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C 型肝炎病毒 E2 蛋白在肝纖維化過程中扮演之角色之探討

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中文摘要

慢性肝炎及肝硬化一直是影響國人健康及造成死亡原因的主要原因之一。除了B型肝炎之外，C型肝炎也是主要引起慢性肝病的原因之一。C型肝炎是由C型肝炎病毒（hepatitis C virus；HCV）感染所引起的慢性肝臟疾病，與肝硬化及肝癌有著因果的關連性。文獻指出C型肝炎病毒套膜蛋白E2與肝臟星狀細胞膜上接受器CD81結合後，會藉由活化ERK/MAPK的途徑活化轉錄因子AP-2的活性，進而導致matrix metalloproteinase（MMP）-2的蛋白表現量以及活性上升，顯示E2蛋白與HCV導致的纖維化有關。另外，之前的文獻也證實肝星狀細胞與肝纖維化的產生具有很重要的相關性。因此，本實驗主要探討C型肝炎病毒中的套膜蛋白E2對肝星狀細胞是否會造成相關纖維化因子表現的情形產生，並進一步的探討和纖維化之間的相關性以及影響的機制。本研究利用real-time PCR、西方墨點法、gelatin zymography、RNA干擾技術（small interfering RNA；siRNA）、流式細胞儀分析（flow cytometry）、免疫螢光染色（in situ immunofluorescence assay）和酵素聯結免疫吸附法（enzyme linked-immunosorbent assay；ELISA）等方法證實，套膜蛋白E2確實造成alpha smooth muscle actin (α-SMA)、collagen α（I）和connective tissue growth factor（CTGF）纖維化因子以及發炎因子interleukin（IL）-6、IL-1β表現量增加，也會促進transforming growth factor beta（TGF-β1）表現量增加，並刺激TGF-β1訊息傳遞路徑的啟動，而MMP-2表現量及活性的增加也更加證實套膜蛋白E2具有刺激肝星狀細胞活化的能力。最後也進一步證實套膜蛋白E2會就由刺激肝星狀細胞產生H_2O_2對細胞產生氧化性傷害，進而活化Janus kinase/signal transducers and activators of transcription (JAK/STAT)傳遞路徑、刺激ERK1/2和p38磷酸化而造成纖維化因子collagen α（I）表現量增加。研究結果證明套膜蛋白E2在C型肝炎病毒誘導纖維化產生的過程當中扮演了重要的角色，希望對於往後在C型肝炎病毒與纖維化的研究與治療上能提供新的研究方向。

關鍵字：套膜蛋白 E2、纖維化、氧化性傷害、肝星狀細胞

Abstract

Chronic infection of hepatitis C virus (HCV) lead 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 metallopeptidase (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 α-smooth muscle actin (α-SMA) and collagen α(I). Furthermore, several pro-fibrosis or pro-inflammatory cytokines, including transforming growth factor (TGF)-β1, connective tissue growth factor (CTGF), interleukin (IL)-6 and IL-1β, 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-β1 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 α(I). Taken together, this study indicated that E2 protein involve in the pathogenesis of HCV-mediated fibrosis via an up-regulation of collagen α(I) and oxidative stress, which is JAK pathway-related.

Keywords: hepatitis C virus E2 protein; fibrosis; collagen; oxidative stress; TGF-β; JAK

報告內容

Introduction

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 **[Beljaars et al., 2002; Iredale, 2001]**, which may be activated via a number of ways, including hepatic injury and several cytokines, such as TNF-α **[Tilg and Diehl 2000]** and TGF-β **[Breitkopf et al., 2006]**. In most cell types, TGF-β1, 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 **[Friedman, 2000; Bissell, 2001; Gressner, 1992]**. 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 MMPs **[Benyon and Arthur, 2001]** and tissue inhibitors of metalloproteinase (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 subsequent unfavourable tissue remodeling and a fibrogenic response.

As one of the major long-term complications of 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 CTGF and TGF-β1. 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 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.

Motivation

Clinically, HCV infection could lead to acute hepatitis, which could be caused by immunological damage and virus-induced apoptosis, while hepatic injury and long-term hepatitis may lead to hepatic fibrosis and eventually cirrhosis. Chronic Hepatitis C infection has been an important health topic worldwide, since no effective vaccine are available for protection, and an early and effective clinical management becomes the most practicable way for blocking the disease progression to cirrhosis or hepatocellular carcinoma. Therefore, the understanding of related pathogenesis will be of great help in developing adequate management for chronic infection. Based on our preliminary data indicating that HCV E2 protein may be involved in the process of hepatic fibrogenesis, this studies project is proposed to investigate the possible underlying pathways regarding E2-induced oxidative damage-related molecules, certain molecular interaction with E2.

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, USA) 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, USA). The cell cultures were maintained at 37 ℃ in a humidified atmosphere of 5% CO_2. At the day prior to transfection, cells were plated on 6-cm tissue culture dishes at a density of 4×10^5 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 hrs, 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, Mass.) and Superscript II enzyme (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. Resultant cDNA was initially dissolved in 20 μl DEPC-H_2O used in the following PCR reactions with primers specific for α-SMA, collagen α(I), IL-6, IL-1β, TGF-β1, CTGF MMP-2 and E2. The thermal cycling conditions comprised an initial denaturation at 94℃ for 5 mins, followed by 20 cycles of denaturation at 94℃ for 1 min, annealing at 60℃ for 1 min, and extension at 72℃ for 2 mins. The final extension step was conducted at 72℃ for 10 mins. The PCR products were analyzed by electrophoresis on 1.5% agarose gel and then quantified using ChemiImager 4400 software (Alpha Innotech, San Leandro, Calif.). 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 1**.

In situ immunofluorescence 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 hrs, cells were fixed with 2% paraformaldehyde (Sigma) for 12 mins and then incubated with 0.5% Triton X-100 (Sigma) for 10 mins. 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 hrs and then with the appropriate primary antibodies at 4℃. 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 mins 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'-GGATCGATCGTTCCGGTGTCCCTACGTATATTCAAGAGA-3' and antisense: 5'-AAGCTAAAAAGATCGTTCCGGTGTCCCTACGTATATTCAAGAGA-3'. To generate siRNA duplex, sense and antisense oligonucleotides (40 μM) were incubated in the PCR thermocycler at 37℃ for 30 mins to anneal and then 65℃ for 15 mins. The completed siRNA duplex was then cloned into the pcDNA3-HU6 vector in frame of the BamHI and HindIII 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, 4×10^5 cells were suspended in 50 μl 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 μg of EGFP-E2 plasmid and 8 μg of SiE2 was added to HSC cells. After the cells were exposed to the DNAs for 6 hrs, 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. Nonfunctional 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 hr 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 α-SMA, C-18 for collagen α(I), 6B13 for CTGF and 8B4 for MMP-2 were purchased from Santa Cruz, California, USA; 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℃, and then with an appropriate peroxidase conjugated secondary antibody for 1 hr. All incubations were carried out at 37℃ 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 nonreducing conditions. The culture media was mixed with non-denatured 5× 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 mins and then

incubated in the activation buffer (5 mM $CaCl_2$, 150 mM NaCl, and 50 mM Tris) at 37℃ for 16 hrs. The gelatinolytic activities were visualized by staining the gel with Coomassie Blue R-250.

Detection of H_2O_2 via Flow cytometry-H_2O_2 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_2O_2/NO^- based radicals and O_2^-, respectively. Activated cells were incubated with 5 μM DCFH-DA in 37℃ for 30 mins 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_2O solution of NAC (5 mM) were added into culture medium before transient transfection and then incubated with cells for 2 hrs, while H_2O 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 μM) for JAK (Calbiochem, San Diego, CA), SB203580 (20 μM) for p38 (Sigma), PD098059 (30 μM) for ERK1/2 (Sigma), or catalase (1,000 U/ml; Sigma) for 1 hr 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 α-SMA and collagen (α)I in HSC were up-regulatedof 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 hours, 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 α-SMA and collagen (α)I. Results indicated that in response to the expression of E2, mRNA levels of α-SMA and collagen (α)I both increased gradually and such increase was time-dependent (**Figure 1A**). Western blot analysis further revealed that protein levels of α-SMA and collagen (α)I were also enhanced in E2-transfected HSC, as compared with that of mock-transfected or untreated HSC (**Figure 1B**). The same results also observed in **Figure 1C** that analyzed by qRT-PCR. To confirm the abovementioned increase of α-SMA was directly related to E2 expression, in situ expression of α-SMA and E2 was detected by immunofluorescence assay to reveal the co-localization of these two molecules (**Figure 1D**). These results 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** and **2B**, the mRNA level of TGF-β1 in HSC at 12 or 24 hrs after E2 transfection was significantly higher than in that in un-transfected 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-β1 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-1β was also proved since mRNA levels of IL-6 and IL-1β were clearly higher in a time-dependent manner (**Figure 2C** and **2D**). 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. This 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 sliencing by siRNA

To further confirm the involvement of E2 during fibrogenesis, TGF-β1, a well-known fibrosis inducer, served as a positive control and 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 (**Figure 3A**). Results of western blotting gave the same observation (**Figure 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 hrs was measured by ELISA. Results indicated that TGF-β1 level in stable Huh7-E2 cells was significantly higher than that in Huh7 cells, suggesting that HCV E2 protein enhances TGF-β1 expression which promotes liver fibrogenesis (**data not show**). 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 show**).

HCV E2 induce the expression of α-SMA and collagen (α)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 hrs. At the end of incubation, DCFH-DA in serum free medium was added to transfected cells and H_2O_2 levels were detect with flow cytometry. The results showed that E2 increase H_2O_2 levels and a co-transfection with SiE2 restore the levels to that of control. A pre-treatment with NAC also has the same effect for reversing the action of E2 (**Figure 4A**). Further investigation observed that the mRNA expression levels of α-SMA and collagen (α)I induced by E2 were decreased by pre-treatment with NAC at 2 hrs before E2 transfection (**Figure 4B**). These data suggested that HCV E2 protein enhance H_2O_2 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 JE, 1997]**. In addition, JAK-STAT pathway has been shown to be activated in response to H_2O_2 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_2O_2 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 (**Figure 5A** and **5C**), 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 (**Figure 5B** and **5D**), suggesting that the capability of E2 to induce phosphorylation of JAK.

HCV E2 increase p38 MAPK phosphorylation

It has been reported that H_2O_2 stimulate the production of Tissue inhibitors of metalloproteinase (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_2O_2 also result in a up-regulation of procollagen (α)I mRNA in HSC cells **[Cao et al., 2004]**. Therefore, if E2-induced H_2O_2 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 (**Figure 6A** and **6B**). However, this E2-enhanced p38 phosphorylation was totally inhibited by a pre-treatment of catalase (**Figure 6C**), demonstrating the involvement of H_2O_2 in E2-related p38 activation.

HCV E2 increases ERK1/2 MAPK phosphorylation

It has been reported that H_2O_2 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 (**Figure 7A** and **7B**) and such increase was abolished by a co-transfection of specific E2 siRNA. Again, the rise was not totally inhibited by a pre-treatment of PD98059, an ERK phosphorylation inhibitor, but completely diminished by a pre-treatment with catalase (**Figure 7C**). These results further demonstrated the vital involvement of H_2O_2 in E2-induced phosphorylation.

HCV E2–induced increase of collagen (α)I was inhibited by JAK inhibitor AG490, catalase, p38 inhibitor SB203580, and ERK1/2 inhibitor PD098059

To further investigate the involvement of H_2O_2 related pathways, the effects of inhibitors of JAKs, H_2O_2, p38, and ERK1/2 on E2–induced collagen (α)I production were studied. Results in **Figure 8** showed that these inhibitors all reduce E2-induced collagen (α)I production to various extents. A pre-treatment 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 (α)I protein was H_2O_2 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 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 hepatic stellate cells, 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 α-SMA and collagen (α)I, were increased in a time-dependent fashion, as shown in **Figure 1**. Co-localization of E2 and α-SMA revealed by fluorescence microscopic observation further confirmed that E2 protein lead to the initialization of fibrosis. Upon the injury of liver, stellate cells secret various cytokines, hormones or oxygen free radicals to lead to hepatic damage, apoptosis or regeneration. TGF-β1 is one of known vital factors in the fibrogenic process and the results in **Figure 2A**, **2B** showed that E2 protein significantly enhanced TGF-β1 mRNA and protein expression levels (**data not show**), 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 show**). Previous studies suggested that TGF-β expression be induced by certain pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, while IL-1β and TNF-α increase the expression and activity of MMP-2 in cardiac fibroblasts **[Siwik et al., 2000; Bennermo et al., 2004; Corbel et al., 2002; Kershenobich Stalnikowitz and Weissbrod, 2003; Ortiz et al., 1994]**. In this study, we also demonstrated that IL-6 and IL-1β 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 **[Falkowska et al., 2007; Heo et al., 2006; Mazzocca et al., 2005]**, 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 hepatic stellate cells 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 fibril collagens replace normal collagen as ECM during fibrogenesis **[Schuppan et al., 2001; Karelina et al., 2000]**. 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_2O_2 and superoxide anions, increased lipid peroxidation. Since H_2O_2 has been shown to serve as a signaling molecule between oxidative stress and collagen (α)I gene up-regulation **[Greenwel et al., 2000]**, this study focused on the effect of HCV E2 protein on H_2O_2 formation and the data indicated that E2 increase the levels of H_2O_2 to promote the expressions of fibrosis-related factors and then involve in liver fibrogenesis (**Figure 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-β **[Meurer et al., 2005]**. Despite that NAC may eliminate the E2-induced H_2O_2 production, as proven in Figure 4A, the possible contribution to these results of NAC-related TGF-β 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 (α)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 (**Figure 7**). In rat HSCs, ERK1/2 signaling was found to involve NF-1 and SP-1 sites in the proximal promoter of the collagen (α)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-β and apoptosis will be further investigated"

In this study, we have demonstrated that HCV E2 protein stimulate collagen (α)I mRNA expression and its protein production in mouse HSC-T6 by the generation of the oxidant H_2O_2, which be via

activation of JAK1 and JAK2. In turn, H_2O_2 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.

Figure and Figure legends

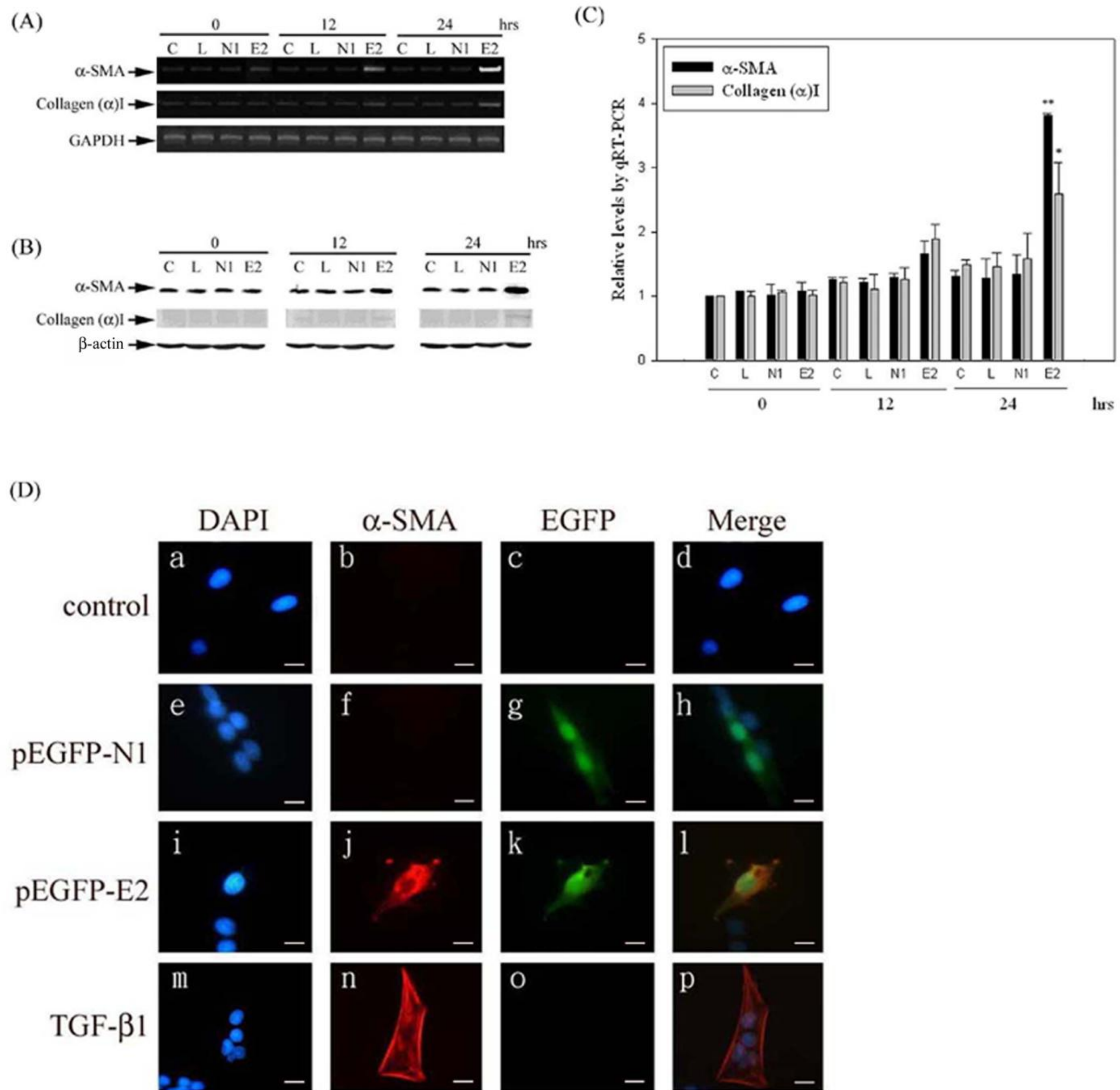

Figure 1. Up-regulation of mRNA and protein levels of α-SMA and collagen (α)I in E2-transfected HSC. At the indicated time after transfection in serum free medium, cells were harvested and then subjected to (A) mRNA extraction followed by semi-quantitative RT-PCR or (B) whole cell lysate preparation followed by western blotting with an antibody against α-SMA and collagen (α)I. (C) The mRNA expression was quantitatively analyzed by qRT-PCR. Results are expressed as averages ± 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 α-SMA and then a secondary antibody with rhodamine (TRITC) conjugate were performed to detect the expression of α-SMA in E2-transfected HSCs (400×) (Scale bars, 10 μm). TGF-β1: HSCs treated with TGF-β1 (5 ng/mL) for 24 hrs in serum free medium, EGFP: enhanced green fluorescent proteins.

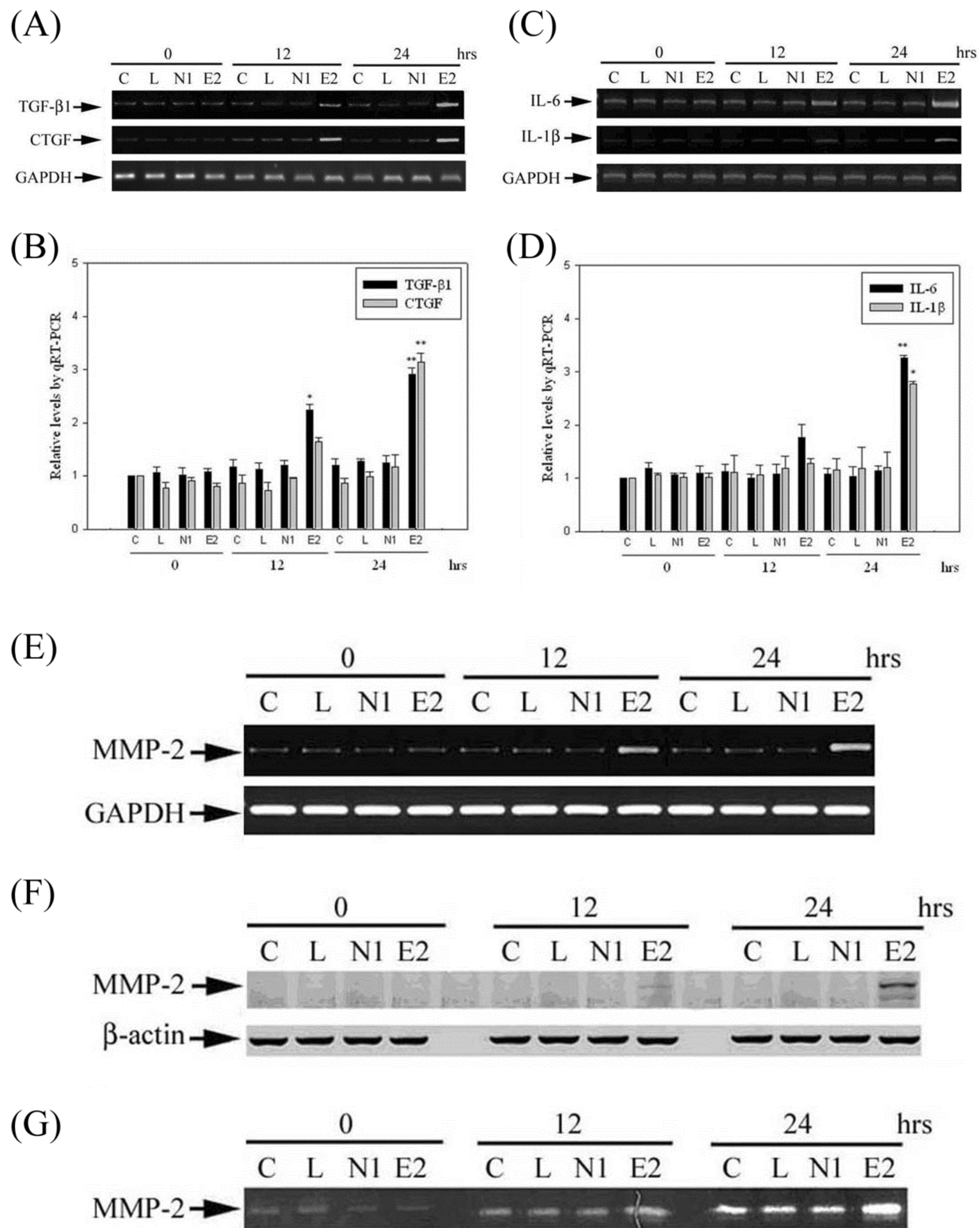

Figure 2. Increased expressions of inflammation-related cytokines and MMP-2 in E2-transfected HSC. At the indicated time after transfection in serum free medium, cells were harvested and then subjected to RNA extraction followed by semi-quantitative RT-PCR and quantitative RT-PCR for certain cytokines, 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.

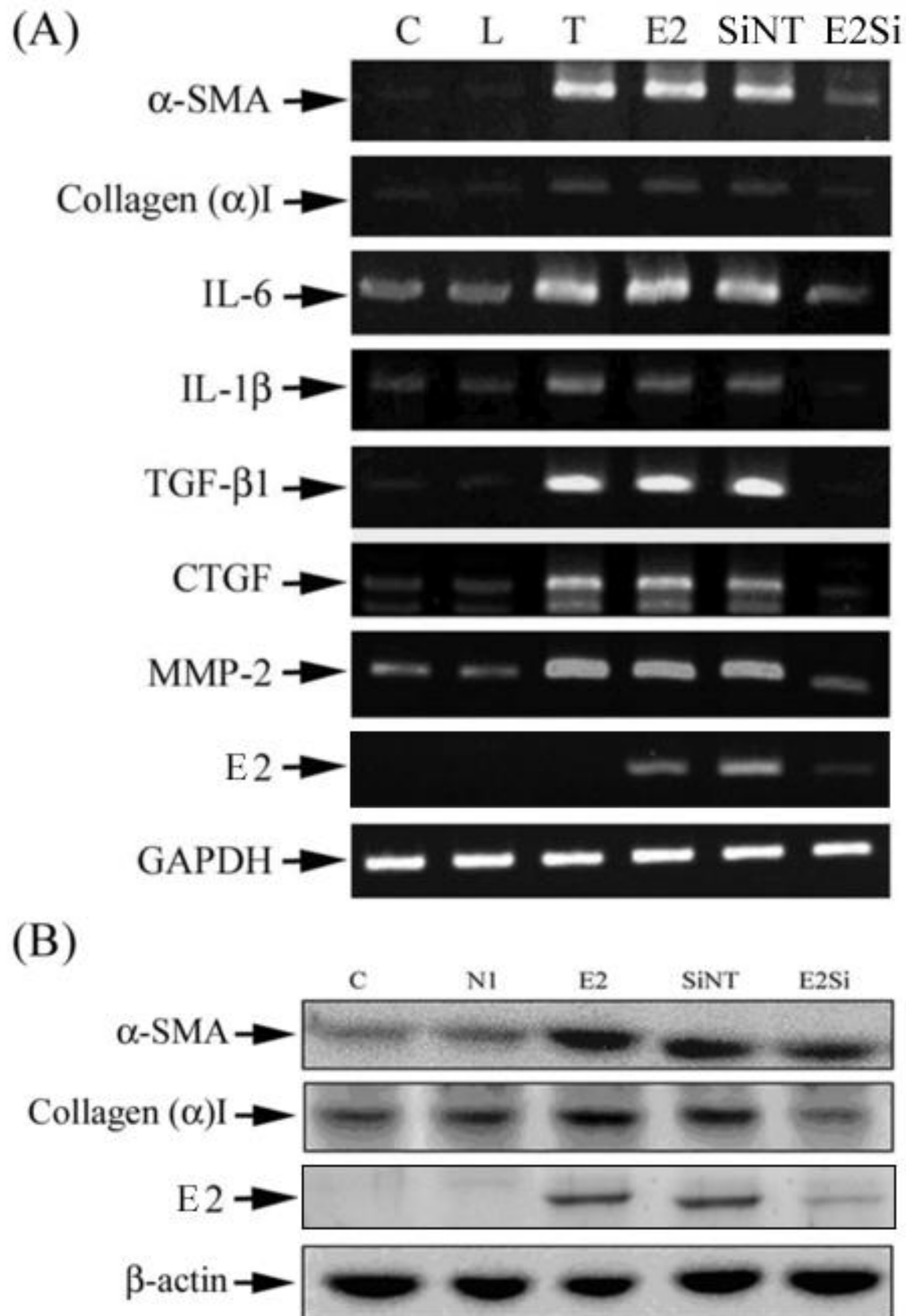

Figure 3. The abolishment of increased expressions of fibrosis-related molecules by E2 silencing. (A) After transfection or TGF-β treatment in serum free medium for 24 hrs, cells were harvested and then subjected to RNA extraction followed by semi-quantitative RT-PCR. (B) After transfection for 24 hrs, cell lysate preparation was subjected to western blotting with an antibody against α-SMA and collagen (α)I. C: control, L: lipofectamine, T: TGF-β (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.

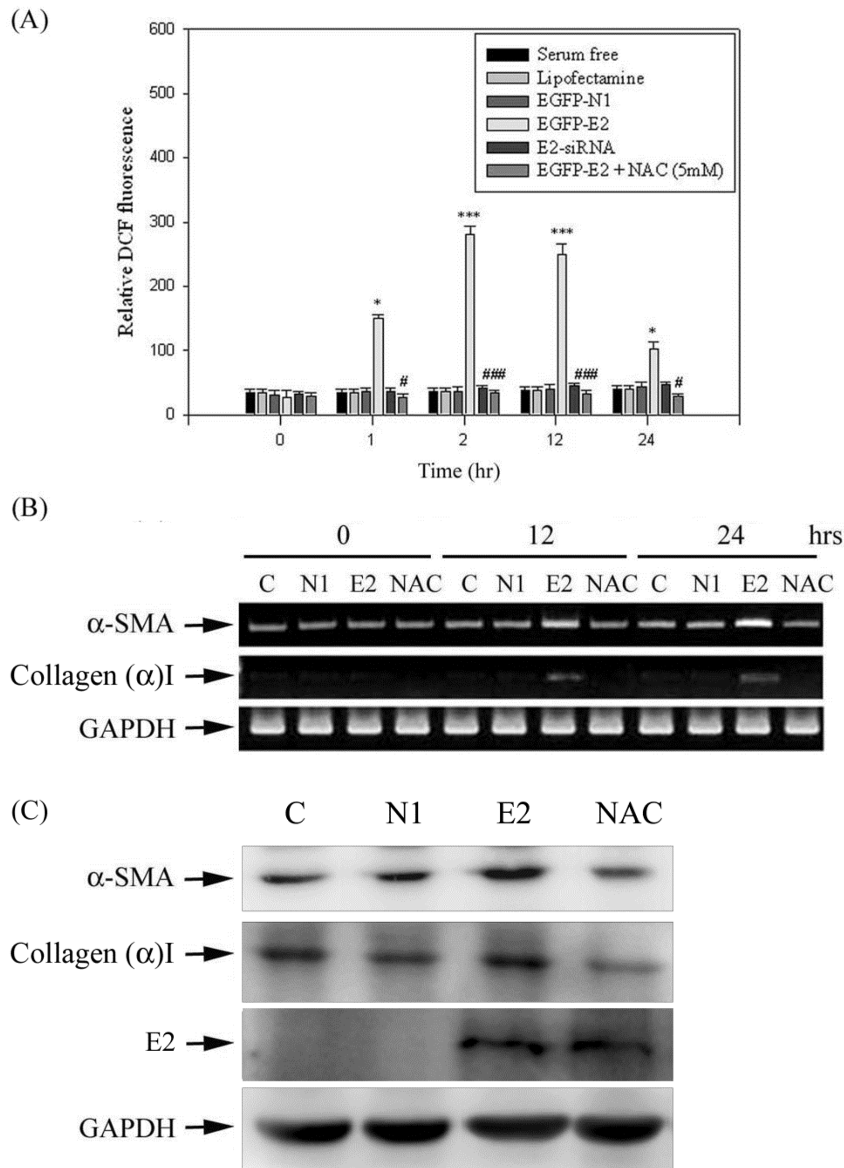

Figure 4. Generation of intracellular ROS by HCV E2 and its prevention by N-acetyl cysteine. (A) HSC-T6 cells transfected with EGFP-E2 or co-transfected with SiE2 for 24 hrs. E2-transfected cells were incubated in serum free medium with 5 μM DCFH-DA at 37℃, 30 mins and then washed twice, resuspended in PBS, and analyzed on a FACSCalibur flow cytometer. For a treatment of NAC, an excellent anti-oxidant, cells were incubated with 5 mM NAC before transfection for 2 hrs. 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 hrs and then harvested and subjected to cell lysate followed by western blotting. C: control, N1: pEGFP-N1, E2: pEGFP-E2, NAC: a pre-treatment of NAC (5 mM) for 2 hrs and then a transfection with pEGFP-E2.

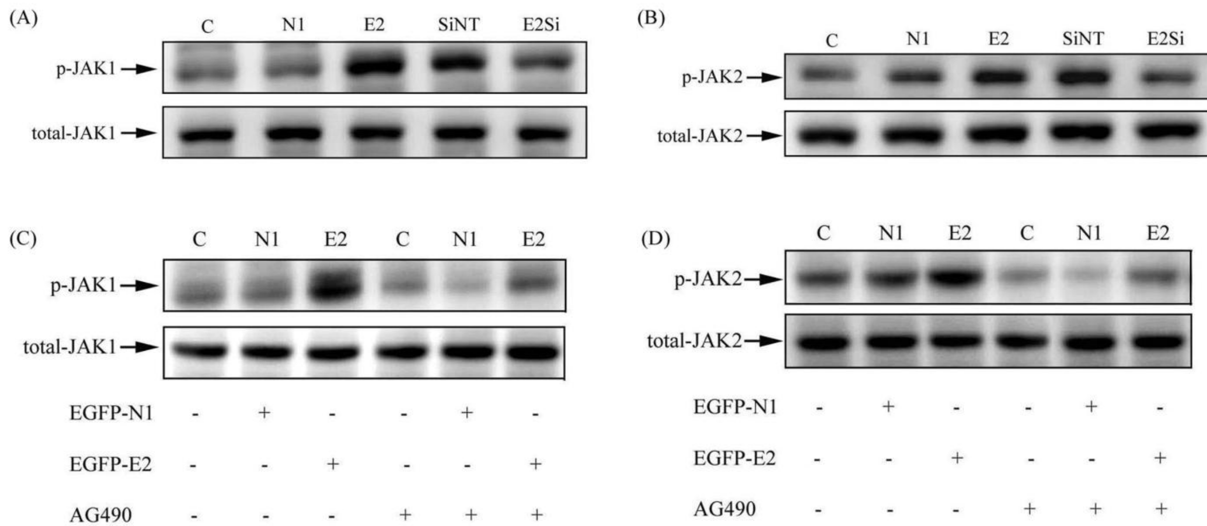

Figure 5. Increased phosphorylation of JAK1 and JAK2 by HCV E2. HSC-T6 cells were transfected with EGFP-E2 alone or co-transfected with SiE2 for 24 hrs. Cells were harvested and the whole cell lysate were prepared for western blotting with an antibody against p-JAK1 (A) and p-JAK2 (B) proteins. For inhibitor treatment, cells were incubated with AG490 (50 μM) in the serum-free medium before transfection for 2 hrs and then transfected with EGFP-E2 alone or co-transfected with SiE2 for 24 hrs 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.

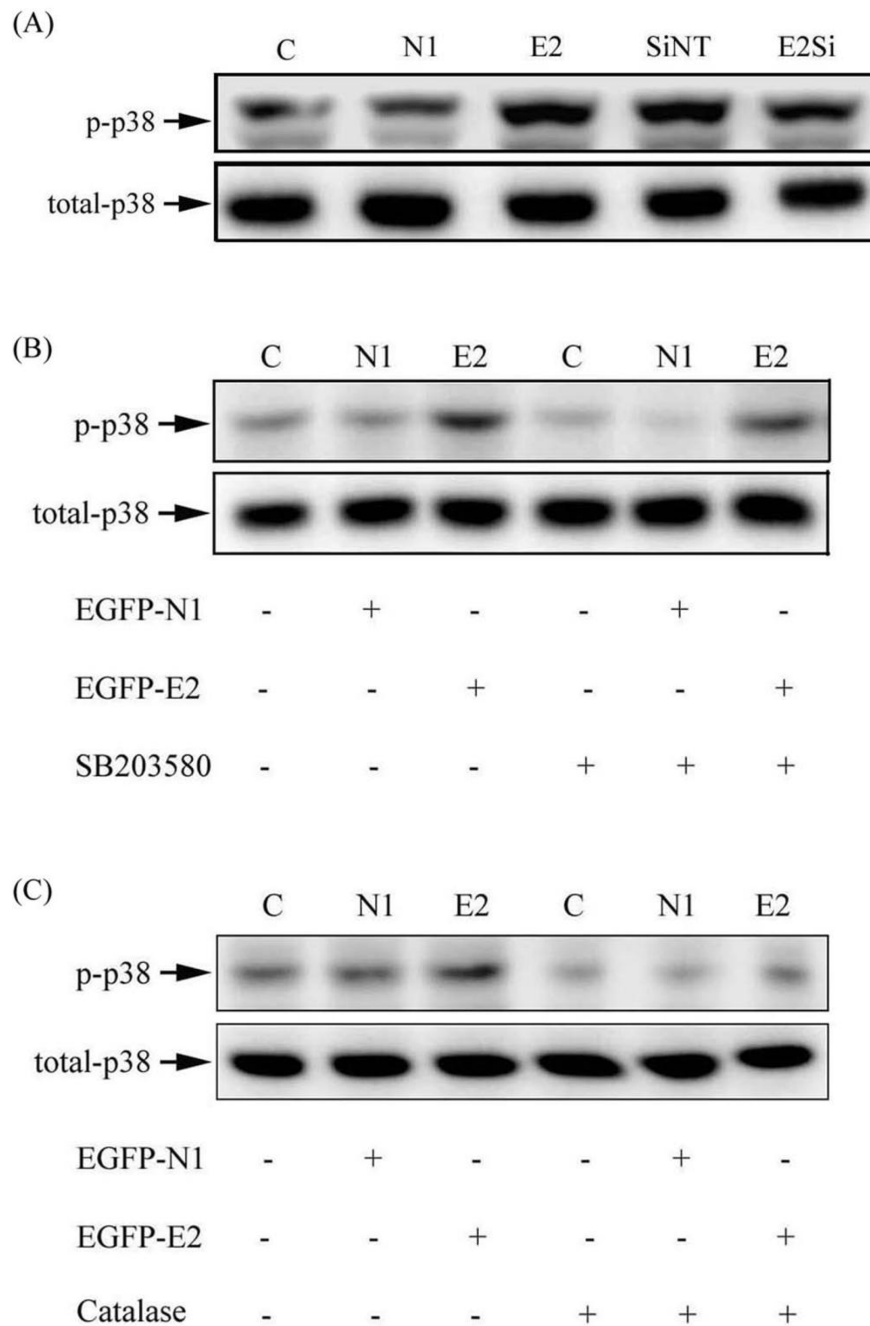

Figure 6. Activation of p38 MAPK by HCV E2 and its inhibition by catalase, p38 inhibitor SB203580. (A) After transfection for 24 hrs, cell lysate were prepared and subjected to western blotting for p-p38 and total p38 proteins. Before transfection, cells were incubated with (B) SB203580 (20 μM) and (C) catalase (1,000 U/ml) in the serum-free medium for 2 hrs and then transfected with pEGFP-E2 alone or co-transfected with SiE2 for 24 hrs 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.

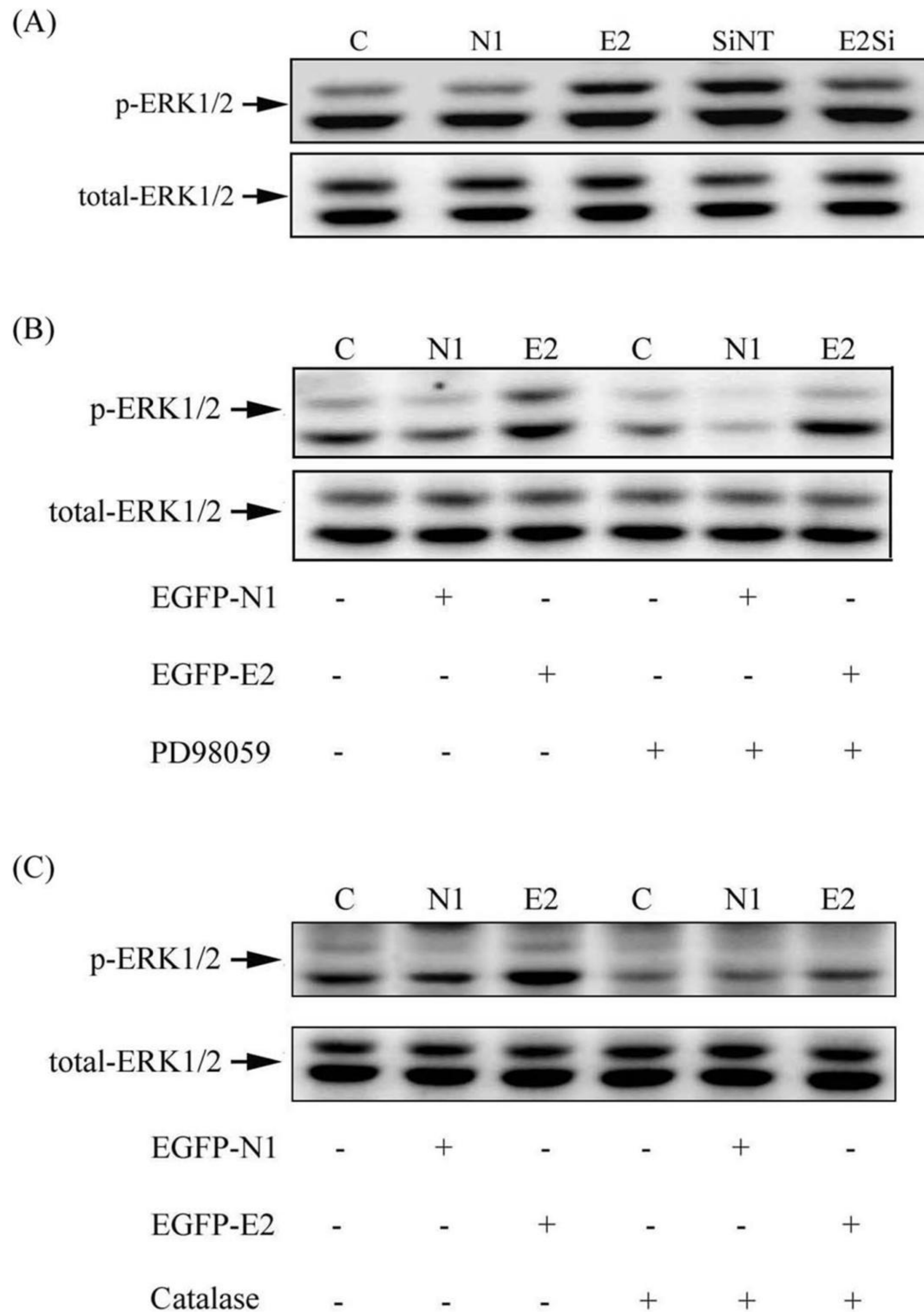

Figure 7. ERK1/2 MAPK phosphorylation by HCV E2 and its inhibition by catalase and ERK1/2 inhibitor PD098059. (A) After transfection for 24 hrs, cell lysate were prepared and subjected to western blotting for p-ERK1/2 and total ERK1/2. Before transfection, cells were incubated with (B) PD098059 (30 μM) and (C) catalase (1,000 U/ml) in the serum-free medium for 2 hrs and then transfected with pEGFP-E2 alone or co-transfected with SiE2 for 24 hrs 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.

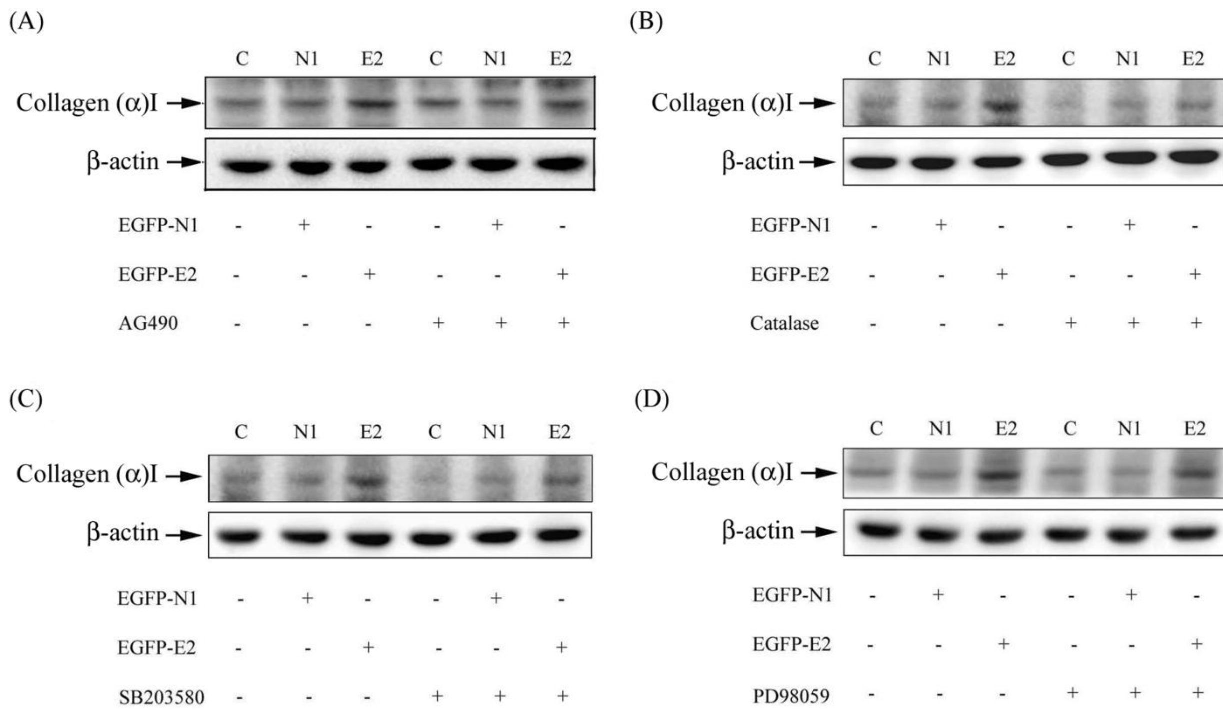

Figure 8. Inhibition of E2-induced collagen (α)I protein by JAK inhibitor AG490, catalase, p38 inhibitor SB203580 and ERK1/2 inhibitor PD098059. Before transfection, cells were incubated with an indicated inhibitor in the serum-free medium for 2 hrs and then transfected with pEGFP-E2 for 24 hrs in the presence or absence of inhibitors. Afterwards, cell lysates were prepared and analyzed by western blotting for collagen (α)I. (A) AG490 (50 μM); (B) catalase (1,000 U/ml); (C) SB203580 (20 μM); (D) PD098059 (30 μM). The protein levels of β-actin were used to show equal protein loading. C: control, N1: pEGFP-N1, E2: pEGFP-E2.

Table 1. Primers employed for RT-PCR

	Sequence	Size (bp)
mCollagen (α)I	5'-GACGCCATCAAGGTCTACTG-3' 5'-ACGGGAATCCATCGGTCA-3'	154
mα-SMA	5'-GTGCTATGTAGCTCTGGACT-3' 5'-ACATCTGCTGGAAGGTAGAC-3'	419
mIL-6	5'-GTGTTCTCAAGGTCTGAGTC-3' 5'-GTAGCCACCTTGTCTTACAG-3'	423
mIL-1β	5'-ATAACCTGCTGGTGTGTGACG-3' 5'-GGTGAAGTCAACTATGTCCC-3'	458
mTGF-β1	5'-GCCCTGGATACCAACTATTGC-3' 5'-GCAGGAGCGCACAATCATGTT-3'	326
mCTGF	5'-CCCGCCAACCGCAAGATT-3' 5'-AGGCGGCTCTGCTTCTCCA-3'	449
mMMP-2	5'-GAGTTGGCAGTGCAATACCT-3' 5'-GCCGTCCTTCTCAAAGTTGT-3'	665
mGAPDH	5'-ACCACAGTCCATGCCATCAC-3' 5'-TCCACCACCCTGTTGCTGTA-3'	451
hCTGF	5'-ACGGCGAGGTCATGAAGAAGAACA-3' 5'-TGGGGCTACAGGCAGGTCAGTG-3'	521
hGAPDH	5'-CGGAGTCAACGGATTTGGTCGTAT-3' 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	306

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※此計畫研究結果內容為參與計畫人員謝明儒的博士研究論文內容

附件二

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
■ 達成目標
□ 未達成目標（請說明，以 100 字為限）
□ 實驗失敗
□ 因故實驗中斷
□ 其他原因
說明：

2. 研究成果在學術期刊發表或申請專利等情形：
論文：■已發表 □未發表之文稿 □撰寫中 □無
專利：□已獲得 □申請中 □無
技轉：□已技轉 □洽談中 □無
其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

探討 C 型肝炎病毒引發肝臟纖維化的病因與相關機制一直是實驗室努力研究的目標，在實驗室之前的研究成果中，我們發現到 C 型肝炎病毒 E2 蛋白除了在病毒侵入宿主細胞扮演著重要的角色之外，E2 蛋白與引發肝臟纖維化的發生過程中也具有相關性。在我們更進一步的研究探討後，除了證明 E2 蛋白具有誘導肝臟星狀細胞活化的能力，並且進一步的發現 E2 蛋白誘導肝臟星狀細胞活化的原因以及相關路徑，因此，在我們的研究結果中證實了 E2 蛋白在 C 型肝炎病毒除了扮演侵入宿主細胞的功能，並且也證明引起的原因以及所牽涉到的相關路徑，這些的研究結果顯示，在未來探討 C 型肝炎病毒引發相關疾病的學術研究上能有進一步的發展，並且也有助於治療方式發展的可能性，未來在臨床的研究治療上不論是利用藥物化學治療或是其他方式的發展上，都可提供一些新的資訊與了解。

附錄

※該計畫已有論文發表

Hepatitis C virus E2 protein induce reactive oxygen species (ROS)-related fibrogenesis in the HSC-T6 hepatic stellate cell line.

Ming-Ju H, Yih-Shou H, Tzy-Yen C, Hui-Ling C.

Journal of cellular biochemistry. 2011; 112(1): 233-43.