiNT: co-transfection with pEGFP-E2 plasmid and siRNA negative control plasmid, E2Si: co-transfection with pEGFP-E2 plasmid and SiE2 plasmid.

elucidated yet. It has been known that replication of the virus and induced inflammation cause the death of hepatocytes, together with the subsequent activation of HSCs [Baroni et al., 1999]. Major resultant outcomes include dys-regulated synthesis of ECM, as well as overproduction of matrix degrading enzymes.

Since hepatic fibrosis is the initial step during cirrhosis, the molecular aspect of HCV-induced fibrogenesis should be investigated. In this study, HCV E2 was transiently expressed in mouse HSCs, the major effector of fibrogenesis, to clarify the impact and underlying mechanism of HCV E2 protein on fibrogenesis. Results indicated that the expression levels of fibrosis-related molecules, including  $\alpha$ -SMA and collagen ( $\alpha$ I), were increased in a time-dependent fashion, as shown in Figure 1. Co-localization of E2 and  $\alpha$ -SMA revealed by fluorescence microscopic observation further confirmed that E2 protein lead to the initialization of fibrosis. Upon the injury of liver, stellate cells secrete various cytokines, hormones, or oxygen-free radicals to lead to hepatic damage, apoptosis or regeneration. TGF- $\beta$ 1 is one of known vital factors in the fibrogenic process and the results in Figure 2A,B showed that E2 protein significantly enhanced TGF- $\beta$ 1 mRNA and protein expression levels (data not shown), which subsequently stimulates the production and deposition of ECM, resulting in liver fibrosis [Tilg, 2001]. CTGF is shown to act as an essential mediator in the hepatic fibrogenesis by stimulating ECM production and mainly derived from HSC during liver fibrogenesis. It has been shown that CTGF be produced by all major cell types in the liver upon initial hepatic injury and damage [Rachfal and Brigstock, 2003]. Furthermore, the expression level of CTGF was also significantly elevated in E2-transfected HSC cell lines and Huh7-E2 stable cells (data not shown). Previous studies suggested that TGF- $\beta$  expression be induced by certain pro-inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, while IL-1 $\beta$  and TNF- $\alpha$  increase the expression and activity of MMP-2 in cardiac fibroblasts [Ortiz et al., 1994; Siwik et al., 2000;

Corbel et al., 2002; Kershenovich and Weissbrod, 2003; Bennermo et al., 2004]. In this study, we also demonstrated that IL-6 and IL-1 $\beta$  mRNA were significantly increased in the E2-transfected HSC cells. HCV E2 glycoprotein has been proposed to be vital during infection cycle by binding to the major loop of CD81 [Mazzocca et al., 2005; Heo et al., 2006; Falkowska et al., 2007], which is expressed in the liver and moreover is increased in the injured liver. Further study showed that HCV E2 glycoprotein binds to the cell surface of human HSCs and increase of MMP-2 gelatinolytic activity by activation of ERK/MAPK phosphorylation [Mazzocca et al., 2005]. Our study demonstrated MMP-2 expression was significantly increased in the E2-transfected HSCs. It has been reported that MMP-2 degrades basement membrane collagen so that denatured fibrillar collagens replace normal collagen as ECM during fibrogenesis [Karelina et al., 2000; Schuppan et al., 2001]. ECM degradation by MMP-2 allow other cells to migrate into the injury milieu. Therefore, the up-regulation of MMP-2 by HCV E2 in HSCs could lead to enhanced penetration of inflammatory cells to sites of injury and further promote fibrogenesis [Yoshizaki et al., 2002].

Previous studies showed that HCV core protein induced oxidative stress and was accompanied by significant changes of several oxidative parameters, including increased formation of the oxidants H<sub>2</sub>O<sub>2</sub> and superoxide anions, increased lipid peroxidation. Since H<sub>2</sub>O<sub>2</sub> has been shown to serve as a signaling molecule between oxidative stress and collagen ( $\alpha$ I) gene up-regulation [Greenwel et al., 2000], this study focused on the effect of HCV E2 protein on H<sub>2</sub>O<sub>2</sub> formation and the data indicated that E2 increase the levels of H<sub>2</sub>O<sub>2</sub> to promote the expressions of fibrosis-related factors and then involve in liver fibrogenesis (Fig. 4). However, NAC, used as an antioxidant in this study, has been proven to be capable of reducing the intramolecular disulfide bond and disrupting the molecular integrity of TGF- $\beta$  [Meurer et al., 2005]. Despite that NAC may eliminate the E2-induced H<sub>2</sub>O<sub>2</sub> production, as proven in



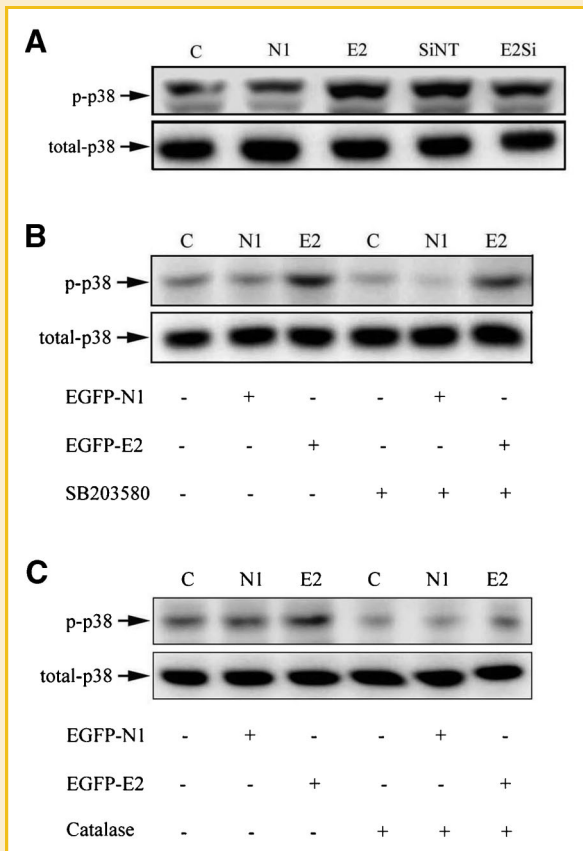


Fig. 6. Activation of p38 MAPK by HCV E2 and its inhibition by catalase, p38 inhibitor SB203580. A: After transfection for 24 h, cell lysate were prepared and subjected to Western blotting for p-p38 and total p38 proteins. Before transfection, cells were incubated with (B) SB203580 (20  $\mu$ M) and (C) catalase (1,000 U/ml) in the serum-free medium for 2 h and then transfected with pEGFP-E2 alone or co-transfected with SiE2 for 24 h in the presence or absence of inhibitors. Afterwards, cell lysates were prepared and analyzed by Western blotting for p-p38 and total p38. C: control, N1: pEGFP-N1, E2: pEGFP-E2, SiNT: co-transfected with pEGFP-E2 and siRNA negative control plasmid, SiE2: co-transfected with pEGFP-E2 and SiE2 plasmid.

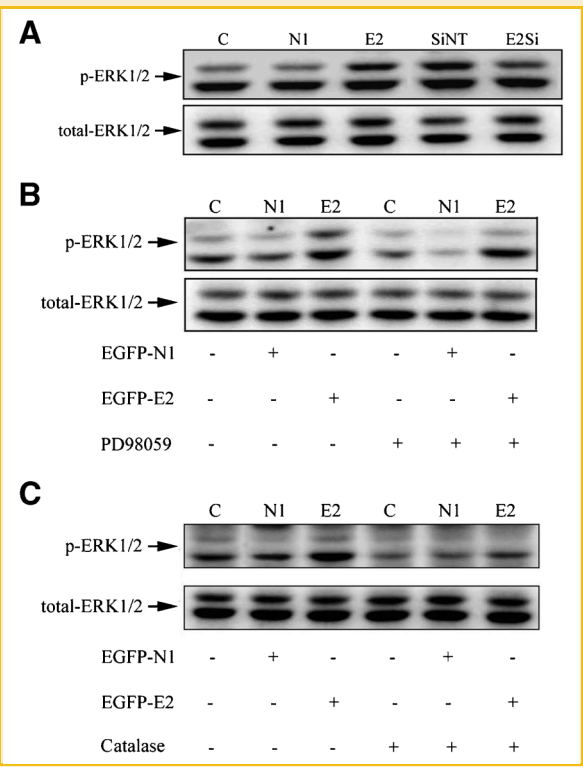


Fig. 7. ERK1/2 MAPK phosphorylation by HCV E2 and its inhibition by catalase and ERK1/2 inhibitor PD98059. A: After transfection for 24 h, cell lysate were prepared and subjected to Western blotting for p-ERK1/2 and total ERK1/2. Before transfection, cells were incubated with (B) PD98059 (30  $\mu$ M) and (C) catalase (1,000 U/ml) in the serum-free medium for 2 h and then transfected with pEGFP-E2 alone or co-transfected with SiE2 for 24 h in the presence or absence of inhibitors. Afterwards, cell lysates were prepared and analyzed by Western blotting for p-ERK1/2 and total ERK1/2. C: control, N1: pEGFP-N1, E2: pEGFP-E2, SiNT: co-transfected with pEGFP-E2 and siRNA negative control plasmid, SiE2: co-transfected with pEGFP-E2 and SiE2 plasmid.

Figure 4A, the possible contribution to these results of NAC-related TGF- $\beta$  disruption should be noted. As shown in Figure 8, a treatment with catalase or an antioxidative enzyme that degrades  $H_2O_2$  prevents the expression of collagen ( $\alpha$ )I protein induced by E2, suggesting an  $H_2O_2$ -dependent regulation. The JAK/STAT pathway is activated in response to cytokines and growth factors and oxidative stress can activate this pathway [Hosui et al., 2003]. In E2-transfected HSCs, levels of p-JAK1 and p-JAK2 were increased, as compared with that of parental cells. Furthermore, treatment with AG490 prevented E2-induced phosphorylation of JAK1 and JAK2 in HSC cells, suggesting a JAK-mediated process. Activation of p38 MAPK in HSC cells after E2-transfected is dependent on  $H_2O_2$  formation, because the process is inhibited by catalase, as shown in Figure 6. ERK1/2 MAPK is activated concurrently with p38 in E2-transfected HSCs [Chin et al., 2001; Cao et al., 2002]. Activation of ERK1/2, like that of p38, is mediated by  $H_2O_2$  formation (Fig. 7). In rat HSCs, ERK1/2 signaling was found to involve NF-1 and SP-1

sites in the proximal promoter of the collagen ( $\alpha$ )I gene [Davis et al., 1996] and ERK1/2 involve in collagen type I production in response to a stimulation with insulin-like growth factor [Svegliati Baroni et al., 1999] or acetaldehyde in human HSCs [Svegliati Baroni et al., 2001]. Still, it is possible that, in addition to p38 and ERK1/2 pathways, HCV E2 can also use the other signaling mechanisms to initiate fibrogenesis in HSC. For example, the involvement of STAT pathway in  $H_2O_2$  signaling will be of future interest, since vital cross-talk among these signaling pathways during collagen production to either enhance or inhibit their effectiveness has been proposed [Korzus et al., 1997; Decker and Kovarik, 2000; Burysek et al., 2002].

Previous reports have demonstrated that E2 induces apoptosis in cultured mammalian cells which is possibly via a mitochondrial-related caspase pathway [Zhu et al., 2004; Chiou et al., 2006]. Since apoptosis is directly linked to the activity of TGF- $\beta$ , we could not exclude the possibility that some findings in this study are related to apoptosis. The association between E2, ROS, TGF- $\beta$  and apoptosis will be further investigated.

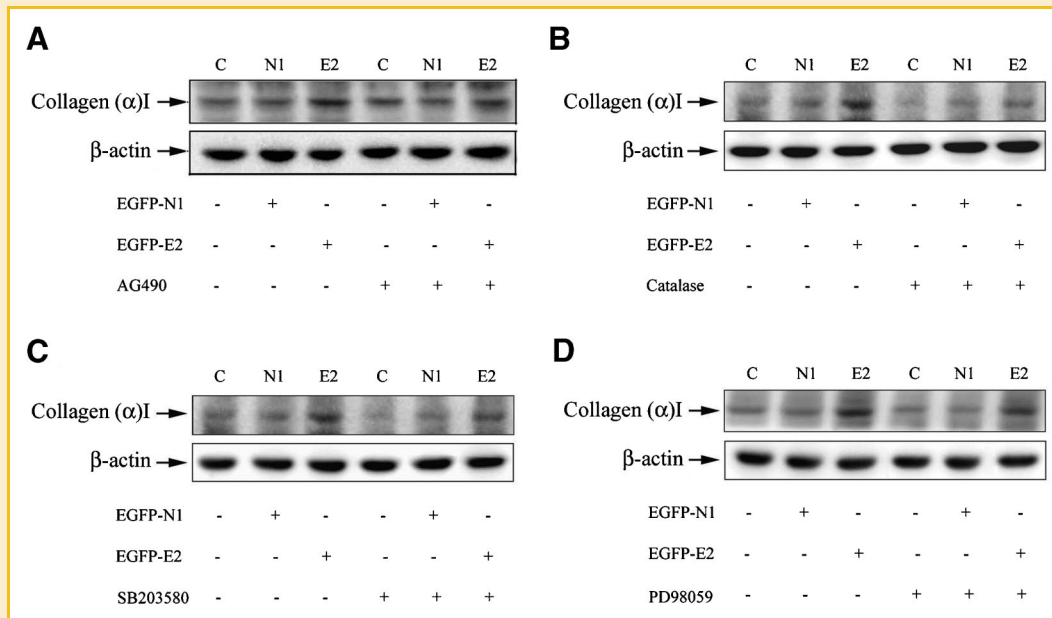


Fig. 8. Inhibition of E2-induced collagen (αI) protein by JAK inhibitor AG490, catalase, p38 inhibitor SB203580 and ERK1/2 inhibitor PD98059. Before transfection, cells were incubated with an indicated inhibitor in the serum-free medium for 2 h and then transfected with pEGFP-E2 for 24 h in the presence or absence of inhibitors. Afterwards, cell lysates were prepared and analyzed by Western blotting for collagen (αI). (A) AG490 (50 μM); (B) catalase (1,000 U/ml); (C) SB203580 (20 μM); (D) PD98059 (30 μM). The protein levels of β-actin were used to show equal protein loading. C: control, N1: pEGFP-N1, E2: pEGFP-E2.

In this study, we have demonstrated that HCV E2 protein stimulate collagen (αI) mRNA expression and its protein production in mouse HSC-T6 by the generation of the oxidant H<sub>2</sub>O<sub>2</sub>, which be via activation of JAK1 and JAK2. In turn, H<sub>2</sub>O<sub>2</sub> signal through the ERK1/2 and p38 pathways. The oxidant generated by HCV E2 protein not only stimulated collagen expression, but it also increased α-SMA expression. However, it must be noted that certain limitations exist for a realistic interpretation of the abovementioned data, such as the lack of viral replication in mouse hepatocytes and the intracellular localization and dynamics of HCV proteins do not mimic the actual HCV infection in humans, as stated in a previous study [Bataller et al., 2004]. Taken together, this study introduced a new insight in the pathogenesis of HCV-mediated fibrosis to state that E2 protein involve via an up-regulation of collagen and α-SMA expression. Nevertheless, the impact of E2 in other pathways related to fibrogenesis, such as TGF-β, TNF-α, and EGF pathways, should also be further examined to clarify the role of E2 in these pathways.

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