

**Binding of Hepatitis C Virus Envelope Protein E2 to CD81 Up-regulates  
MMP-2 in Human Hepatic Stellate Cells**

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## SUMMARY

The Hepatitis C virus (HCV) envelope E2 glycoprotein is a key molecule regulating the interaction of HCV with cell surface proteins. E2 binds the major extracellular loop of human CD81, a tetraspanin expressed on various cell types including hepatocytes and B lymphocytes. Regardless, information on the biological functions originating from this interaction are largely unknown. Since human hepatic stellate cells (HSC) express high levels of CD81 at the cell surface, we investigated the E2/CD81 interaction in human HSC and the possible effects arising from this interaction. Matrix metalloproteinase 2 (MMP-2, gelatinase A), a major enzyme involved in the degradation of normal hepatic extracellular matrix, was up-regulated following the interaction between E2 and CD81. In particular, by employing zymography and western blot, we observed that E2 binding to CD81 induces a time-dependent increase in the synthesis and activity of MMP-2. This effect was abolished by pre-incubating HSC with an anti-CD81 neutralizing antibody. Similar effects were detected in NIH3T3 mouse fibroblasts transfected with human CD81 with identical time-course features. In addition, E2/CD81 interaction in human HSC induced the up-regulation of MMP-2 by increasing AP-2/DNA binding activity via ERK/MAPK phosphorylation. Finally, suppression of CD81 by RNA interference in human HSC abolished the described effects of E2 on these cells, indicating that CD81 is essential for the activation of the signaling pathway leading to the upregulation of MMP-2. These results suggest that HSC may represent a potential target for HCV. The interaction of HCV envelope with CD81 on the surface of human HSC induces an increased expression of MMP-2. Increased degradation of the normal hepatic ECM in areas where HCV is concentrated may favour inflammatory infiltration and further parenchymal damage.

## INTRODUCTION

HCV is the most common cause of chronic liver disease, leading to hepatic fibrosis and ultimately to cirrhosis [1]. The HCV major envelope E2 glycoprotein exposed on the surface of virions is likely to be involved in the interactions with the host and has been identified as responsible for binding of HCV to target cells [2]. Truncated, secreted versions of E2 glycoprotein have been utilized as soluble mimics of viral particles in order to study virus-cells interactions [3,4]. In particular, E2 glycoprotein has been reported to bind to the major loop of human transmembrane molecule CD81, a member of tetraspanin protein superfamily, expressed on various cell types including hepatocytes and B lymphocytes, and, accordingly, CD81 has been proposed as a putative receptor for HCV [2].

The most striking feature of CD81, as well as other tetraspanin molecules, is a propensity to associate with a wide variety of membrane proteins, acting as a molecular facilitator for downstream intracellular signaling [5]. The wide range of complexes into which CD81 assembles suggests that the association of CD81 with different partners may have important and diverse effects on different cell types [6]. Accordingly, binding of E2 glycoprotein to CD81 may be relevant for explaining the development and the persistence of HCV infection in the liver, as well as in extrahepatic tissues [7]. A preliminary study performed in our laboratory on sections of normal human liver indicates that CD81 is expressed at the cell surface of hepatocytes, as well as within sinusoidal structures (**Figure 1**). HCV E2 binds different cell types present in liver tissue samples, although with different intensities, and this binding is prevented by pre-incubation of liver tissue samples with anti CD81 antibodies [8]. In addition, we have recently reported that human HSC, liver-specific pericytes and key effectors of hepatic fibrogenesis, express high levels of CD81 protein at the cell surface [9].

An essential step in the progression of HCV-related hepatic fibrogenesis is the degradation of normal liver extracellular matrix (ECM), mediated by increased expression of proteolytic enzymes, such as matrix metalloproteinases (MMPs). These enzymes are involved in the acute phases of liver injury, as well as during chronic hepatic wound healing and fibrogenesis [10], and may contribute to the inflammatory and fibrogenic response to many stimuli [11,12], including viral proteins [13]. The activity of MMPs, secreted mainly by connective tissue cells as proenzymes, is controlled by transcriptional regulation, zymogen activation and specific tissue inhibitors (TIMPs) [14].

MMP-2 (also known as gelatinase A or collagenase IV), the most relevant MMPs component involved in remodelling of normal liver ECM during hepatic fibrogenesis, efficiently degrades collagen type IV and type I, fibronectin, and laminin [15]. Secretion of active MMP-2 may be stimulated by several mediators, including pro-inflammatory cytokines, during the inflammatory phase of wound healing. Moreover, the same mediators are able to activate the proenzymatic form of MMP-2 by inducing a membrane type matrix metalloproteinase (MT1-MMP), which subsequently activates MMP-2 [16]. It is conceivable that activation of MMP-2 represents a key event in the wound healing process following acute or chronic tissue damage. Degradation of the normal ECM in areas of necrosis allows penetration of inflammatory cells exerting phagocytic and/or immunological activities. Along these lines activation of MMP-2 can be viewed as a relevant pro-inflammatory event contributing to tissue injury.

In the present study, we investigated the interaction of E2/CD81 in human HSC and the expression of MMP-2 following this interaction as a possible host cellular response to viral infection. Our results indicate that human HSC could represent a novel potential cellular target for HCV within the liver and show that binding of E2 to CD81 on HSC surface leads to an increased expression of MMP-2, mediated by ERK/MAPK activation followed by AP-2 transcriptional activity.

## EXPERIMENTAL PROCEDURES

### *Reagents*

The HCV E2 protein used throughout this study was a clinical-grade batch prepared by Chiron Co. (Emeryville, CA). Briefly, the protein was prepared from a CHO cell line stably transfected with plasmid pCMVa120 [17] in which the E2 sequence from amino acids 384 to 715 was fused to the tissue plasminogen activator leader sequence. After cell disruption and debris removal by microfiltration, the protein was purified by three subsequent chromatographic steps: lectin affinity chromatography, hydroxyapatite chromatography, and ion-exchange chromatography. As described elsewhere, all the HCV envelope E2 proteins were ~90% pure after purification [4].

The mAb 291 (IgG1) was obtained from mice immunized with CHO/E2<sub>715</sub> and screened for the ability to recognize E2 bound to target cells as previously described [4]. The antibodies against CD81 JS81 and 1.3.3.22 were purchased from Pharmingen (San Diego, CA) and from Santa Cruz Biotechnology (Santa Cruz Biotech. CA), respectively. The rabbit polyclonal anti-MMP-2 (AB809) was purchased from Chemicon International (Temecula, CA). The Immobilon-P was purchased from Millipore (Bedford, MA). The Enhanced Chemiluminescence system (ECL) was from Amersham Pharmacia (Buckinghamshire, U.K.). Gelatin-Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). Protein concentration in samples was determined using the bicinchoninic acid kit from Sigma (St. Louis, MO). PD-98059 was purchased from Calbiochem (La Jolla, CA).

### *Human tissues and immunohistochemistry*

These experiments were conducted on frozen surgical sections of human liver as described in detail elsewhere [18]. Dried sections were sequentially incubated with the primary anti-CD81

mAb and, after washing, with affinity-purified rabbit anti-mouse antibodies. At the end of the incubation, sections were washed twice in TBS and then incubated with APAAP and developed. A non-immune mouse IgG primary antibody was used as negative control.

### ***Isolation and culture of human HSC***

Human HSC were isolated from wedge sections of normal human liver unsuitable for transplantation as previously reported [19]. Briefly, after a combined digestion with collagenase/pronase, HSC were separated from other liver nonparenchymal cells by ultracentrifugation over gradients of stractan (Cellsep™ isotonic solution, Larex Inc., St. Paul, MN). Extensive characterization was performed as described elsewhere [19]. Cells were cultured on plastic culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ) in Iscove's modified Dulbecco's medium supplemented with 0.6 U/ml insulin, 2.0 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, antibiotic antifungal solution (all provided by Gibco Laboratories, Grand Island, NY) and 20% fetal bovine serum (Imperial Laboratories, Andover, U.K.). Experiments described in this study were performed on cells between first and third serial passages (1:3 split ratio) using three independent cell lines. At these stages of culture, cells show functional and ultrastructural features of fully activated HSC. In particular, analysis of cell surface markers indicates that, at these stages of culture *in vitro*, HSC present with a cell marker profile identical to the so-called “interface” myofibroblasts detected in liver tissue specimens at the border between “active” fibrotic septa and the parenchyma of the liver lobule [20]. In all experiments, confluent HSC were left for 24 h in Iscove's SFIF-medium before the addition of the different stimuli.

### ***Flow Cytometry Analysis***

For flow cytometry (FACS) analysis, cells were trypsinized, harvested and washed with 2% fetal calf serum in Iscove's medium. E2 protein was bound to cells for 30 min on ice. Cells were

washed and incubated with anti-E2 mAb for further 30 min on ice. After three washes, cells were resuspended in goat anti-mouse-fluorescein isothiocyanate (FITC, 1:40 dilution), and incubated 30 min on ice. Cells were washed again, resuspended in serum free medium and fluorescence analyzed using a FACScan flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Cells incubated with BSA/PBS 1% were used as negative controls. For CD81 analysis, cells were stained with anti-CD81 mAb (JS81) or with the isotype mouse IgG as control, washed and incubated with an anti-mouse FITC-conjugated, washed again and analyzed as previously described [9].

### ***Immunofluorescence and Confocal Microscopy***

For immunofluorescence analysis, cells were plated in Iscove's medium supplemented with 1% FCS on glass coverslips for 2-4 h. Once spread, cells were fixed for 5 min with 2% paraformaldehyde in PBS, pH 7.2. Coverslips were blocked for 1 h with 3% heat-inactivated BSA/PBS. Cells were then incubated with E2 diluted in 3% BSA-PBS and after three washes stained with anti-E2 mAb, incubated, and washed again. Staining was subsequently visualized with FITC-goat anti-mouse antibody (Calbiochem-Novabiochem, San Diego, CA) before the coverslips were mounted with anti-fading Vectashield (Vector Laboratories, Inc. Burlingame, CA). Immunofluorescence was examined by a series of optical sections, obtained with a confocal scanning laser microscope (MRC-1024, Bio-Rad Laboratories, Hercules, CA) and images were captured, processed, and superimposed by using the LaserSharp software (Bio-Rad Laboratories). An anti-CD81 mAb staining was included as a positive control. A solution of BSA/PBS 3% and a non-immune mouse IgG were employed as a negative control for E2 and CD81, respectively.

### ***E2 binding assay***

Binding of E2 to HSC was evaluated by a competition assay. HSC were incubated with increasing concentrations of anti-CD81 mAbs (JS81 and 1.3.3.22) or anti-HLA-I (clone W6/32, Sigma) as negative control and seeded onto 96-well E2 protein coated plates for 1 h at 37°C. To remove unbound cells, wells were then filled with PBS, and gently washed three times. Adherent cells were fixed in 3% paraformaldehyde/PBS and then stained with 0.5% crystal violet in 20% methanol/80% H<sub>2</sub>O. Wells were then washed with water to remove excess dye, cells were then solubilized in 1% SDS and the amount of dye was quantified by using Bio-Rad Multiscan plate reader at 595 nm. The specificity of E2 protein binding to CD81 on human HSC was further confirmed by the displacement of E2 from the binding to CD81 in the presence of the competitive anti- human CD81 mAb (JS81). For these experiments, HSC were preincubated with increasing concentrations of E2 protein (0.1, 1.0, 10.0, 50.0, 100 µg/ml) prior plating onto 96-well plates coated with immobilized anti-CD81 mAb (JS81) for 1 h at 37°C. Adherent cells were then washed, fixed, stained and solubilized in 1% SDS, and the amount of dye was quantified as described above.

### ***Gelatinolytic zymography***

Conditioned media from the cell cultures were analyzed for gelatin degradation activity by SDS-PAGE under non-reducing conditions. The gel contained 5.8 mg/ml gelatin and 8 % acrylamide. Electrophoresis was carried out at 4°C. After a brief wash with water, the SDS in the acrylamide gel was extracted by incubation with 2% Triton X-100/PBS solution. Gelatinolytic activities were developed in a buffer containing 5 mM CaCl<sub>2</sub>, 150 mM NaCl and 50 mM Tris at 37°C for 16 hours. The gelatinolytic activities were visualized by staining the gel with Coomassie Blue R-250. The amount of 66 kDa MMP-2 was determined using scanning densitometry with NIH Image analysis software.

### ***Western blot***

Samples for Western blot were prepared differently for conditioned media and for cells. Cell monolayers were extracted with buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.5, and 1 mM PMSF. Cell debris were removed by centrifugation at 14,000 x g. The supernatant was subjected to 8% SDS-PAGE. In the conditioned media the MMP-2 enzyme was enriched by incubation of 500 ml conditioned medium with 30 ml gelatin-conjugated Sepharose 4B. The sepharose was washed with buffer containing 400 mM NaCl, 50 mM Tris, pH 7.5, and the bound protein was eluted with SDS-sample buffer. All samples were resolved by reducing SDS-PAGE and transferred to Immobilon-P. Filters were blocked with 5% non-fat milk in TBS (150 mM NaCl, 50 mM Tris, pH 7.5) for 2 hours at room temperature, then incubated overnight at 4°C with individual antibodies in TBS + 0.05% Tween-20 (TBS-T). The polyclonal anti-MMP-2 was used at 1:5000 dilution. Blots were incubated for 2 hours with a horseradish peroxidase-conjugated goat anti-rabbit antibody at 1:5000 dilution in TBS-T and visualized with enhanced chemiluminescence. In some experiments, cells were treated with 1 µM of the protein synthesis inhibitor cyclohexymide (Sigma, St. Louis, MO). For AP-2 blotting, a mouse mAb anti-human AP-2α (Santa Cruz Biotechnology) was used to detect AP-2 protein.

### ***Generation of human CD81 stable cell lines***

NIH3T3 cells were maintained in complete medium [90% minimal essential medium, 10% (w/v) fetal-bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine] (Life Technologies) at 37 °C in a 5% CO<sub>2</sub> atmosphere, and cells at 50–60% confluence were used in transfection assays. Transfection into cells was done by the LipofectAMINE<sup>TM</sup> method with the 2000 reagent according to the manufacturer's instructions (Life Technologies, Inc.) with the use of 4 µg/60-mm dish of pcDNA 3.1-CD81 or 1.5 µg/35-mm dish of pcDNA 3.1-CD81 vector. The stably transfected cell clones were generated culturing the cells in DMEM with 10%

FBS and G418 (0.2 mg/mL). A stable clone expressing high level oh human CD81 (clone 42) was selected by flow cytometry analysis and used for the experiments.

### ***Immunoprecipitation and lipid kinase assay***

For immunoprecipitation, HSC monolayers were lysed in buffer containing 1% CHAPS, 20 mM Hepes (pH 7.5), 200 mM NaCl, 5 mM MgCl<sub>2</sub>, 200 μM Na<sub>3</sub>VO<sub>4</sub>, 2 mM NaF, 10 mM Na<sub>4</sub>P<sub>3</sub>O<sub>7</sub>, 2 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and immunoprecipitates were prepared using protein G-Sepharose (Pharmacia, Upsala, Sweden). After immunoprecipitation with anti-CD81 mAb (1.3.3.22), anti-E2 (mAb 291)-E2 protein or with non-immune mouse IgG as a control, immune complexes were washed four times in lysis buffer and one time in 10 mM HEPES + 5 mM MgCl<sub>2</sub> before phosphoinositide kinase reactions were performed directly on beads. Briefly, the reaction mixture included 20 mM HEPES (pH 7.5), 10 mM MgCl<sub>2</sub>, 50 μM ATP (Pharmacia Biotech), 0.3% Triton X-100, 10-15 μCi [<sup>32</sup>P]-ATP (ICN Biomedicals, Inc.), and 200 μg/ml sonicated L-α-phosphatidylinositol (Sigma) as a substrate. Adenosine (200 μM, Sigma) was employed in these experiments as an established in vitro inhibitor of type II PI 4-K reactions [21]. Reactions were carried out for 5 min at room temperature and stopped with 2 M HCl. Lipids were extracted with 1:1 (vol/vol) chloroform-methanol, and the organic layer was resolved by TLC on potassium oxalate-treated Silica gel 60 silica plates (EM Science, Darmstadt, Germany). Beta-emitting radioactivity corresponding to PIP was quantified using a Betascope 603 Blot Analyzer (Betagen, Waltham, MA). Lipid kinase activity is expressed as counts per min within a defined area representing the PI-4-[<sup>32</sup>P] spot.

### ***ERK phosphorylation***

Confluent cell monolayers on 100-mm dishes were growth-arrested in serum-free medium for 24 h prior to treatment with E2. Cells were washed twice with PBS, scraped, resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 20 μg/ml aprotinin,

20 µg/ml leupeptin, and 20 µg/ml pepstatin), and clarified by centrifugation at 14,000 × g for 10 min. Thirty µg of protein/sample was separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blocked for 2 h at 25 °C with 5% nonfat milk in PBS buffer (20 mM Tris, 500 mM NaCl, and 0.01% Tween 20). The membrane was then incubated overnight at 4 °C with an appropriate dilution of anti-phospho-ERK or anti-ERK (New England Biolabs Inc.) polyclonal antibody at 4 °C overnight, washed, and followed by incubation for 2 h with a 1:2000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody. The immunoblot signal was visualized through enhanced chemiluminescence.

#### ***Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)***

For the nuclear extract preparation HSC were grown in 100-mm dishes and incubated in serum-free medium for 24 h prior to exposure to E2 protein at different time. Cells were then washed with cold PBS, harvested by scraping, and pelleted. Cells were resuspended in 1 ml of buffer A (10 mM KCl, 20 mM HEPES, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.4 mM PMSF, 1 mM sodium fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>), incubated on ice for 10 min, and pelleted at 1000 × g for 10 min. Pellets were resuspended in 0.5 ml of buffer A plus 0.1% Nonidet P-40, incubated on ice for 10 min, and centrifuged at 3,000 × g for 10 min. The nuclear pellet was resuspended in 1 ml of buffer B (10 mM HEPES, 400 mM NaCl, 0.1 mM EDTA, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.4 mM PMSF, 15% glycerol, 1 mM sodium fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>) and incubated for 30 min at 4 °C with constant gentle mixing. Nuclei were then pelleted at 40,000 × g for 30 min, and extracts were dialyzed for 2 h at 4 °C against 1 liter of buffer C (20 mM HEPES, 200 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, 15% glycerol, 1 mM sodium fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>). Extracts were cleared by centrifugation at 14,000 × g for 15 min at 4 °C. Protein concentrations were determined using a Bio-Rad protein assay. For EMSA four micrograms of nuclear extract were mixed with 20 µg of poly (dI:dC) in 20 µl of a reaction buffer consisting of

(in mM) 25 HEPES, pH 7.5, 1.2 dithiothreitol, 4 MgCl<sub>2</sub>, and 150 NaCl, as well as 5% glycerol, 0.005% bromphenol blue, and 0.05% Nonidet P-40 (Sigma). The mixture was incubated on ice for 15 min followed by the addition of 10 fmol of  $\gamma$ -<sup>32</sup>P end-labeled AP-2 consensus binding sequence oligonucleotide (nucleotide sequence: 5'-GAT CGA ACT GAC CGC CCG CGG CCC GT-3'; Promega, Madison, WI). Incubation was continued for 30 min. The incubation mixture was subjected to electrophoresis on a 6% polyacrylamide gel in Tris-glycine buffer. The gels were dried, and autoradiography was performed at -70°C with an intensifying screen. Bands were quantitated by laser densitometry (model 300S, Molecular Dynamics). Competition experiments were performed with a 200-fold excess of unlabeled AP-2 consensus binding sequence oligonucleotide.

#### ***CD81 silencing by small RNA interference***

The siRNA sequence targeting human CD81 (GenBank accession number NM\_004356) spans nucleotides 138–156 (target = 5'-ATCTGGAGCTGGGAGACAA-3') and is specific for human CD81 based on BLAST search (NCBI database). Sense siRNA sequence was 5'-AUCUGGAGCUGGGAGACAAdTdT-3' and antisense was 5'-UUGUCUCCCAGCUCCAGAUdTdT. A siRNA sequence corresponding to nucleotides 695–715 of the firefly luciferase (U31240) was used as a negative control. The siRNAs were chemically synthesized by Qiagen-Xeragon (Germantown, MD) and for annealing, 40  $\mu$ M of siRNA single strands were incubated in the annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate) for 1 min at 90 °C followed by 1 h at 37 °C. For transfection, the Amaxa nucleofection technology™ (Amaxa, Koeln, Germany) was employed. HSC were resuspended in the nucleofector T solution, available as part of the Amaxa cell optimization kit, following the Amaxa guidelines for cell line transfection. Briefly, 100  $\mu$ l of 2–5 $\times$ 10<sup>6</sup> cell suspension mixed with 2  $\mu$ g of pmaxGFP vector (to evaluate the transfection efficiency) plus 1  $\mu$ l of 40  $\mu$ M siRNA or negative control was transferred to a cuvette and nucleofected with an Amaxa

Nucleofector apparatus. Cells were transfected using the U-25 pulsing parameter and were immediately transferred into wells containing 37 °C pre-warmed culture medium in 6-well plates. 72 h after transfection, the CD81 protein expression levels at the cell surface were analyzed on a BDLSRII cytofluorimeter, using the DIVA software (BD Biosciences, Franklin Lakes, NJ). The area of positivity was determined using an isotype-matched control Ab.  $10^4$  events for each sample were acquired.

### ***Statistical analysis***

Results, relative to the number of experiments indicated, are expressed as mean+SD. Statistical analysis was performed by one-way ANOVA, and, when the F value was significant by Duncan's test.

## RESULTS

### *Expression and distribution of CD81 in normal and pathologic human liver tissue*

In agreement with a previous study exploring the binding of E2 in normal human liver [8], expression of CD81 was widespread in the liver lobule. Although a definite staining was present on hepatocyte membranes, a much stronger expression of CD81 was detected in sinusoidal structures (**Figure 1**, panel A). In human liver with extensive fibrosis and cirrhosis, the overall expression of this tetraspanin was increased and particularly abundant in correspondence with capillarized sinusoidal structures and interhepatocellular fibrosis (**Figure 1**, panel B).

### *HCV E2 glycoprotein specifically binds CD81 on human HSC surface*

Binding of HCV E2 glycoprotein to human HSC surface was evaluated by flow cytometry analysis (**Figure 2**, panel A). The level of E2 glycoprotein present on HSC surface was high and comparable to CD81 expression by human HSC, as detected by flow cytometry using the anti-CD81 mAb (**Figure 2**, panel B). The cellular distribution of E2 glycoprotein in human HSC was investigated by indirect immunofluorescence and confocal microscopy. E2 staining delineated the cell surface of individual HSC showing the typical distribution pattern of plasma membrane molecules (**Figure 2**, panel C). A similar distribution was observed by using the anti-CD81 mAb (**Figure 2**, panel D).

To demonstrate the specificity of E2 binding to CD81 on human HSC, we performed E2 binding competition assay. Incubation of HSC with increasing concentrations of anti-CD81 mAbs (JS81 and 1.3.3.22) inhibited HSC attachment on E2 coated wells in a mAb concentration-dependent manner, as detected by spectrophotometry using crystal violet staining (**Figure 3**, panel A). The anti human CD81 mAb JS81 showed to be more efficient in inhibiting E2-CD81

binding than anti-CD81 mAb 1.3.3.22. (**Figure 3**, panel B). The anti-HLA-I mouse mAb (W6/32) was used as negative control.

The specificity of E2 binding to CD81 on human HSC was further corroborated by the pre-incubation of HSC with E2 glycoprotein at increasing concentrations. The cells were then plated on wells coated with the immobilized anti-CD81 mAb JS81. As shown in **Figure 3**, panel C and D, the attachment of HSC was inhibited in a concentration-dependent manner.

***HCV E2/CD81 interaction induces the synthesis of MMP-2 in time- and dose-dependent manners in HSC***

Incubation of HSC with HCV E2 glycoprotein induced a time-dependent increase of MMP-2 gelatinolytic activity detected in HSC conditioned medium (**Figure 4**, panel A). A significantly increased activity was already observed after 2 hours of incubation. In addition, the increased MMP-2 activity induced by E2 glycoprotein in HSC was dose-dependent, and peaked at 10 µg/ml (**Figure 4**, panel B). The effects of E2 on the expression of MMP-2 were further investigated. Western blot analysis of both HSC conditioned medium and whole cell lysate confirmed a time-dependent increase of MMP-2 expression (**Figure 5**, panels A and B). Remarkably, MMP-2 expression was already maximally evident as early as 2 hours. In order to assess whether the increased expression of MMP-2 was due to de-novo synthesis or to mobilization of the protein from intracellular compartments, the above experiments were repeated by pre-incubating the cells with the protein synthesis inhibitor cyclohexymide. As shown in **Figure 5**, panel C, cyclohexymide induced the complete abrogation of E2-induced increase in MMP-2 expression, thus indicating that the up-regulation of MMP-2 upon E2 stimulation is due to de-novo protein synthesis.

### ***Binding of HCV E2 to CD81 directly mediates the expression of MMP-2 in human HSC***

To evaluate whether or not E2 protein is able to directly mediate synthesis and activity of MMP-2 through the binding to CD81, HSC were incubated with two different anti-CD81 mAbs and then stimulated with 10 µg/ml of E2 protein. As shown in **Figure 6**, the effect of E2 on MMP-2 activity was abolished by pre-incubating HSC with the neutralizing anti-CD81 mAb JS81 [2], but not with anti-CD81 mAb 1.3.3.22, in agreement with the results of the E2 binding assay (see Figure 3). Incubation with anti-CD81 mAbs alone, was without any effect.

### ***HCV E2-induced MMP-2 expression is dependent on human CD81***

The HCV E2 glycoprotein is able to bind CD81 only in human and chimpanzee, but not other species [2]. In order to confirm that the observed effects were truly dependent on the binding of E2 to human CD81, identical experiments were performed in NIH3T3 mouse fibroblasts stably transfected with human CD81. Accordingly, since E2 is not capable of binding murine CD81, incubation of non transfected NIH3T3 mouse fibroblasts with E2 did not cause any increase in MMP-2 gelatinolytic activity (**Figure 7**, panel B). On the contrary, this effect was clearly detected in human CD81-NIH3T3 stable transfectants (hCD81-NIH3T3: clone 42) with identical time-course features previously observed in human HSC (**Figure 7**, panel A). No effect was observed in NIH3T3 cells transfected with the vector alone.

### ***HCV E2/CD81 binding stimulates PI 4-kinase activity***

It has been reported that a cell surface molecular link occurs between CD81 and the type II lipid kinase PI 4-K followed by ERK/MAPK activation [21-23]. We therefore evaluated the activity of type II PI 4-K following E2/CD81 binding. As shown in **Figure 8**, incubation of HSC with E2 induced activation of type II PI 4-K. In order to rule out the possibility that E2 could immunoprecipitate with other lipid kinases, and particularly PI 3-K, we performed experiments in the presence of adenosine, which has been shown to be an *in vitro* specific inhibitor of type II

PI 4-K [21-23]. Following this approach a significant reduction of PI 4-K activity was observed. Immunoprecipitation with an anti-CD81 mAb was also performed as a positive internal control.

### ***HCV E2/CD81 binding induces ERK/MAPK activity***

We have previously shown that clustering of CD81 by mAbs stimulates ERK/MAPKinase activity and tyrosine phosphorylation of the adapter protein Shc in liver cancer cells [23]. Accordingly, we investigated the role of ERK/MAPK activation following the binding of HCV E2 to CD81 in human HSC. Immunoblotting with an antibody directed against the dually phosphorylated activated form of ERK1/2 revealed that phosphorylation of these proteins was increased in HSC stimulated by E2 glycoprotein (**Figure 9**, panel A *top*). In particular, the time course of ERK/MAPK activation showed that the maximal ERK1/2 phosphorylation occurred within 15-60 min after stimulation with E2. Moreover, E2-induced ERK/MAPK phosphorylation levels were similar to those induced by PDGF, a potent ERK/MAPK activator in HSC. Blotting with a phosphorylation state-independent antibody showed similar levels of ERK1/2 (**Figure 9**, panel A *bottom*), confirming that the increase in ERK1/2 phosphorylation was due to an increase of the activity rather than an increased in the abundance of ERK1/2. In order to validate the role of ERK/MAPK in mediating the expression of MMP-2 induced by E2, we performed experiments employing PD-98059 (25  $\mu$ M; Calbiochem, La Jolla, CA), an inhibitor that blocks the ERK/MAPK signalling pathway. As shown in **Figure 9**, panel B, treatment of HSC with PD-98059 was able to completely abolish the phosphorylation of ERK induced by E2/CD81 binding. More importantly, the ability of E2 to stimulate gelatinase activity and increase MMP-2 protein levels was also reversed by PD-98059 (**Figure 9**, panel C), thus confirming the essential role of ERK/MAPK activity in mediating MMP-2 up-regulation upon E2 stimulation.

***HCV E2-mediated ERK/MAPK activity is required for the synthesis of MMP-2 by increasing AP-2 protein expression and DNA binding activity***

In order to gain further insight into the regulation of MMP-2 gene by the HCV E2/CD81 interaction, we evaluated the expression and DNA binding activity of AP-2, a transcription factor regulated by ERK/MAPK and involved in MMP-2 gene transcription [24-28]. Indeed, once phosphorylated, ERK1/2 can enter the nucleus and up-regulate the expression of AP-2 transcription factor. AP-2 then binds to the consensus binding sequence of the MMP-2 promoter and activates expression of MMP-2. As shown in **Figure 10** panel A, AP-2 protein levels were markedly increased by E2 stimulation. The stimulatory effect of E2 on AP-2 protein levels peaked at 2 h, but levels returned to baseline by 6 h. The capacity of E2 to increase AP-2 protein levels at 2 h was reversed by PD-98059 (25  $\mu$ M) (**Figure 10**, panel B), thus confirming its dependence on ERK/MAPK activation. PD-98059 alone had no effect on AP-2 protein levels (data not shown). In addition, nuclear extracts from HSC exposed to E2 for 4 h showed markedly increased binding to an AP-2 consensus binding sequence oligonucleotide (**Figure 10**, panel C). This effect was no longer present after 6 h of exposure. No band was observed in the absence of nuclear extracts, and binding was competitively inhibited by a 200-fold excess of unlabeled AP-2 consensus binding sequence oligonucleotide. Finally, the ability of E2 to increase the binding of HSC nuclear extracts to the AP-2 consensus binding sequence oligonucleotide was completely reversed by PD-98059 (25  $\mu$ M).

***Silencing of CD81 gene abolishes the effect of HCV E2 on CD81 signaling pathway leading to MMP-2 expression***

To further validate the role of CD81 in initiating the intracellular signalling leading to the upregulation of MMP-2 upon E2 stimulation, a functional knock down of CD81 was performed by the RNA interference strategy. HSC were transiently transfected by nucleofection technology with a synthetic small interfering RNA (siRNA) duplex, targeting a specific sequence of CD81

mRNA. The expression levels of CD81 protein at cell surface were then analyzed by flow cytometry. **Figure 11** panel A, shows the quantitative analysis of the mean fluorescence intensity of CD81. The CD81 protein expression was reduced to ~ 90 % of control at 72 h after exposure to siRNA in HSC. Afterwards, the protein expression levels of AP-2 transcription factor, the MMP-2 protein expression and the enzymatic activity were evaluated in CD81-silenced HSC stimulated with 5 µg/ml of E2 glycoprotein. As shown in **Figure 11** panel B, the synthesis of AP-2 transcription factor (*top*) upon E2 stimulation was abolished in CD81-silenced HSC when compared to control, as well as the MMP-2 expression (*middle*) and the gelatinolytic activity (*bottom*), thus indicating that CD81 is essential in mediating these effects in E2-stimulated HSC.

## DISCUSSION

The HCV envelope E2 glycoprotein has been proposed as a key molecule regulating the interaction of HCV with surface proteins of target cells. E2 binds the major extracellular loop of human CD81, a tetraspanin expressed on various cell types, including hepatocytes and B lymphocytes. Regardless, the interaction between E2 and CD81 is not sufficient for cell infection and, overall, the biological functions originating from this interaction are still unknown [4]. In addition, while attention has been focused on the interaction between HCV and hepatocytes, no information is available concerning the possible direct effects of HCV on non-parenchymal liver cells. This latter possibility is suggested by the very prominent expression of CD81 in sinusoidal areas in normal and pathologic human liver and in areas of mesenchymal expansion and fibrogenesis typical of chronic liver diseases. In particular, expression of this tetraspanin is evident in cultured human HSC expressing the phenotype of “interface” myofibroblasts [9]. This cell type is known to play a key role in the fibrogenic progression of chronic liver diseases through the deposition of fibrillar extracellular matrix, synthesis and secretion of chemokines and other soluble factors involved in the amplification of the inflammatory response, and the establishment of a pathologic tissue tension due to its contractile features [29]. Accordingly, the aim of the present investigation was to start characterizing the possible effects induced by E2 binding to CD81 expressed on the surface of human HSC. We report that the interaction between the HCV E2 glycoprotein and CD81 expressed on the surface of human HSC leads to potentially relevant biologic effects to be framed in the pro-inflammatory and pro-fibrogenic role of this cell type during chronic liver disease and, particularly, in the presence of chronic HCV infection.

ECM degradation by MMP-2 is pivotal to inflammation and tumor invasion. Unlike other MMPs, the activation of MMP-2 occurs at the cell surface, which confers to this metalloproteinase a key role in cell migration [30]. MMP-2 activity at the cell surface has been

linked to its co-localization with caveolae, microdomains of the cell membrane referred to as organizing centers for surface receptors, integrins, signaling molecules and proteases [31]. Activation of the precursor form pro-MMP-2 is thought to be consequent to the formation of a macromolecular complex including MMP-2, membrane type 1 MMP (MT1-MMP) and TIMP2 [32,33]. However, evidence derived from studies performed in different cell types, including HSC [34], suggests that this process is more complex than previously proposed. Although activated HSC express MMP-2, MT1-MMP and TIMP2 [35-37], only the precursor form of MMP-2 (pro-MMP-2) is detectable in conventional HSC cultures. The presence of the active form of MMP-2 becomes evident when HSC are co-cultured with hepatocytes [34] or are plated on collagen type I [38]. It is therefore conceivable that the cleavage of pro-MMP-2 into the active form requires a complex microenvironmental setting allowing conformational changes of the molecules involved [39]. Expression of MMP-2 by activated HSC is usually seen as an event leading to the development of irreversible fibrosis. However, given the key role of this cell type in the recruitment of infiltrating inflammatory cells through the synthesis and release of chemokines and other soluble factors [40], the concomitant overexpression of MMP-2 should be viewed also as a mechanism favoring the penetration of the inflammatory infiltrate at sites of tissue damage. In this context, the observed increase in the expression and activity of MMP-2 following the interaction between the HCV E2 glycoprotein and CD81 in human HSC appears of particular relevance.

The specificity of E2 binding to CD81 responsible for the above biologic effects was substantiated by competitive binding experiments. Binding of E2 to CD81 caused a time-dependent increase in the release of the pro-enzymatic form of MMP-2, with a peak at 24 hrs of incubation, as demonstrated by zymography and Western blot techniques. Most reports on the expression of MMP-2 in conditioned medium of HSC deal with incubation times of 24-48 hours. In these conditions no changes in the expression of MMP-2 are observed following any stimuli using conventional zymography. It is noteworthy that a time-dependent increase was more

evident for incubation times of 2 and 6 hours. The early induction of MMP-2 release was confirmed by Western blot analysis both in conditioned media and cell monolayers. This effect was due to de novo protein synthesis rather than release of pro-MMP-2 from intracellular compartments since it was abolished by pre-incubating the cells with cycloheximide. In addition, the time-dependency of this effect suggests that up regulation of MMP-2 occurs through gene transcription regulated by intracellular signals elicited by the interaction of E2 with CD81.

The results of experiments performed by pre-incubating HSC with an anti-CD81 mAb (JS81) that neutralize the binding of E2 to CD81 further confirmed that the increased expression of MMP-2 was due to the interaction between E2 and CD81. In order to confirm that this effect was truly dependent on the binding of E2 with human CD81, identical experiments were performed in NIH3T3 mouse fibroblasts transfected with human CD81. As expected, since E2 is not able to bind murine CD81, incubation of non transfected NIH3T3 cells with E2 did not cause any increase in MMP-2 expression, whereas this effect was clearly detected in NIH3T3 cells transfected with human CD81 with identical time-course features. This possibility was further validated by experiments in human HSC in which a functional knock down of CD81 was performed by the RNA interference strategy.

An important aspect of tetraspanins is their ability to aggregate with one another and with various other transmembrane receptors into multimeric clusters. It has recently been shown that CD81 can homodimerize [41] and could serve as “molecular platform” on the cell surface that could play an important role in regulating signal cascade. Since we found that E2 was able to activate type II PI-4K through the binding with CD81 in HSC, we hypothesize that the clustering of CD81 induced by E2 at the plasma membrane increases PtdIns 4-kinase activity and phosphoinositides synthesis (i.e. phosphatidylinositol 4-phosphate, PtdIns 4-P). Phosphoinositides play an essential role in regulating assembly of signaling proteins at the plasma membrane [42]. In particular, membrane recruitment of adapter proteins is an important mechanism that regulates activation of ERK/MAPK pathway. Accordingly, we have previously

shown that CD81 stimulates synthesis of phosphoinositides with the recruitment of Shc to the plasma membrane via PTB domain, and this sequence of events leads to the activation of ERK/MAPKinase [23]. Along this line, we investigated the effect of E2/CD81 binding on ERK/MAPK signalling pathway in human HSC. We found that the binding of E2 to CD81 activates ERK/MAPK phosphorylation similarly to PDGF in cultured human HSC. In addition, the ability of E2 to up-regulate MMP-2 enzyme activity and protein levels was blocked by PD-98059, a selective inhibitor of the ERK signaling pathway, thus demonstrating that ERK/MAPK activity is essential for MMP-2 expression upon E2 stimulation.

The human MMP-2 gene contains a potent enhancer element in its 5'-flanking region, which includes an AP-2 binding sequence that regulate MMP-2 gene expression [24-26]. Recent evidence suggests that the transcription factor AP-2 is implicated as an important regulator of MMP-2 gene transcription in other organ-specific pericytes, such as glomerular mesangial cells [43]. Importantly, activation of the ERK/MAPK cascade has previously been shown to up-regulate AP-2/DNA binding activity [27, 28]. Our results show that HCV E2 binding to CD81 results in increased AP-2 protein levels in human HSC. This increase is mediated through the ERK/MAPK pathway since the ability of E2 to raise AP-2 levels is reversed by PD-98059, an inhibitor of the ERK/MAPK cascade. In addition, increased AP-2 levels induced by E2-CD81 binding are associated with an increase in the binding of HSC nuclear extracts to an AP-2 consensus binding sequence oligonucleotide. The enhanced AP-2/DNA binding activity as well as the increase in MMP-2 protein levels and gelatinase activity are reversed by PD-98059. These data suggest that HCV E2 glycoprotein enhances MMP-2 activity by up-regulating AP-2 transactivation of the MMP-2 promoter and that E2-induced AP-2 transcriptional activation is mediated by ERK/MAPK signaling pathway, as in the proposed model illustrated in **Figure 12**.

The results of the present study highlight the possible interaction between HCV and liver nonparenchymal cells leading to pro-inflammatory and pro-fibrogenic events. Along these lines, Bataller and coworkers recently reported that HCV core and the nonstructural proteins NS3 and

NS5, transduced into HSC by adenoviral vectors, induce pro-fibrogenic effects in this cell type including an increase of TGF- $\beta$ 1 bioactivity and of pro-collagen 1(I) expression [44]. Independently from the possibility that HCV could enter into HSC and exert the above effects, our data suggest that it may cause an increased MMP-2 synthesis and activity, simply by interacting with the plasma membrane of HSC. Taken together, all these recent advances introduce a new aspect of HCV biology potentially relevant for the understanding of the pathogenesis of damage, inflammation and fibrosis occurring in the presence of chronic HCV infection.

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**FOOTNOTES:**

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**The abbreviations used are:** ECM: extracellular matrix; ERK/MAPK: extracellular regulated kinase/mitogen activated kinase; HCV: hepatitis C virus; HSC: hepatic stellate cells; MMPs: metalloproteinases; PI 4-K: phosphatidylinositol 4-kinase; AP-2: activator protein-2; SFIF: serum-free/insulin-free; CHX: cyclohexymide; PMSF: phenylmethylsulfonyl fluoride; TIMPs: tissue inhibitor of metalloproteinases; FACS: fluorescence-activated cell sorter; mAb: monoclonal antibody; IgG: immunoglobulin G; HLA-I: human leukocyte antigen class I; TBS: Tris-buffered saline; TLC: thin-layer chromatography; APAAP: alkaline anti-alkaline phosphatase; SDS: sodium dodecyl sulfate; PAGE: polyacryl-amid-gel-elctrophoresis; PBS: phosphate-buffered saline; FBS: fetal bovine serum; BSA: bovine serum albumine; si RNA: small interfering RNA.

## FIGURE LEGENDS

Fig.1. ***Distribution of CD81 in normal and pathologic human liver tissue.*** [A,B] Immunohistochemical staining with anti-CD81 mAb (APAAP): [A] Normal human liver, original magnification X200, inset X400; [B] Cirrhotic human liver (HCV-related), original magnification X200, inset X400. Solid arrow indicates capillarized sinusoidal structures, hatched arrow indicates an area of interhepatocellular fibrosis. [C,D] Standard histology counterstaining (hematoxylin and eosin) in serial sections: [C] normal liver; [D] cirrhotic liver. [E,F] Serial sections treated with non-immune mouse IgG as primary antibody (negative control): [E] normal liver; [F] cirrhotic liver.

Fig. 2. ***Binding of HCV E2 glycoprotein on the surface of human HSC.*** [A] Flow cytometry analysis: cells were incubated with E2 glycoprotein and then with anti-E2 mAb (left) or with anti-CD81 mAb (right) . Cells were then resuspended in goat anti-mouse-fluorescein isothiocyanate. Fluorescence was analyzed using a FACScan flow cytometer. [B] Immunofluorescence and confocal microscopy: cells plated on glass coverslips, were incubated with E2 and then stained with anti-E2 mAb (left) or stained with anti-CD81 mAb as a positive control (right). Staining was subsequently visualized with FITC-goat anti-mouse antibody. Immunofluorescence was examined by a series of optical sections, obtained with a confocal scanning laser microscope and images were captured, processed, and superimposed by using the LaserSharp software.

Fig. 3. ***HCV E2 specifically binds CD81 expressed on the surface of human HSC.*** [A,B] Binding of E2 to HSC was evaluated by a competition assay. HSC were incubated with increasing concentrations of anti-CD81 mAbs (JS81 and 1.3.3.22) or anti-HLA-I (clone W6/32,

Sigma) as negative control and seeded onto 96-well E2 protein coated plates for 1 h at 37°C. To remove unbound cells, wells were then filled with PBS, and gently washed three times. Adherent cells were fixed in 3% paraformaldehyde/PBS and then stained with 0.5% crystal violet in 20% methanol/80% H<sub>2</sub>O. Wells were then washed with water to remove excess dye, cells were then solubilized in 1% SDS and the amount of dye was quantified by using Bio-Rad Multiscan plate reader at 595 nm. [C,D] The specificity of E2 protein binding to CD81 on human HSC was further confirmed by the displacement of E2 from the binding to CD81 in the presence of the competitive anti- human CD81 mAb (JS81). For these experiments, HSC were preincubated with increasing concentrations of E2 protein (0.1, 1.0, 10.0, 50.0, 100 µg/ml) prior plating onto 96-well plates coated with immobilized anti-CD81 mAb (JS81) for 1 h at 37°C. Adherent cells were then washed, fixed, stained and solubilized in 1% SDS, and the amount of dye was quantified.

Fig. 4. ***HCV E2 induces MMP-2 expression in human HSC.*** Gelatinolytic zymography. [A] Cell conditioned media were obtained after stimulating human HSC with E2 glycoprotein for 2, 6 and 24 hours, and [B] after incubation with increasing concentrations of E2 glycoprotein. HSC conditioned medium obtained after incubating cells with SFIF-medium in the presence of 0.2% BSA (E2 vehicle) was used as negative control (C). Conditioned media from the cell cultures were analyzed for gelatin degradation activity by SDS-PAGE under non-reducing conditions. The gel contained 5.8 mg/ml gelatin and 8 % acrylamide. Developed gelatinolytic activities were visualized by staining with Coomassie Blue R-250. The amount of 66 kDa proenzymatic form of MMP-2 was determined using scanning densitometry with the NIH Image analysis software. Barograms are relative to the mean+SD of four experiments. \*=  $P < 0.05$  or higher degree of significance when compared to unstimulated conditions.

Fig. 5. ***HCV E2 induces de-novo synthesis of MMP-2 in human HSC.*** Cells were stimulated with E2 glycoprotein (10 µg/ml) for 2, 6 and 24 hours. HSC conditioned media (HSC-CM) [A]

or HSC lysate [B] were harvested. Samples for Western blot were prepared differently for conditioned media and for cells (see *Materials and Methods*). All samples were resolved by reducing SDS-PAGE and transferred to Immobilon-P. Filters were blocked, then incubated with polyclonal anti-MMP-2 at 1:5000 dilution. After incubation with a horseradish peroxidase-conjugated goat anti-rabbit antibody, the blot was visualized with enhanced chemiluminescence. [C] Cells were preincubated for 12 hours with 1  $\mu$ M of cycloheximide and then stimulated for 2 and 6 hours with E2 glycoprotein. Samples obtained after incubating cells with SFIF-medium were used as negative control. Blots anti- $\alpha$ -smooth muscle actine to evaluate equal protein loading are also shown.

**Fig. 6. *HCV E2/CD81 binding induces MMP-2 expression in human HSC.*** Gelatinolytic zymography. HSC conditioned media were obtained after stimulating HSC with E2 glycoprotein (10  $\mu$ g/ml) in the presence or absence of two distinct anti-CD81 mAb (JS81 and 1.3.3.22). HSC conditioned medium obtained after incubating the cells with SFIF-medium containing 0.2 % BSA (E2 vehicle) was used as negative control (C). Conditioned media from the cell cultures were analyzed for gelatin degradation activity by SDS-PAGE under non-reducing conditions. The gel contained 5.8 mg/ml gelatin and 8% acrylamide. Developed gelatinolytic activities were visualized by staining with Coomassie Blue R-250. The amount of 66 kDa proenzymatic form of MMP-2 was determined using scanning densitometry with the NIH Image analysis software. Barograms are relative to the mean+SD of three experiments. \*=  $P < 0.01$  when compared to cells stimulated with E2 alone.

**Fig. 7. *MMP-2 expression induced by HCV E2 is dependent on binding with human CD81.*** Gelatinolytic zymography. Conditioned media were obtained from non transfected NIH3T3 cells [B] and human CD81 stable transfected NIH3T3 mouse fibroblasts [A] following stimulation with E2 glycoprotein (10  $\mu$ g/ml) for 2, 6 and 24 hours. Cell transfections were performed with

LipofectAMINE™ 2000 reagent using pcDNA 3.1-CD81 vector. A stable and high level CD81 expressed clone (indicated as *clone 42* in panel A) was selected by flow cytometry analysis and used for the experiments. Cells transfected with pcDNA 3.1 vector alone (indicated as *neo* in panel A) were used as control. Conditioned media from cell cultures were analyzed for gelatin degradation activity by SDS-PAGE under non-reducing conditions. Gels contained 5.8 mg/ml gelatin and 8 % acrylamide. Developed gelatinolytic activities were visualized by staining with Coomassie Blue R-250.

Fig. 8. **PI 4-K activity in HCV E2/CD81 complex.** PI 4-K immunoassay. HSC lysates were immunoprecipitated with a non-immune mouse IgG (negative control), with E2 previously bound to an anti-E2 mAb (clone 291), or with an anti-CD81 mAb (clone 1.3.3.22). Phosphoinositidyl kinase activity in the immunoprecipitates was assayed as described under *Experimental Procedures*. [A] Representative experiment. Adenosine (200  $\mu$ M, Sigma) was employed in these experiments as an established *in vitro* inhibitor of type II PI 4-K reactions. [B] Barograms are relative to mean+SD of three experiments. Beta-emitting radioactivity corresponding to PIP was quantified using a Betascope 603 Blot Analyzer (Betagen, Waltham, MA). Lipid kinase activity is expressed as counts per minute (cpm) within a defined area representing the PI-4-[<sup>32</sup>P] spot. \*=  $P < 0.05$  or higher degree of significance when compared to conditions without co-incubation with adenosine.

Fig. 9. **HCV E2 induces ERK/MAPK activity in human HSC.** [A] Confluent HSC monolayers on 100-mm dishes were growth-arrested in serum-free medium for 24 h prior to time course-treatment with E2 and PDGF (positive control). HSC conditioned medium obtained after incubating the cells with SFIF-medium was used as negative control (C). Cells were washed twice with PBS, scraped, resuspended in lysis buffer and clarified by centrifugation. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and blocked for 2 h with

0.5% nonfat milk in PBS buffer. The membrane was then blotted with anti-phospho-ERK polyclonal antibody or anti-ERK (bottom) to evaluate equal protein loading. [B] E2-stimulated ERK/MAPK activity is reversed by PD-98059 (25  $\mu$ M), an inhibitor of the ERK/MAPK signalling cascade. [C] Treatment with PD-98059 was also able to prevent the increase of MMP-2 protein levels and gelatinase activity induced by E2 stimulation. All panels refer to one representative experiment out of three.

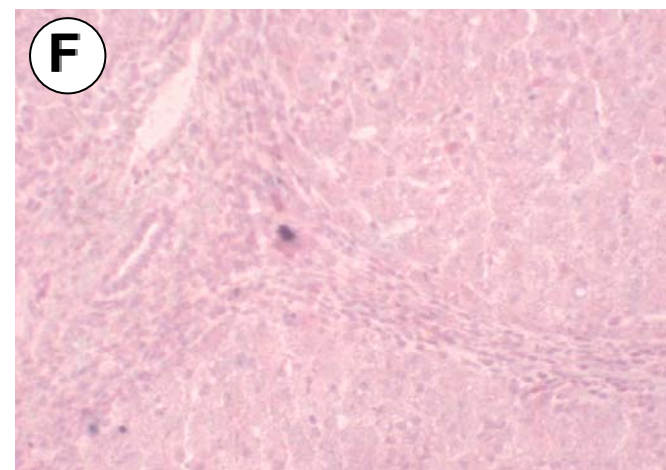
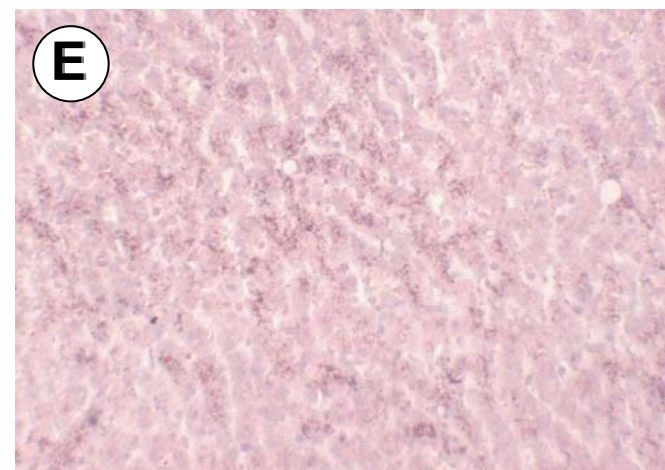
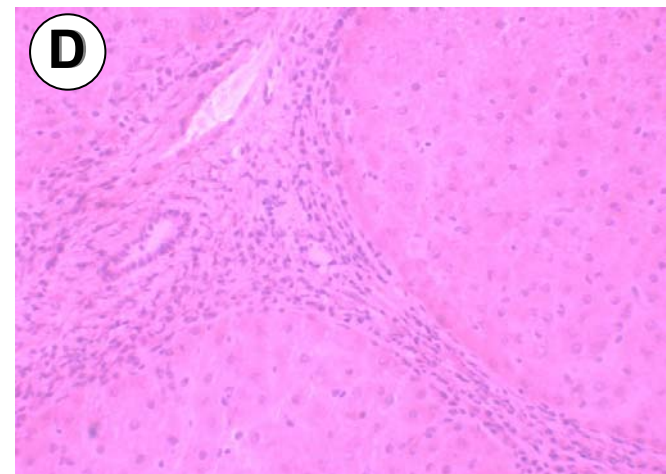
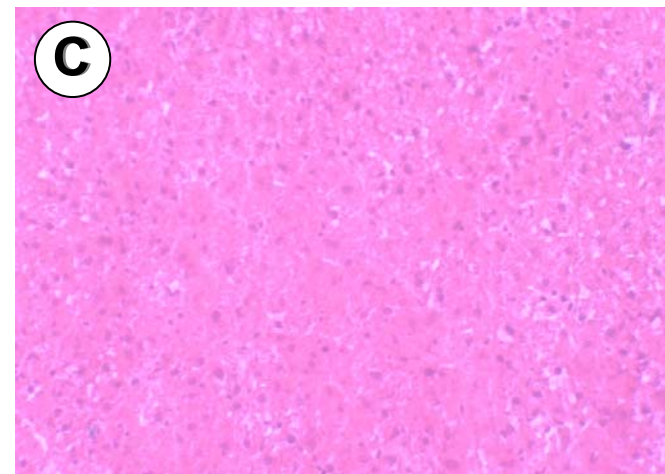
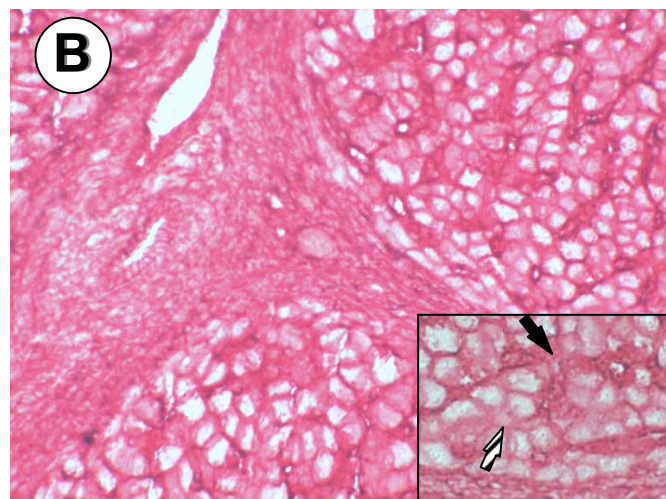
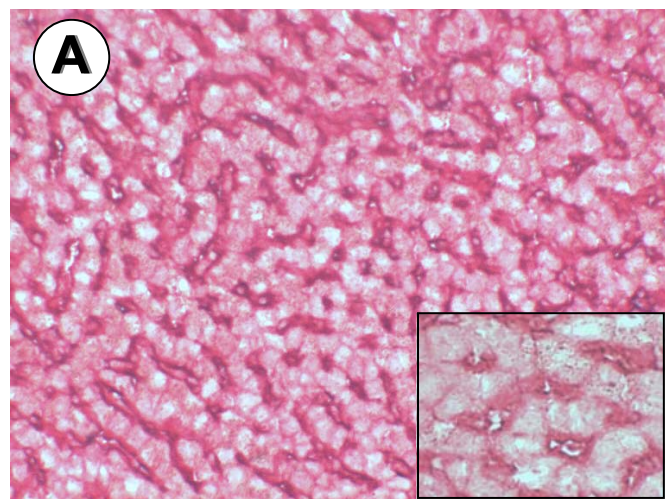
Fig. 10. ***HCV E2 induces the expression of MMP-2 by the synthesis and DNA binding of the transcription factor AP-2.*** [A] Time course of E2-stimulated AP-2 protein levels. [B] The stimulatory effect of E2 on AP-2 protein levels is reversed by PD-98059 (25  $\mu$ M), an inhibitor of the ERK/MAPK signaling cascade. A representative Western blot is shown. [C] Nuclear extracts isolated from human HSC show increased binding to an AP-2 consensus binding sequence oligonucleotide after exposure to E2 for 4 h. *Lane 1*, nuclear extracts from control cells grown in serum-free medium in the absence of E2. No nuclear extracts were added to *lane 2*. A 200-fold excess of cold AP-2 consensus binding sequence oligonucleotide was added to *lane 3*. *Lane 4*, nuclear extracts from HSC treated with E2 for 2 h; *lane 5*, nuclear extracts from HSC treated with E2 for 4 h; *lane 6*, nuclear extracts from HSC treated with E2 for 6 h; *lane 7*, nuclear extracts from HSC treated with E2 + PD-98059 for 2 h. *lane 8*, nuclear extracts from HSC treated with E2 + PD-98059 for 4 h. A representative gel-shift experiment is shown.

Fig. 11. ***Effect of CD81 RNAi on the MMP2 expression induced by HCV E2-CD81 interaction***

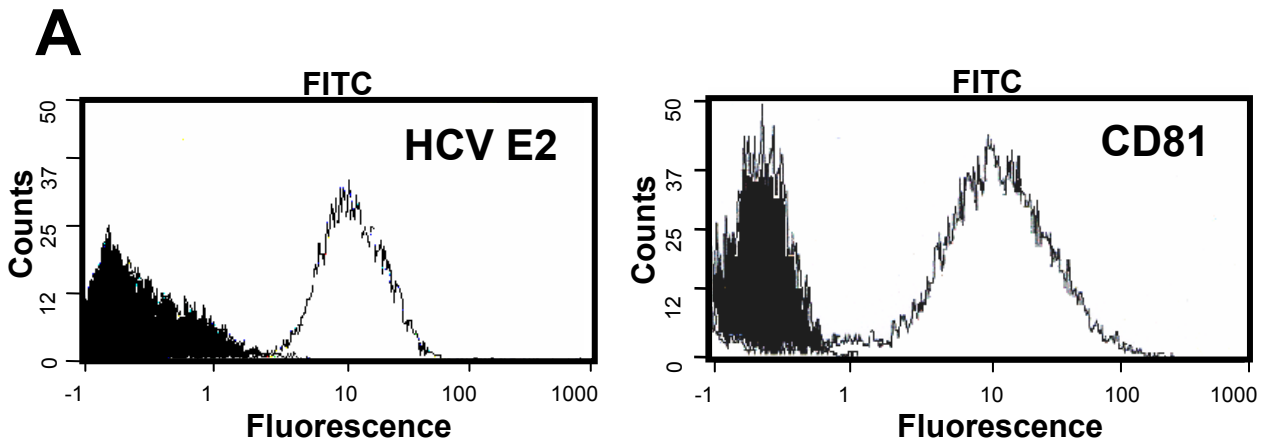
[A] Flow cytometry analysis of CD81 expression in HSC after exposure to siRNA. 72 h after transfection cells were harvested, washed, and incubated with an anti-CD81 mAb. Cells were then resuspended in goat anti-mouse-phycoerythrin conjugated and analyzed on a BDLSRII cytofluorimeter, using the DIVA software (BD Biosciences). Black lines represent the staining obtained with an anti-CD81 mAb, whereas dotted lines represent the staining obtained with an

isotype matched mAb, in both silenced and non-silenced HSC. A representative experiment out of three is shown. [B] CD81-silenced HSC and the cells transfected with the negative control were starved in SFIF-medium and then stimulated with E2 glycoprotein (5  $\mu\text{g/ml}$ ) for 2 h to evaluate the AP-2 protein expression levels (*top*), and for 6 h to evaluate MMP-2 protein expression and the gelatinolytic activity (*middle* and *bottom*, respectively). Samples for Western blot were resolved by reducing SDS-PAGE and blotted using an anti-AP-2 mAb and anti-MMP-2 polyclonal antibodies to detect AP-2 and MMP-2 proteins, respectively. Conditioned media from the cell cultures were analyzed for gelatin degradation activity in a gel zymography assay as described under *Experimental Procedures*. Equal amount of loaded protein/sample was also evaluated by immunoblotting against  $\alpha$ -smooth muscle actin (data not shown).

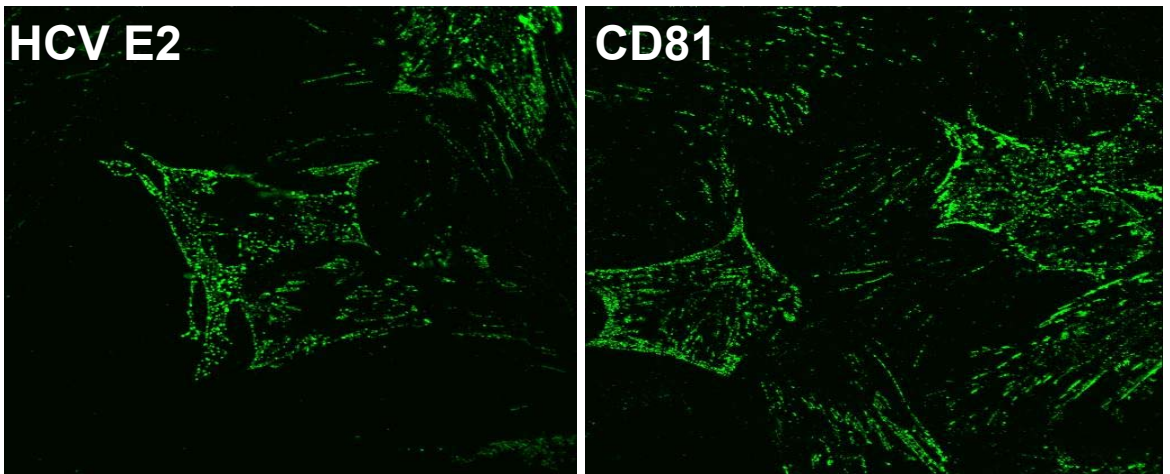
Fig. 12. ***Proposed model of HCV E2 mediated MMP-2 upregulation in HSC.*** The HCV envelope E2 glycoprotein binds the major extracellular loop CD81, a tetraspanin highly expressed on the human HSC membrane. By stimulating CD81 signalling pathway, E2 induces ERK/MAPK phosphorylation which translocate into the nucleus and upregulate the expression of AP-2 transcription factor. AP-2 then binds to the consensus binding sequence of the MMP-2 promoter and activates expression of MMP-2.



**Fig. 1** Mazzocca et al.

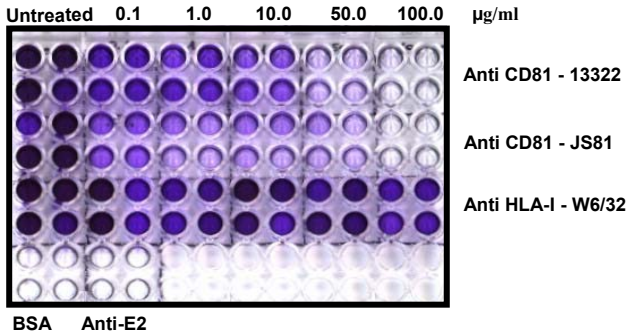


**B**

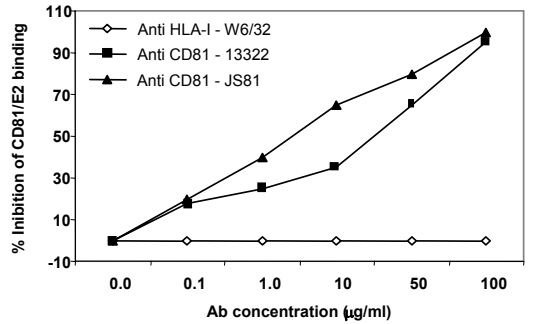


**Fig. 2** Mazzocca et al.

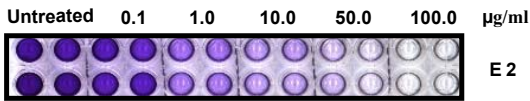
### A. E2 (20 µg/ml) coating



### B. E2 (20 µg/ml) coating



### C. CD81 mAb JS81 (20 µg/ml) coating



### D. CD81 mAb JS81 (20 µg/ml) coating

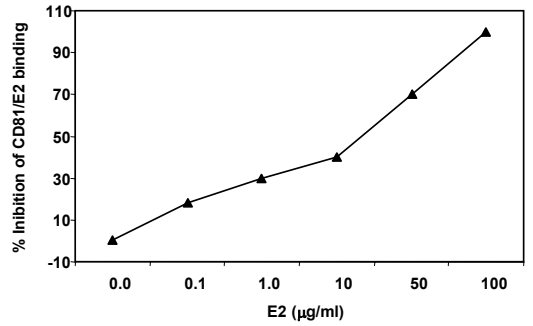
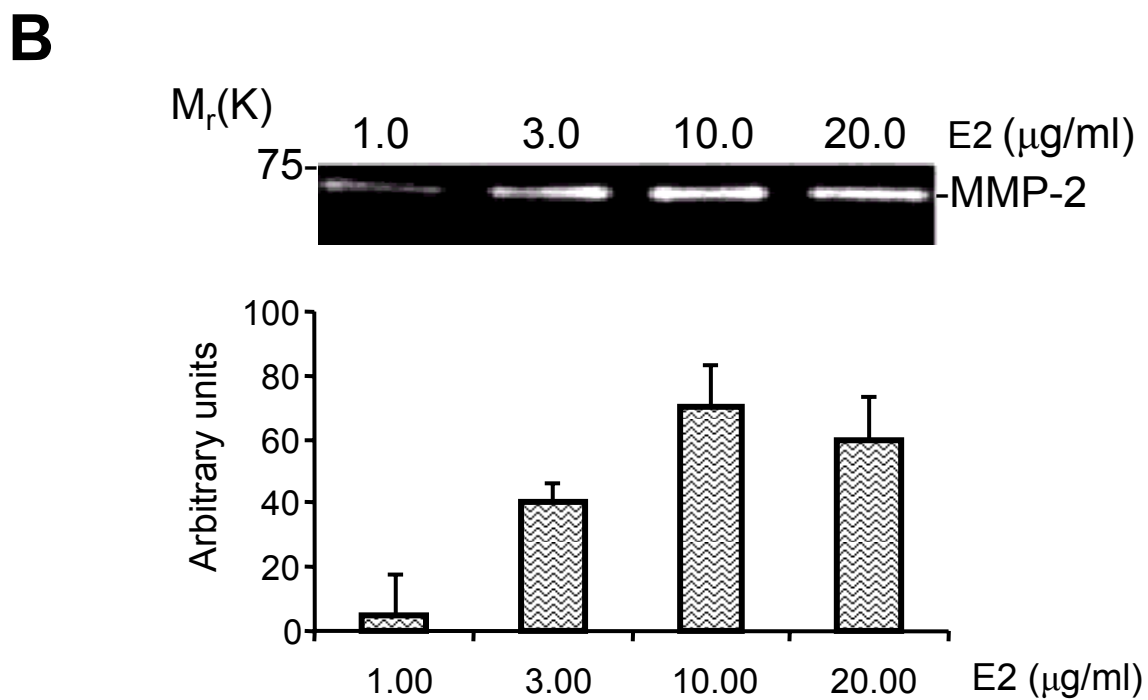
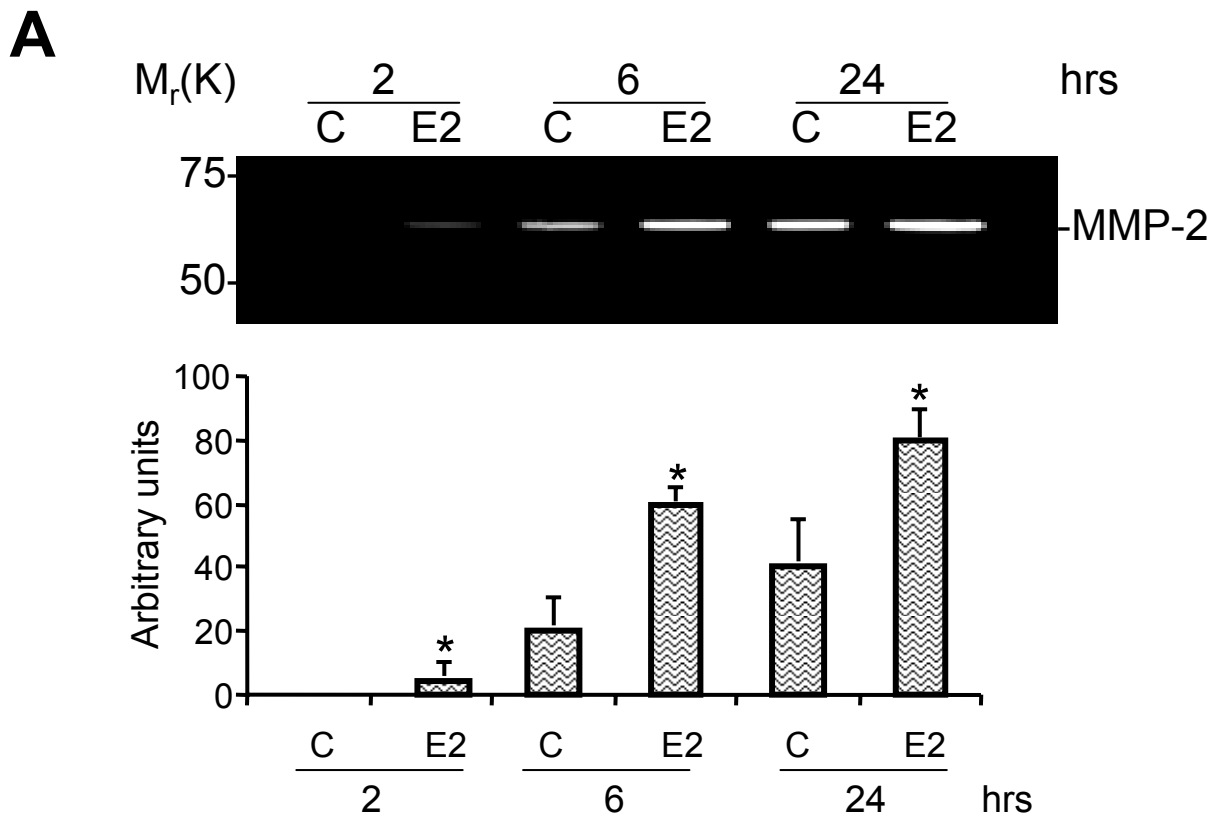
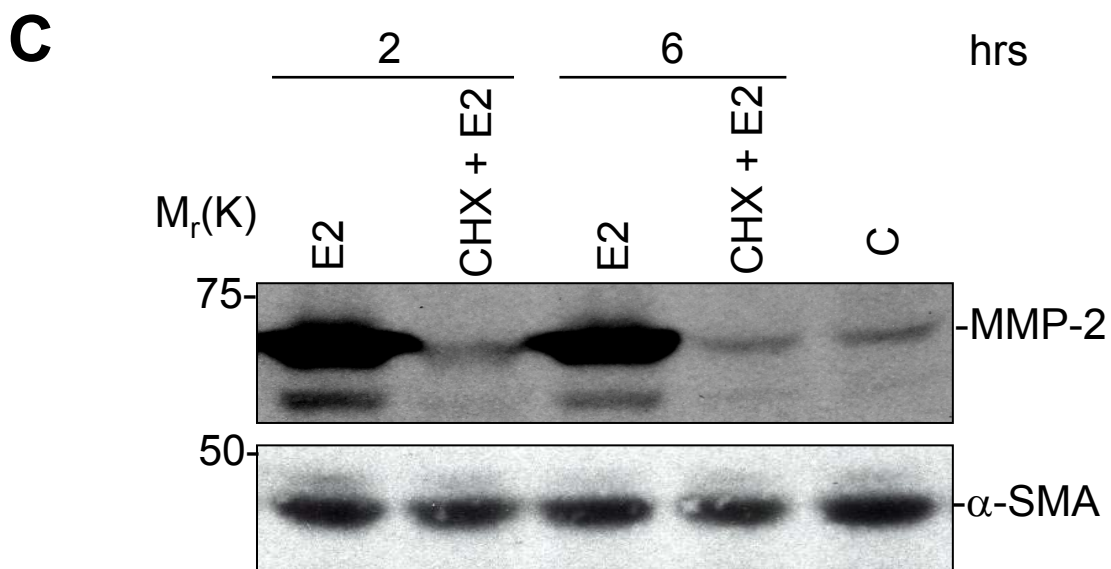
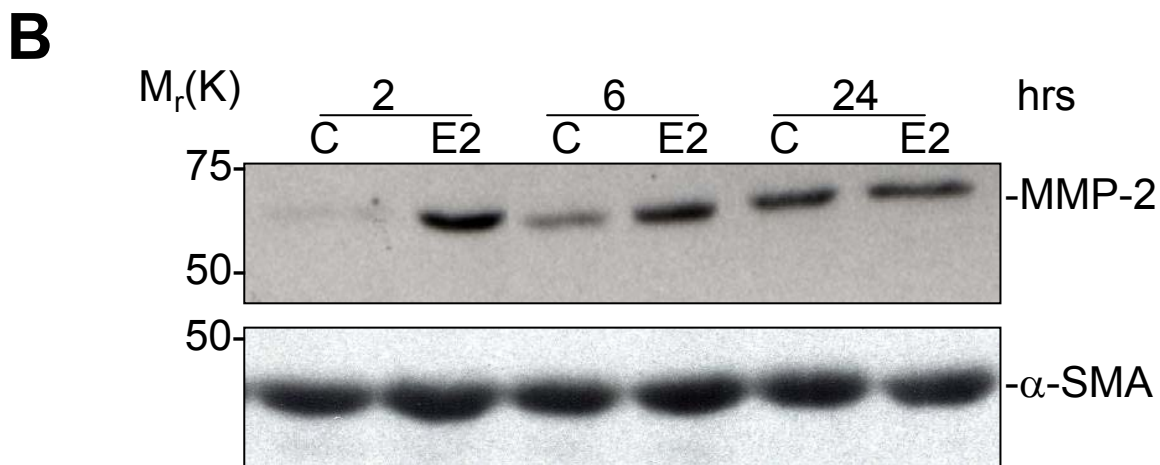
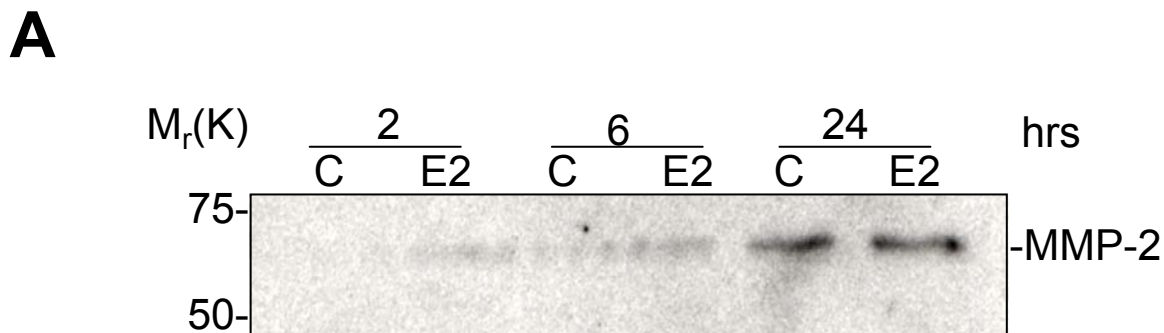


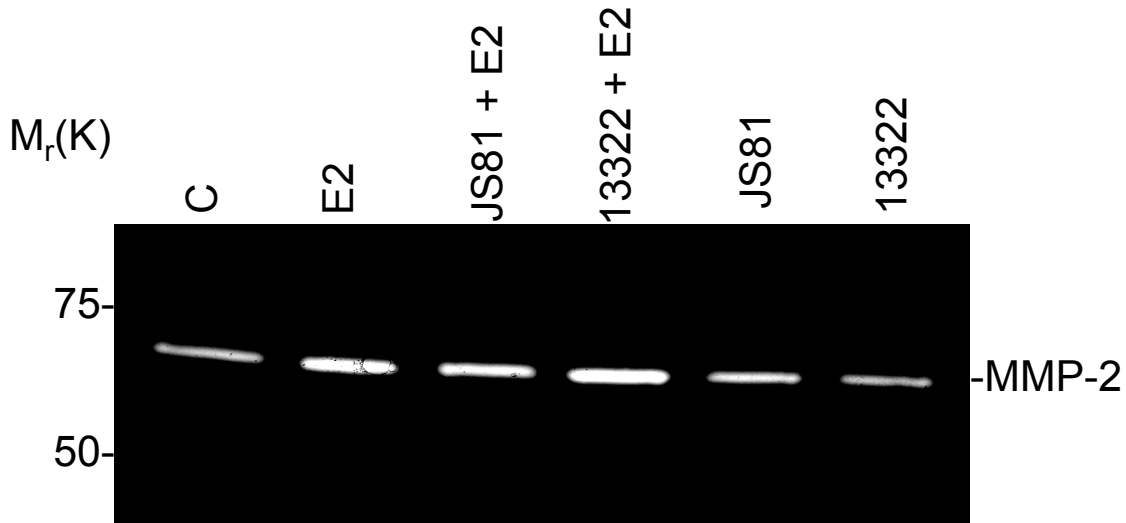
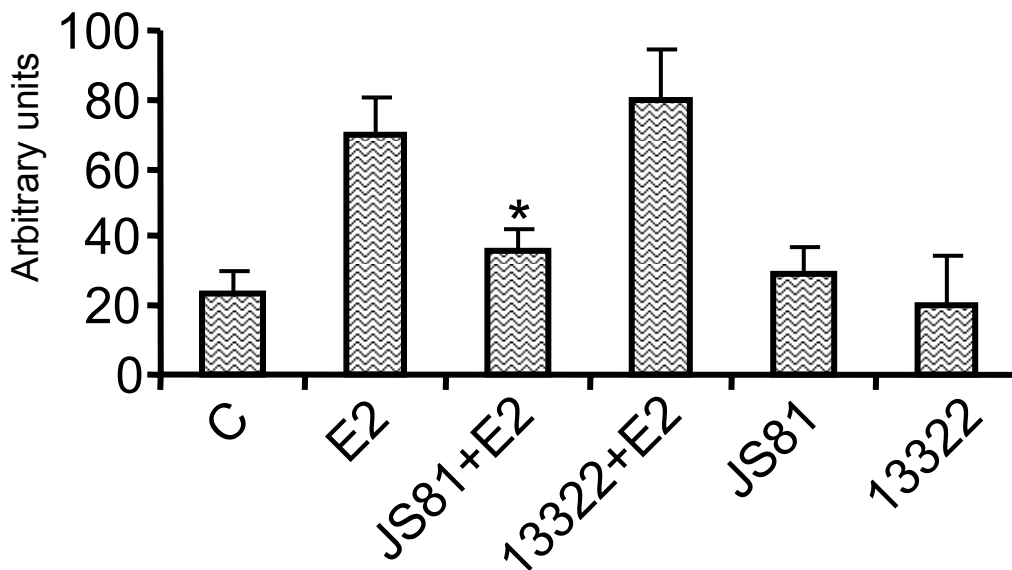
Fig. 3 Mazzocca et al.



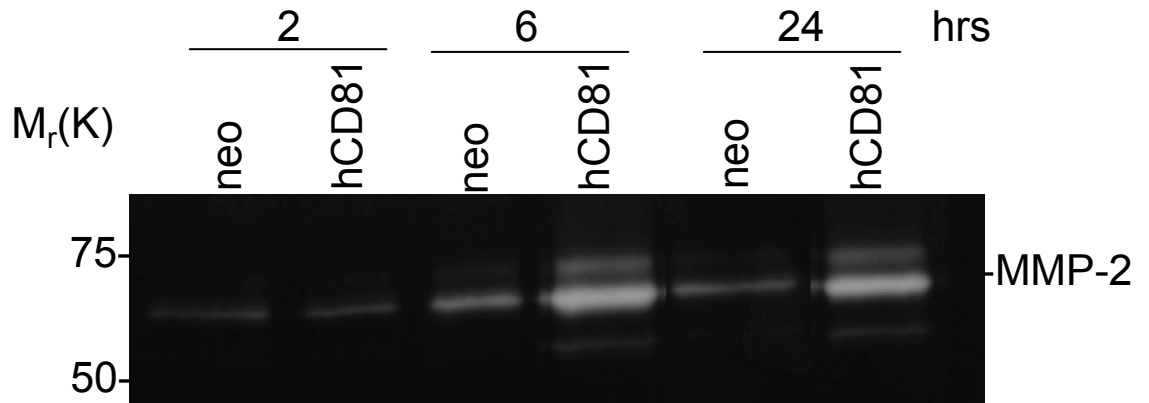
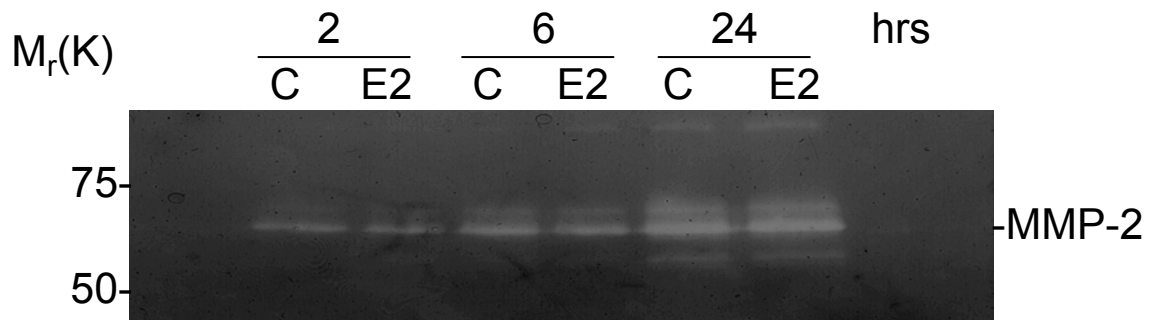
**Fig. 4** Mazzocca et al.



**Fig. 5** Mazzocca et al.

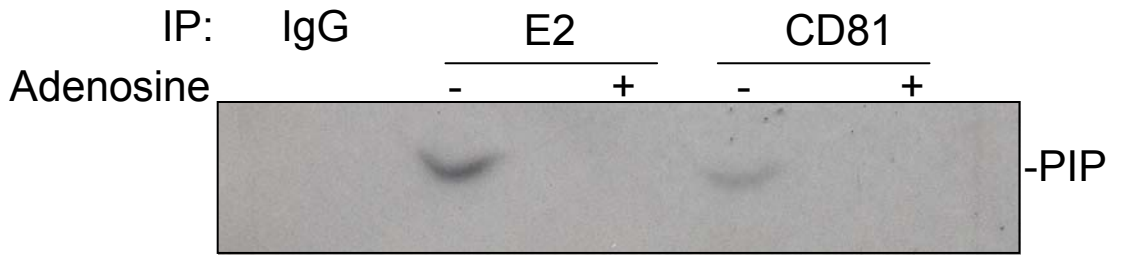
**A****B**

**Fig. 6** Mazzocca et al.

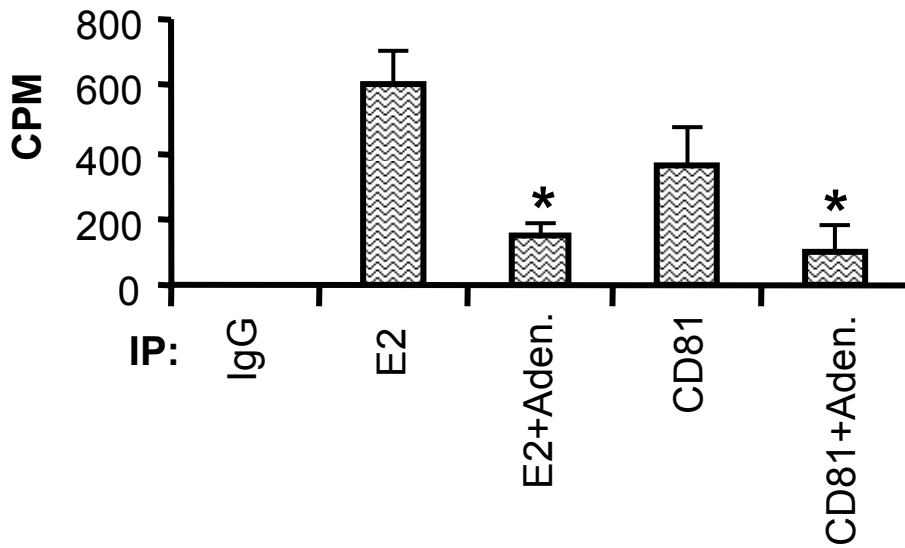
**A****B**

**Fig. 7** Mazzocca et al.

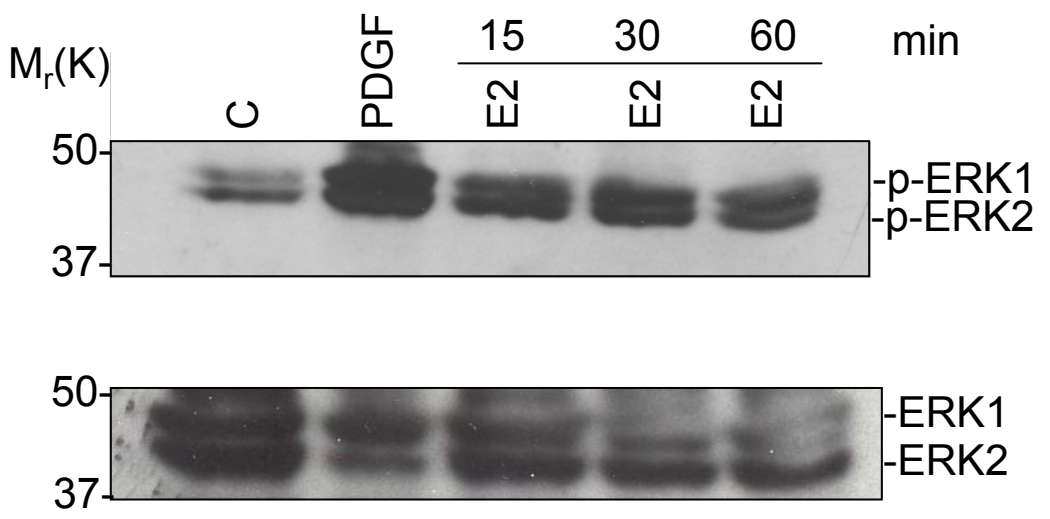
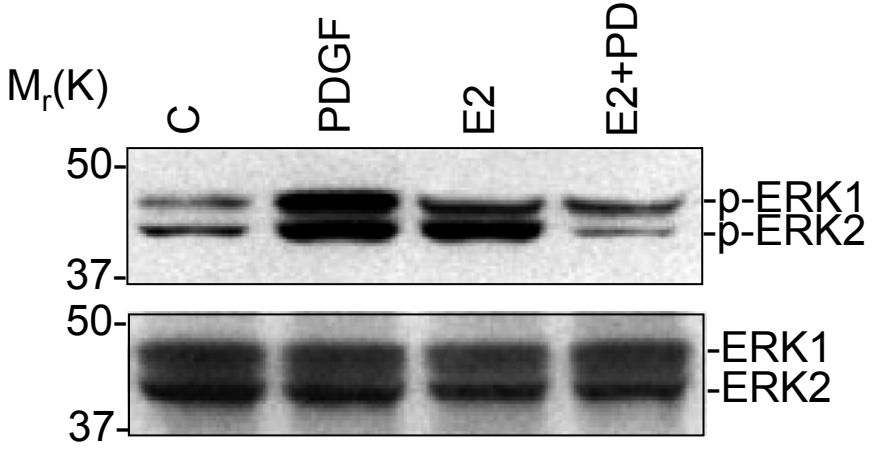
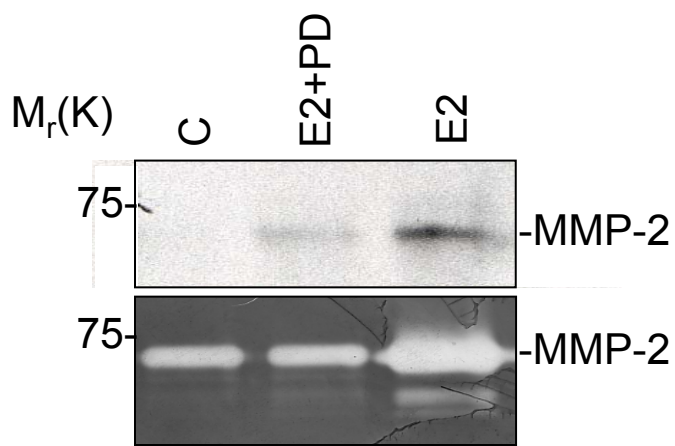
**A**

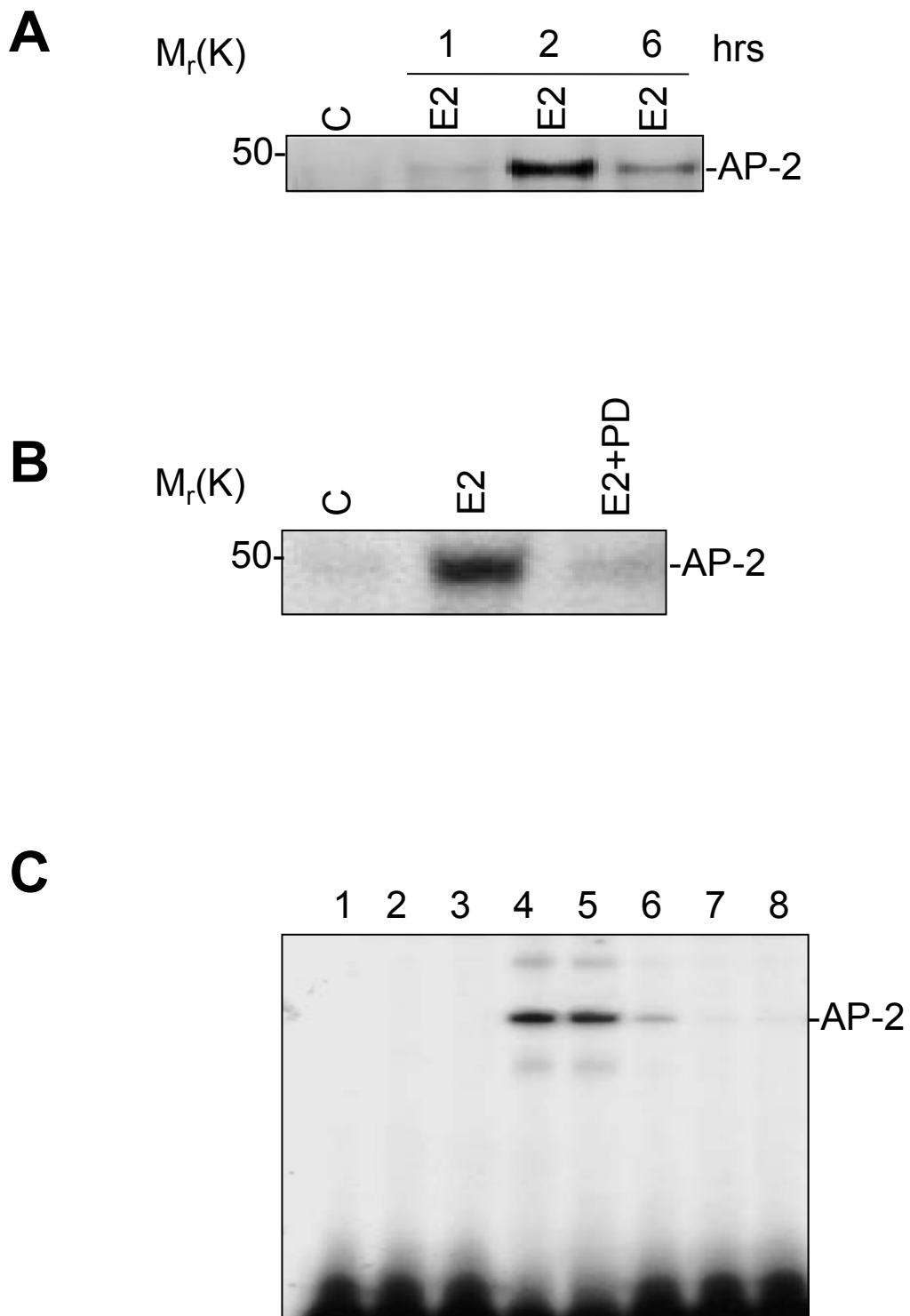


**B**

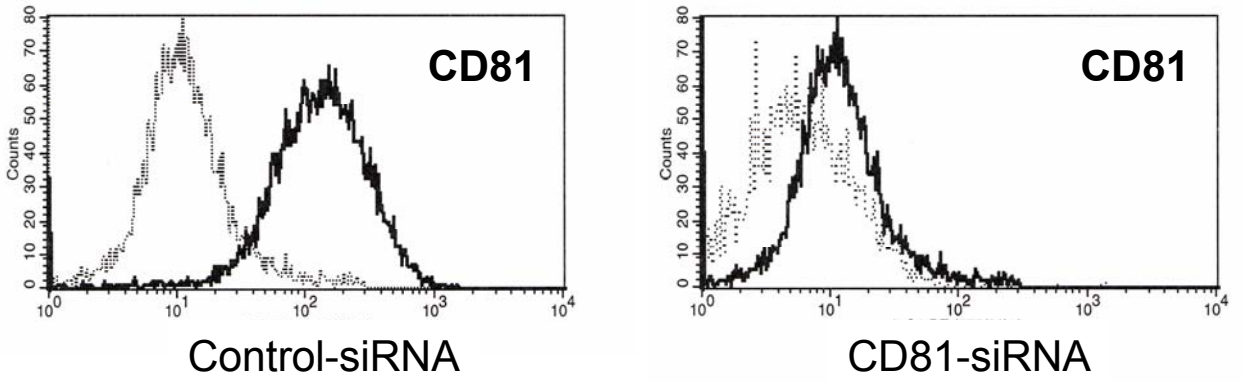
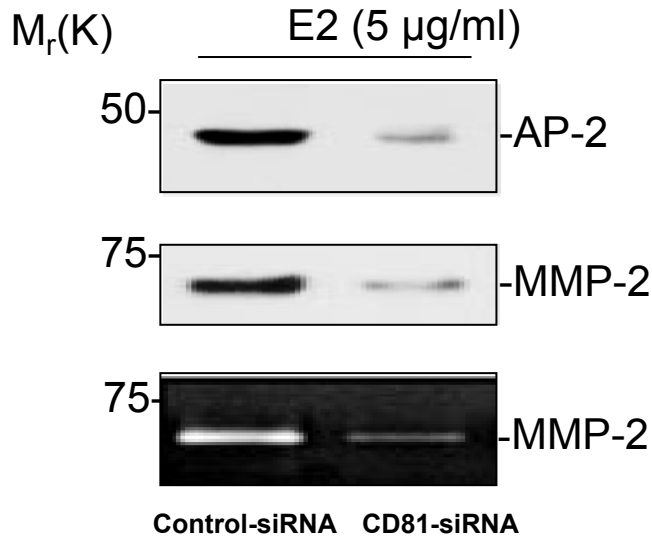


**Fig. 8** Mazzocca et al.

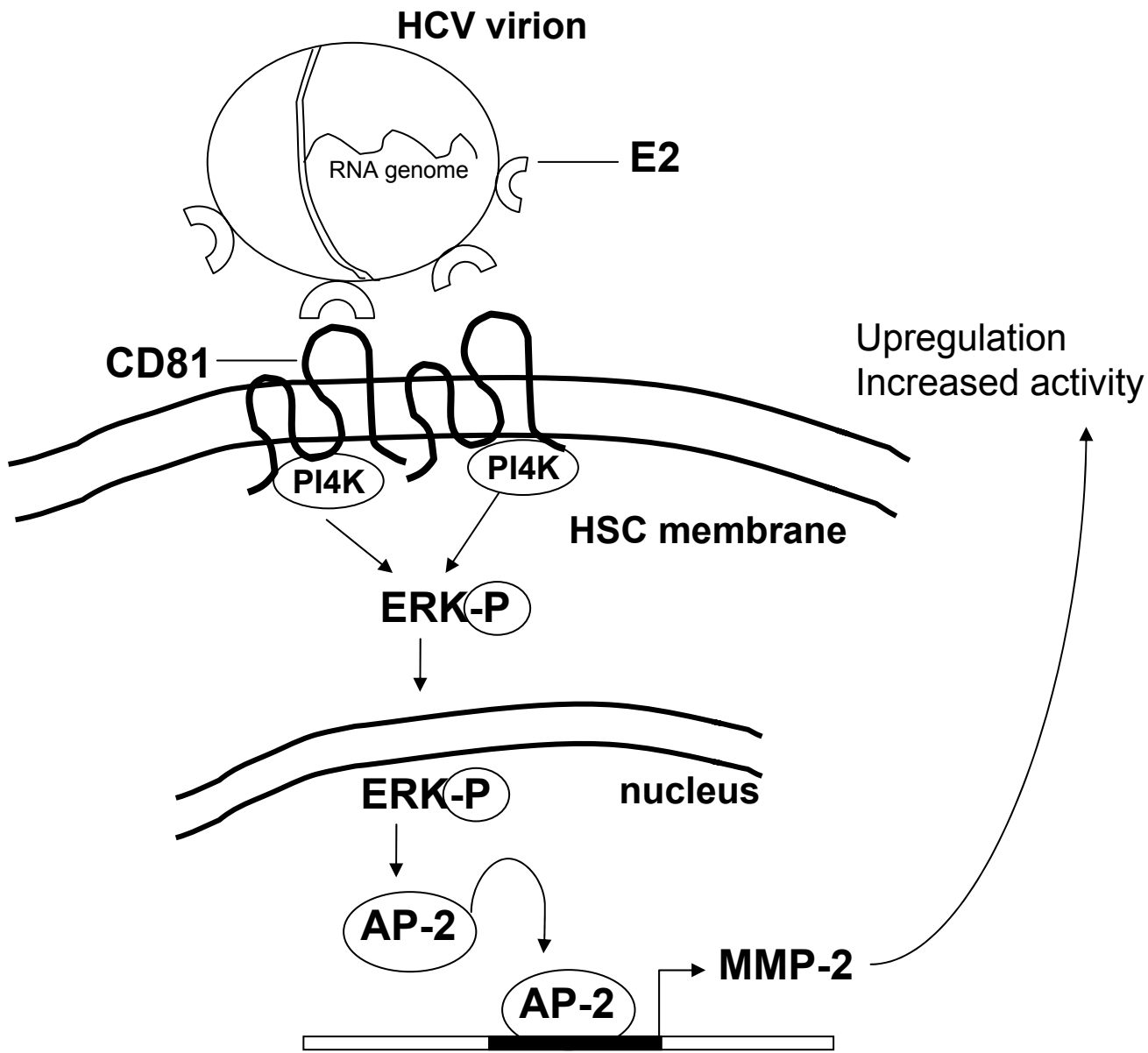
**A****B****C****Fig. 9** Mazzocca et al.



**Fig. 10** Mazzocca et al.

**A****B**

**Fig. 11** Mazzocca et al.



**Fig. 12** Mazzocca et al.