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Upregulation of Epac-1 in hepatic stellate cells by Prostaglandin E_2 in liver fibrosis is associated with reduced fibrogenesis.

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Running title page

a. Running title: PGE2 attenuates EPAC expression in fibrosis

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d. Abbreviations: α-SMA: α-Smooth muscle actin, 6-Bnz-cAMP : N^6-Benzoyladenosine-3',5'-cyclic monophosphate sodium salt, CCL_4: carbon tetrachloride, CE3F4 : 5,7-Dibromo-6-fluoro-3,4-dihydro-2-methyl-1(2*H*)-quinolinecarboxaldehyde, COX: cyclo-oxygenase, CYP2E1: cytochrome P450 2E1 subfamily, EIA: enzyme immunoassay, ELISA: enzyme-linked immunosorbent assay, Epac-1: Exchange protein activated by cAMP, HSA: Human Serum Albumin, HSC: Hepatic Stellate Cell, LH: lactosylated Human Serum Albumin, LPS: lipopolysaccharide, MH: mannosylated Human Serum Albumin, NFA: Niflumic acid, NO: Nitric Oxide, 8-pCPT-2'-O-Me-cAMP: 8-(4-Chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclic monophosphate acetoxymethyl ester, PDGF-BB: Platelet Derived Growth Factor BB, PGE_2: prostaglandin E_2, PH: pPB-coupled to Human Serum Albumin, PKA: Protein Kinase A, PLH: PGE_2 coupled to lactosylated Human Serum Albumin, PMH: PGE_2 coupled to mannosylated Human Serum Albumin, PMLC: phosphorylated myosin light chain, pPB: peptide derived from Platelet Derived Growth Factor B chain, PPH: PGE_2 coupled to pPB-Human Serum Albumin, pSMAD: phosphorylated SMAD, Rap1: Ras-related protein 1, Rt-PCR: reverse transcription

polymerase chain reaction, TGFβ: Transforming growth factor-β, VASP: Vasodilator-stimulated phosphoprotein, ALT: Alanine transferase, AST: Aspartate transaminase

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ABSTRACT

Exchange protein activated by cAMP (Epac-1) is an important signaling mechanism for cAMP-mediated effects yet factors that change Epac-1 levels are unknown. Such factors are relevant because it has been postulated that Epac-1 directly affects fibrogenesis. Prostaglandin E_2 (PGE_2) is a well-known cAMP-activator and we therefore studied the effects of this cyclo-oxygenase product on Epac-1 expression and on fibrogenesis within the liver. Liver fibrosis was induced by 8 weeks CCL_4 administration to mice. In the last 2 weeks, mice received vehicle, PGE_2, the COX-2 inhibitor Niflumic acid (NFA), or PGE_2 coupled to cell-specific carriers to hepatocytes, Kupffer cells or hepatic stellate cells (HSC). Results showed anti-fibrotic effects of PGE_2 and pro-fibrotic effects of NFA in CCL_4-mice. Western blot analysis revealed reduced Epac-1 protein expression in fibrotic livers of mice and man compared to healthy livers. PGE_2 administration to fibrotic mice completely restored intrahepatic Epac-1 levels, and also led to reduced Rho-kinase activity, a downstream target of Epac-1. Cell-specific delivery of PGE_2 to either hepatocytes, Kupffer cells or hepatic stellate cells identified the latter cell as the key player in the observed effects on Epac-1 and Rho-kinase. No significant alterations in PKA expressions were found. In primary isolated HSC, PGE_2 elicited Rap1 translocation reflecting Epac-1 activation, and Epac-1 agonists attenuated PDGF-induced proliferation and migration of these cells. These studies demonstrate that PGE_2 enhances Epac-1 activity in HSC which is associated with significant changes in (myo)fibroblast activities in vitro and in vivo. Therefore, Epac-1 is a potential target for antifibrotic drugs.

INTRODUCTION

Liver fibrosis is caused by an imbalanced remodeling process due to chronic inflammation associated with excessive scar tissue formation (Seki and Schwabe, 2015). Hepatic stellate cells (HSC), key players in this process, transform from quiescent cells into proliferative, fibrogenic and contractile myofibroblast-like cells in response to growth factors such as transforming growth factor β (TGFβ) and platelet derived growth factor BB (PDGF-BB) (Seki and Schwabe, 2015). The differentiation and activation of HSC is tightly controlled by the activity of cAMP (Mallat et al., 1998). Increased intracellular cAMP levels inhibit fibroblast migration and proliferation and block the phenotype switch into myofibroblasts, leading to less scar tissue formation (Swaney et al., 2005).

Protein Kinase A (PKA) is a well-known second messenger system that mediates the effects of cAMP. More recently, Exchange Protein Activated by cAMP (Epac) was identified as an important signaling mechanism for cAMP-mediated effects (Insel et al., 2012; Lezoualc'h et al., 2016; Schmidt et al., 2013). Epac is involved in the regulation of key cellular processes, such as calcium handling, neural signaling, inflammation, proliferation and migration by promoting the exchange of GTP and GDP in the GTPase cycle (Schmidt et al., 2013; Yang et al., 2015; Yu et al., 2016). Two isoforms of Epac have been identified: Epac-1, found in heart, vasculature, brain, kidney and lungs, and Epac-2, mostly found in brains and adrenal glands. Of interest, Epac-1 is reported to be involved in fibrogenesis. In several reports significant lower Epac-1 expressions were observed during activation of myofibroblasts (Insel et al., 2012; Schmidt et al., 2013; Yokoyama et al., 2008). However to date, nothing is known about mechanisms or factors that can enhance Epac-1 expression and the consequences of this enhancement for fibrogenic processes *in vivo* (Insel et al., 2012).

The cyclooxygenase (COX) product prostaglandin E_2 (PGE_2) affects cAMP levels directly in most cell types. In fibroblasts, PGE_2 exerts anti-fibrotic activities through cAMP activation by binding to EP2 or EP4 receptors (Mallat et al., 1998; Ruwart et al., 1989; Weinberg et al., 2009). We therefore investigated the effects of PGE_2 and the COX-inhibitor niflumic acid (NFA) on Epac-1 expression levels in the CCl_4-induced mouse model for liver fibrosis. In addition, we used a cell-specific delivery approach to deliver PGE_2 to several resident hepatic cells to identify its most important target cell *in vivo.* Our studies show a decreased Epac-1 protein expression in fibrotic livers relative to normal

livers. These Epac-1 levels were restored by PGE_2 delivery to HSC, but not by its delivery to hepatocytes or Kupffer cells. In HSC this was associated with reduced fibrogenic activity both in vitro and in vivo. Our studies indicate a significant role for Epac-1 during liver fibrosis and delineate a pathway to enhance these Epac-1 levels.

MATERIALS & METHODS

Synthesis of drug carriers and PGE_2-constructs.

PDGFβ-receptor recognizing peptides (pPB; prepared by Ansynth Service BV, Roosendaal, The Netherlands), mannose (p-aminophenyl-a-D-mannopyranoside, Sigma-Aldrich, St. Louis, MO) and lactose (Merck, Darmstadt, Germany) were covalently coupled to Human Serum Albumin (HSA) to yield respectively pPBHSA (PH), lactosylated HSA (LH) or mannosylated HSA (MH) as previously described (Beljaars et al., 1998; Beljaars et al., 2003).

PGE_2 (Cayman Chemicals, Ann Arbor, MI) was coupled to PH, LH and MH yielding PGE_2-PPBHSA (PPH), PGE_2-lactosylated HSA (PLH) and PGE_2-mannosylated HSA (PMH). First, the carboxylic acid group of PGE_2 was activated by N,N-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS). This PGE_2/DCC/NHS solution was reacted overnight at room temperature with PH, LH or MH (molar ratio HSA:PGE_2=1:60). All products were subsequently dialyzed for at least 24 h against PBS using a 10 KDa dialysis cassette (Slide-A-Lyzer 10K, Pierce Biotechnology, Rockford, IL), and further purified using chromatographic techniques. Conjugates were lyophilized and stored at −20°C. The amount of PGE_2 coupled to each carrier was assessed by mass spectrometry.

Cell experiments

Primary rat HSC were isolated from livers of male Wistar rats (Harlan, The Netherlands; >400g) as described (Beljaars et al., 2003) and cultured on plastic for 10 days to obtain an activated cell type.

HepG2, RAW264.7, NIH/3T3 fibroblasts (all derived from ATCC) and LX2 cells were used as model cells for respectively hepatocytes, macrophages and HSC. Data are presented as the mean of three experiments, each performed in duplicate.

Concentrations used (unless otherwise stated): 5 μM PGE_2, 50 μM Epac-1 agonist (8-pCPT-2'-O-Me-cAMP: Biolog, Bremen, Germany), 500 μM PKA agonist (6-Bnz-cAMP; Biolog), 50 ng/ml PDGF-BB (PeproTech, Rocky Hill, NJ) and 5 ng/ml TGFβ (Roche Diagnostics, Mannheim, Germany).

Proliferation Assay: Primary rat HSC ($2x10^5$ cells/well) were seeded in 12-wells plates and grown for 2 days. After 24hr starvation, PGE_2, Epac-1- or PKA-agonist was added along with PDGF-BB. After

18hr, ^{3}H-thymidin (0.25 µCi/ml) was added for 6 hr. Cells were washed, fixed with 5% TCA, lysed with 1M NaOH, and radioactivity was counted.

Migration Assay: Primary rat HSC ($6x10^4$ cells/well) were cultured in Transwell chambers (8 µm pore size; Costar) with PGE_2 or agonists. PDGF-BB was added to the lower chamber. After 24hr, membranes were fixed and stained with hematoxylin. Cells on both sides of the membrane were counted in at least five fields/membrane (magnification 40x). Migration was calculated as percentage of cells in the lower chamber relative to total cell number.

In vitro effects of PGE_2-conjugates: HepG2 cells (150.000 cells/well) were incubated with PGE_2 and equimolar amounts of PLH for 60 min. Cells were processed and used for Western Blot analysis using VASP-1 antibody. RAW264.7 cells were incubated with 100 ng/ml LPS plus 10 µM PGE_2, equimolar amounts of PMH, or vehicle. After 24 hr, medium was harvested and total NO production was assessed (Melgert et al., 2001). LX2 cells were grown to confluency in 12-wells plates. A standard scratch was made after which cells were incubated for 24hr with 10 ng/ml PDGF-BB plus 5 µM PGE_2, equimolar amounts of PPH, or vehicle. The scratch size was measured using imaging techniques at t=0 hr and t=24 hr yielding the % wound healing.

Epac-1 and PKA expression: 3T3 fibroblasts (100.000 cells/well) were incubated with PGE_2 (0.1, 1, 5, 10 µM) for 6, 24 and 48 hr. Cells were homogenized and processed for Western Blot and PCR analysis.

Rap1 expression: 3T3 cells (25.000 cells/well), seeded in glass labteks, were incubated with 10 µM PGE_2 and 20 µM Epac-1 antagonist CE3F4 (Courilleau et al., 2012) for 60 min. After fixation in acetone/methanol (1:1), Rap1 was stained (O/N,4ºC) using the primary antibody (Santa Cruz), and subsequently visualized using Alexa488-goat anti rabbit IgG (Invitrogen) and DAPI.

Animal Experiments

All animal experiments were approved by the Animal Ethics Committee of the University of Groningen, the Netherlands.

CCl_4 Model: Male Balb/c mice (20-22g, Harlan, Zeist, Netherlands) received CCl_4 for 8 weeks, according to standard protocols (Beljaars et al., 2003). At week 7 and 8, mice also received (iv, 3

times/week) 0.5 mg/kg PGE_2, 5 mg/kg NFA, vehicle (PBS), PPH, PH, PMH, MH, PLH, or LH (n = 6-9/ group). All animals received the same PGE_2 dose either free or bound to the albumin drug carrier. Mice were sacrificed at week 8. Two animals from each group received i.v. a PGE_2-loaded carrier 15 min prior to sacrifice to check whether the PGE_2-carrier conjugate still accumulated in the designated target cell. The liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST), reflecting liver damage, were measured in plasma according to routine methods at the clinical chemistry lab of the University Medical Center Groningen, the Netherlands.

Human tissue

Human liver tissue samples were obtained from the Department of Surgery and Liver Transplantation (University Medical Center Groningen, the Netherlands). All patients signed an informed consent for the use of residual tissue for research purposes. Experimental protocols were approved by the Medical Ethical Committee and anonymized tissue samples were used according to Dutch guidelines (http://www.federa.org/gedragscodes-codes-conduct-en). Normal human liver tissue was obtained from donor livers discarded for transplantation for technical reasons. Cirrhotic human liver tissue was obtained from patients undergoing liver transplantation and indications for transplantation were a.o. primary sclerosing cholangitis, primary biliary cirrhosis, congenital cirrhosis, alcohol-induced liver disease, acute liver failure and Wilson's cirrhosis. All human liver material was anonymized and available patient characteristics are listed in Table 1.

Tissue analysis

Immunohistochemistry. Cells and cryostat sections were stained according to standard methods. Primary antibodies used in this study: mouse anti-α-SMA and anti-Desmin from Sigma-Aldrich (St. Louis, MO), goat anti-collagen I from Southern Biotechnology Associates (Birmingham, AL), anti-PMLC2 (Ser19) from Cell Signaling Technology (Danvers, MA), rat anti-CD68 (AbD Serotec, Dusseldorf, Germany), rabbit anti-CYP2E1 (Millipore Corporation, Darmstadt, Germany), and goat anti-HSA (Cappel, USA). Species-specific horseradish peroxidase and FITC-/TRITC-conjugated

secondary antibodies were from Dako (Glostrup, Denmark). Stainings were quantified by analyzing complete sections from 3 different liver lobes of each animal at magnification 10x10 using Cell D image analyzing software (Olympus, Hamburg, Germany).

Western Blot analysis was performed according to standard methods with 100 µg protein loaded on a 12% gel and using the primary antibodies: goat anti-pSMAD 2/3 and goat anti-Epac-1 (Santa Cruz Biotechnology, Heidelberg, Germany), goat anti-PKA [Cα] (BD Transduction Laboratories), mouse anti-α-SMA, goat anti-collagen I, and β-actin (Sigma). Signals were quantified with Genetools (Syngene, Cambridge, UK) using ECL reagent (Perkin-Elmer, Boston, MA). Of each animal, samples from 3 different liver lobes were analyzed and corrected for the house-keeping gene β-actin.

Quantitative Real Time PCR: Total RNA was isolated by RNeasy mini kit (Qiagen, Hilden, Germany) and reverse transcribed using cDNA synthesis kit (Promega). Rt-PCR was performed using SYBR green PCR Master Mix (Applied Biosystems) or SensiMix SYBR kit (Bioline, UK) according to standard methods with β-actin as house-keeping gene. Primers (Sigma Genosys, Haverhill, UK) used: αSMA (forward: ACTACTGCCGAGCGTGAGAT, reverse: CCAATGAAAGATGGCTGGAA), Çollagen 1a1 (forward: TGACTGGAA- GAGCGGAGAGT, reverse: ATCCATCGGTCATGCTCTCT), Epac-1 (forward: CAGTGCTGCTCTGGCCGGGA, reverse: GTTCCTGCAGGCTGGGGCTC), human EPAC-1 (forward: CATGTGAAACACGACTGGGC, reverse: GAGGTCCAGCTCTTCATCCG) PKA (catalytic subunit) (forward: GGTTCAGTGAGCCCCACGCC, reverse: GGGGGTCCCACA- CAAGGTCCA) and β-actin (forward: GGCATCCTGACCCTGAAGTA, reverse: GGGGTGTTGAAGGTCTCAAA).

PGE_2 ELISA: Liver samples were homogenized and PGE_2 amounts were assessed using the monoclonal PGE_2 EIA kit (Cayman Chemicals) according to manufacturer's instructions.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analyses were performed using the Mann Whitney-test and considered significant at $p < 0.05$.

RESULTS

PGE_2 and NFA affect liver fibrosis in CCL_4-treated mice

Mice received CCl_4 for 8 weeks to induce liver fibrosis. During the last 2 weeks of CCl_4 administration, the mice were treated with PGE_2 (n=9), the COX-2 inhibitor NFA (n=6) or vehicle (PBS; n=9). Normal mice, receiving only PBS, served as control (n=9). Plasma levels for alanine transaminase (ALT) and aspartate transaminase (AST) indicated significant liver damage in all CCl_4-treated mice (see table 2). No major differences in damage were seen between fibrotic animals receiving different compounds in the final two weeks, although a reduced average in plasma ALT level was seen in NFA-treated animals ($p<0.05$). Markers reflecting HSC activation (αSMA) and matrix deposition (collagen I) were examined both at the mRNA (Supplemental Figure 1) and protein level (Fig 1). Immunohistochemistry and rtPCR analysis showed that PGE_2-treated mice displayed significantly less intrahepatic αSMA and collagen I expression levels compared to CCl_4-mice receiving no treatment (fig. 1A-D). In contrast, NFA induced a significant increase in αSMA and collagen I protein levels in fibrotic mice compared to CCL_4-mice receiving no treatment. pSMAD2/3 expression levels, indicating TGFβ signaling, were strongly enhanced in CCL_4-receiving mice treated with PBS (Fig 1E). Treatment with PGE_2 significantly reduced these levels, whereas NFA administration enhanced pSMAD2/3 levels in fibrotic animals.

Measurement of intrahepatic PGE_2 levels revealed that CCL_4-mice displayed increased intrahepatic PGE_2 levels relative to healthy animals ($p< 0.01$, fig. 1F). PGE_2 treatment significantly reduced these levels compared to untreated fibrotic mice ($p<0.01$). NFA treatment completely abolished intrahepatic PGE_2 production in fibrotic animals.

PGE_2 enhances Epac-1 expression and attenuates Rho- activity in vivo

Previous in vitro studies showed that PGE_2 activates cAMP in myofibroblasts (Huang et al., 2008). We now examined intrahepatic expression levels of both cAMP mediators PKA and Epac-1 in CCL_4-treated mice. In fibrotic livers a significant reduction in Epac-1 protein expression levels was observed relative to healthy mice livers (fig 2A), in agreement with previous reports (Insel et al., 2012; Schmidt et al., 2013; Yokoyama et al., 2008). PGE_2-treatment completely restored Epac-1 levels in fibrotic livers. In contrast, NFA-treatment further reduced Epac-1 levels in fibrotic animals. Western blot

analysis did not reveal significant changes in PKA expression levels in CCl_4-treated animals as compared to normal mice and expression levels for PKA were also not affected by PGE_2 or NFA treatment.

We also examined human livers for Epac-1 expression. Western blots revealed that Epac-1 expression levels were significantly reduced also in human fibrotic livers, irrespective of etiology, compared to control livers (fig. 2C).

Rho-kinase is reported to be a downstream target of Epac-1 (Roscioni et al., 2011; Schmidt et al., 2013). Rho-kinase in turn stimulates fibroblast-to-myofibroblast transdifferentation, migration and contraction of these cells via phosphorylation of the Myosin Light Chain (i.e. formation of PMLC) (Mack et al., 2001). This PMLC stimulates Myosin II ATP-ase activity and contraction of actin fibers in HSC, leading to portal hypertension, one of the hallmarks of fibrosis (Klein et al., 2012). Recently, reports showed that Epac-1 inhibits phosphorylation of MLC. In fibrotic mice, clear staining for PMLC in the fibrotic bands was visible, while little or no expression was seen in healthy animals (Fig 2D). PMLC staining co-localized with desmin staining, the marker for HSC. Fibrotic animals treated with PGE_2 displayed no PMLC staining in fibrotic bands or in any desmin positive cell at all.

Collectively, our data show that PGE_2 attenuated αSMA and Collagen I expression in fibrotic mice which is associated with an increase in Epac-1 levels and inhibition of its downstream target Rho-kinase compared to untreated fibrotic mice. In contrast, NFA increased HSC activation and collagen deposition and further reduced Epac-1 expression in fibrotic animals.

Cell-specific delivery of PGE_2 to resident hepatic cells.

Epac-1 proteins are expressed in many resident and circulating cells. We therefore used a drug targeting approachto assess which cell-type is involved in the observed effects on intrahepatic Epac-1 levels and fibrogenesis. We therefore coupled PGE_2 to cell-specific carriers directed at either HSC, Kupffer cells or hepatocytes (fig. 3A). Lactosylated human serum albumin (LH) which binds to the asialoglycoprotein receptor is a well-known carrier to hepatocytes (Beljaars et al., 1998). PDGFβ-receptors and mannose-receptors are highly expressed on respectively activated HSC (Seki and Schwabe, 2015) and macrophages (Beljaars et al., 1998; Melgert et al., 2001), which both are

abundantly present in fibrotic livers. These receptors are suitable targets for respectively HSC- and KC delivery. Therefore, we coupled PDGFβ-receptor recognizing peptides to HSA (pPB-HSA) to obtain a HSC-selective carrier and attached mannose to HSA (MH) to reach macrophages. The cell-specificity of all these carriers has been described *in vivo* in earlier reports (Beljaars et al., 1998; Beljaars et al., 2003; Melgert et al., 2001). PGE_2 was subsequently coupled to each carrier. Cell-specificity of carriers was retained after attachment of PGE_2: PGE_2 attached to pPB-HSA (=PPH) was found in Desmin positive-cells, reflecting HSC, 15 min after its administration to fibrotic mice. Mannosylated HSA (=PMH) was found in CD68-posive KC cells and lactosylated HSA (=PLH) co-stained with the hepatocyte marker (CYP2E1) (fig. 3C). All three PGE_2-conjugates, i.e. PPH, PMH and PLH, also were pharmacologically active compounds as tested in cell cultures: PPH significantly attenuated PDGF-induced activities of fibroblasts *in vitro*, PMH significantly attenuated the LPS-induced NOx production of RAW264.7 macrophages, and PLH induced VASP phosphorylation in HEPG2 cells, all similar to free PGE_2 (fig. 3B).

Fibrotic mice treated for 2 weeks with the HSC-selective PGE_2 conjugate PPH displayed a significant reduction in collagen I expression compared to untreated fibrotic mice (fig. 3D). Mice receiving the carrier alone (i.e. PH) displayed no such reduction. Treatment of fibrotic mice with the KC-selective PMH conjugate or the carrier MH alone led to the opposite; upregulation of collagen I compared to the vehicle group was found. Treatment with the hepatocyte-selective PLH conjugate or the carrier alone (LH) did not induce significant alterations in collagen I deposition.

Subsequently, we studied Epac-1 and PKA expression levels in mice treated with PGE_2-conjugates or their respective control compounds. Only in livers of PPH-treated mice we found significant upregulation of Epac-1 expression compared to PBS-treated fibrotic mice (fig. 3E). All other treatments didn't induce any alteration in Epac-1 expression. PKA expression was not different from untreated fibrotic mice in any of the groups (fig. 3E). The increase in Epac-1 expression in mice treated with the HSC-selective PGE_2 conjugate was, similar to PGE_2 alone (see also fig 2E), associated with a significant reduction in PMLC expression in HSC (desmin-positive cells) in fibrotic livers (fig. 4). Other treatments (i.e PLH and PMH) and controls did not induce a change in hepatic pMLC

staining relative to untreated fibrotic mice (data not shown). This indicates that HSC's are the key players of PGE_2-mediated effects on Epac-1 levels and fibrogenic processes within fibrotic livers.

Collectively, these drug targeting studies show that hepatic stellate cells, rather than Kupffer cells, hepatocytes or circulating immune cells are the effector cells of PGE_2-induced changes in hepatic Epac-1 levels. These data also confirm the association between Epac-1 and Rho-kinase activity in HSC *in vivo*.

PGE_2 induces Rap1 translocation in vitro

To test whether PGE_2 is able to directly activate Epac-1 in fibroblasts we added PGE_2 to 3T3 cells and examined Epac-1 expression at the mRNA and protein level as well as Epac-1 activation. No change in Epac-1 levels at mRNA nor protein levels was found (data not shown). However, a change in Rap1 staining was noted (fig 5). Epac-1 is known to induce translocation of Rap1 from the cytosol to plasma membranes (Yang et al., 2015). Figure 5 shows Rap1 staining evolving from a general weak cytoplasmatic staining within fibroblasts to a more concentrated intense staining along membranes after addition of PGE_2 indicating re-localization and concentration of Rap1. In turn, the PGE_2-induced relocalisation was inhibited by the Epac-1 inhibitor CE3F4 (Courilleau et al., 2012). This indicates the involvement of Epac-1 in this PGE_2-mediated effect.

Effects of Epac-1 and PKA agonists in primary HSC

Our *in vivo* data implicate that fibrogenic mediators reduce Epac-1 expression whereas Epac-1 enhancement leads to inhibition of HSC activities. We tested this in vitro using primary HSC, isolated from rats. In these primary cells, PDGF-BB and TGFβ significantly reduced Epac-1 mRNA expression levels compared to unstimulated cells, whereas no changes in PKA mRNA levels were seen (fig. 6A). Subsequently, the PKA agonist 6-Bnz-cAMP and the Epac-1 agonist 8-pCPT-2'-O-Me-cAMP (Roscioni et al., 2009; Roscioni et al., 2011) were used to study the effects of the Epac-1 and PKA signaling pathway on PDGF-induced migration and proliferation in rHSC. Both agonists attenuated PDGF-induced migration of rHSC (fig 6B). The Epac-1 agonist also inhibited PDGF-induced proliferation in rHSC, whereas the PKA-agonist had no effect (fig. 6B).

DISCUSSION

This study demonstrates the involvement of the cAMP effector Epac-1 in the regulation of hepatic fibrogenesis *in vitro* and *in vivo*. Epac proteins, alone or in concert with PKA, are closely involved in the regulation of pivotal processes including cell survival, proliferation and differentiation, immune responses, and signaling cascades (Lezoualc'h et al., 2016; Roscioni et al., 2011; Yokoyama et al., 2008). Despite these activities, little is known about Epac during liver fibrosis *in vivo.* The relevance of Epac-1 during fibrogenesis has been inferred from studies showing a reduced Epac-1 expression after fibroblast activation in several organs (Insel et al., 2012; Lezoualc'h et al., 2016; Roscioni et al., 2011; Schmidt et al., 2013). It was therefore suggested that upregulation of Epac-1 might attenuate fibrosis (Insel et al., 2012; Schmidt et al., 2013). However, methods to achieve such an upregulation were unknown (Insel et al., 2012). Our *in vivo* experiments in fibrotic mice now show that Epac-1 can be upregulated in HSC by PGE_2. This is associated with reduced fibrogenesis *in vivo*.

PGE_2 was found to exert significant antifibrotic effects in mice with liver fibrosis, as reflected in this study by reduced collagen I, αSMA and pSMAD2/3 levels. The COX-2 inhibitor NFA induced the opposite effects on these parameters. This profibrotic effect cannot be explained by increased damage induced by NFA (see table 2). Also others found a profibrotic effect of COX-2 inhibitors (Gilroy et al., 1999) but the mechanism behind this always has been unclear. Our studies suggest a role for Epac-1 in this cascade of cyclooxygenase-induced effects.

PGE_2 signals through intracellular cAMP inducing PKA or Epac-1 activation. Whereas PKA levels did not change in our studies, Epac-1 dropped significantly during hepatic fibrogenesis in mice and after HSC activation in vitro, confirming earlier reports (Insel et al., 2012). A limited survey in human tissue samples indicates that this reduction also occurs in patients with different liver diseases. PGE_2 and NFA displayed opposite effects in fibrotic livers: a strong up-regulation of Epac-1 was seen after PGE_2 treatment, and a down-regulation after NFA treatment. NFA treatment was shown to abolish intrahepatic PGE_2 production, confirming effective inhibition. These observations suggest that the anti-fibrotic effects of PGE_2 are, at least partly, mediated by Epac-1.

Our data seemingly reveal a contradiction. We showed that PGE_2 administration enhanced Epac-1 expression in fibrotic livers, yet liver fibrosis is associated with high hepatic PGE_2 levels (fig 1F) and

reduced Epac-1 expression (fig 2C). Moreover, PGE_2-treatment attenuated intrahepatic PGE_2 production, yet enhanced Epac-1 levels. It is clear that total intrahepatic PGE_2 content does not correlate with Epac-1 or fibrosis. Of note, the plasma t½ of the administered PGE_2 (5 min) is too short to be detectable at the time of sacrifice (24 hr after injection). PGE_2 and NFA are both anti-inflammatory compounds and consequently can both attenuate PGE_2 production by inflammatory cells (Gilroy et al., 1999). At the same time they have opposite effects on fibrogenesis, HSC activation and Epac-1 levels (see fig 1 and 2A). Cell-specific delivery of PGE_2 to HSC did enhance intrahepatic Epac-1 levels and attenuated fibrogenesis and HSC activation. Therefore, it can be deduced that PGE_2 affects fibrosis via an effect on Epac-1 levels specifically within HSC, irrespective of the total PGE_2 content within livers.

One of the downstream targets of Epac-1 is Rho-kinase (Yu et al., 2016), which was found to be downregulated by Epac-1 (Rajagopal et al., 2013; Roscioni et al., 2011; Schmidt et al., 2013). Rho-kinase stimulates the transdifferentiation of HSC into collagen-producing myofibroblast-like cells via phosphorylation of its downstream substrate, myosin light chain (MLC) (Mack et al., 2001). This prompted us to examine PMLC levels in fibrotic livers. Indeed, PMLC was abundantly expressed in desmin-positive cells in fibrotic septa in livers of untreated mice. In contrast, in fibrotic mice treated with PGE_2, PMLC formation was completely absent, indicating a lower Rho-kinase activity at these sites. Rho-kinase has profound effects on portal pressure via actin-filament contraction in HSC (Klein et al., 2012) and portal hypertension is a key factor in clinical complications during cirrhosis, so the modulation of Rho-kinase activity by PGE_2 in HSC is quite relevant.

PGE_2 is pleiotropic molecule affecting many cell types including (circulating) inflammatory cells, smooth muscle cells, hepatocytes, and macrophages (Haag et al., 2008; Hui et al., 2004). To investigate which cell type was responsible for the observed alterations we used different albumin-based cell-selective carriers. Cell-selectivity of the applied carriers has been extensively demonstrated in previous studies (Beljaars et al., 1998; Beljaars et al., 2003). Cell specificity was verified for all PGE_2 constructs: all constructs were taken up by the designated target cells as illustrated by double staining for albumin and specific cell markers. Also pharmacological activity of constructs was verified in cell lines expressing the designated target receptor (i.e. the PDGFβ-R, Mannose-R, or the

asialoglycoprotein-R) and we started therapies in a late stage of disease, when target receptor expression is high. In vivo, only HSC-selective PGE_2 delivery inhibited the CCl_4-induced scar tissue formation, normalized Epac-1 expression levels and abolished PMLC protein expression, thereby excluding a role for KC, hepatocytes and circulating or infiltrating inflammatory cells. We did not test the effect of PGE_2 on sinusoidal endothelial cells yet. Studies with PGE_2 coupled to endothelial-specific carriers need to be performed in order to exclude a role for this cell type. Our approach however led to insight in a very complex situation, where inflammation and fibrosis interact and PGE_2 exerts many effects in multiple cell types. Collectively, our cell-selective approach shows that PGE_2 increases Epac-1 within HSC, which is associated with local inhibition of Rho-kinase activity and reduced fibrogenesis in CCl_4-treated mice.

In vitro studies confirmed Epac-1-mediated effects by PGE_2 on fibroblasts: Rap1 translocation to the plasma membrane, reported to be induced by Epac-1 (Yang et al., 2015), was seen after addition of PGE_2 to fibroblast and this was inhibitable by the Epac-1 inhibitor CE3F4 (Courilleau et al., 2012). Although PGE_2 is known for its role in inflammation and fibrosis (Haag et al., 2008; Huang et al., 2008; Mallat et al., 1998; Weinberg et al., 2009), literature on the effects of PGE_2 on liver fibrosis *in vivo* is scarce. One study (Ruwart et al., 1989) demonstrated reduced collagen deposition in cirrhotic rats treated with PGE_2, although the mechanism of action behind this was unknown. This lack of clarity is most likely due to the pleiotropic effects of PGE_2 and its poor pharmacokinetic profile; it is rapidly cleared by the liver and kidneys, or inactivated by plasma proteins. In fact, it's surprising that despite its short half-life, significant effects of PGE_2 were found on liver fibrosis. Similar to PGE_2, the effect of COX-activity on fibrogenesis in vivo is unclear. COX-inhibitors are reported to be either pro- (Gilroy et al., 1999) or anti-fibrotic (Chavez et al., 2010) which may indicate a dual effect on fibrogenesis; their anti-inflammatory effects may lead to anti-fibrogenic effects, whereas effects on fibrogenic cells, for instance via inhibition of PGE_2 production, may stimulate fibrogenesis. This remains to be elucidated but the present study provides clues for such a dual effect of PGE_2 and COX-inhibitors.

In summary, our study shows the involvement of the cAMP effector Epac-1 in the regulation of liver fibrosis. Fibrogenesis is associated with a reduced Epac-1 expression in vivo. Restoration of Epac-1

levels by PGE_2 is associated with attenuated fibrogenic activity. In contrast, the COX inhibitor NFA attenuates hepatic Epac-1 expression, which is paralleled by an increased HSC activation and fibrogenesis. In HSC, but not in hepatocytes or KC, PGE_2 restores Epac-1 expression levels and strongly affects Rho activity in these cells. Our studies reveal a pathway to modulate Epac-1 levels and thereby fibrogenesis, although our studies also show that cell-specificity is important when interfering with Epac-1 levels. Detailed insight into this pathway is essential to identify drug targets and understand the effects of COX inhibitors in chronic liver diseases.

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Authorship Contributions

Participated in research design: Schippers, Beljaars, Lotersztajn, Schmidt, Poelstra

Conducted experiments: Schippers, Post, Reker-Smit, Han, Munoz-Llancao, Boxum, Spijkers

Performed data analysis: Schippers, Beljaars, Spijkers, Poelstra

Wrote or contributed to the writing of the manuscript: Schippers, Beljaars, Schmidt, Poelstra

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Footnotes

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Figure legends

Figure 1. Effect of PGE_2 and NFA (COX inhibitor) on fibrosis-related parameters in mice with CCl_4-induced liver fibrosis. Representative pictures of (*A.*) αSMA and (*C.*) collagen type I staining in healthy and CCl_4-mice receiving treatment with PBS, PGE_2 or NFA (magnification 40x). Quantitative analysis of (*B.*) αSMA- and (*D.*) collagen-stained liver sections. *E.* Expression of pSMAD2/3 as analyzed by western blot. *F.* Analysis of hepatic PGE_2 levels measured by ELISA in healthy mice and fibrotic mice treated with PBS, PGE_2 or NFA. (* $p<0.01$ compared to CCL_4; # $p< 0.05$ compared to healthy; n=6-9 per group).

Figure 2. Expression of EPAC-1, PKA and PMLC in fibrotic livers. Effects of PGE_2 and NFA on intrahepatic expression of EPAC-1 (*A.* and *C.*) and PKA (*A.* and *B.*) in fibrotic mice, as determined by Western blot analysis. *D.* Epac-1 expression in healthy and cirrhotic human livers. *E.* Rho-kinase activity, as reflected by PMLC staining, and desmin staining, as marker for HSC, in fibrotic livers. Note the colocalization of desmin and PMLC and the absence pf PMLC staining after PGE_2 treatment (magnification 100x). *= $p< 0.05$, **=$p<0.01$

Figure 3. Cell-specific effects of PGE_2 targeted to HSC (PPH), to Kupffer cells (PMH) or to Hepatocytes (PLH) on fibrogenic markers and on Epac-1 and PKA expressions in fibrotic livers in mice. Figure *A.* schematically outlines the different constructs and target cells. Figure *B.* Pharmacological effects of PGE_2 coupled to pPB-HSA (PPH), to mannosylated HSA (PMH), or to lactosylated HSA (PLH) in cell cultures. A wound healing assay in cultures of fibroblasts was used to test the activity of PPH. The biological activity of PMH was assessed by LPS-induced NO-production in RAW264.7 macrophages and the phosphorylation of VASP in HEPG2 cells was used to assess the activity of PLH. Figure *C.* Double staining for the PGE_2-conjugate (with an antibody directed against HSA) and the cellular marker desmin (marker for HSC), CD68 (marker for KC) or CYP2E1 (hepatpocyte marker). Arrow heads show double staining for desmin and PPH, double staining of

CD68 and PMH and double staining for CYP2E1 and PLH in livers of fibrotic mice, 30 minutes after injection of the compounds (original magnification 20x10, inserts 40x10). Figure *D.* Protein levels for collagen type I in livers of fibrotic mice treated with PPH (n=9/group), PMH (n=6/group), or PLH (n=6/group) measured by western blot analysis. Figure *E.* Hepatic protein levels for cAMP effectors Epac-1 and PKA in mice after treatment with the different PGE_2 constructs. Vehicle (PBS) or drug carriers carriers without PGE_2 (i.e. PH, MH or LH) served as controls.

Figure 4: Effects of PGE_2 targeted to HSC (PPH), free PGE_2, or carrier (PH) on intrahepatic pMLC expression (reflecting Rho-kinase activity) in fibrotic livers *in vivo*. Representative pictures of immunofluorescent staining for phosphorylated-myosin light chain (PMLC = Rho-kinase marker: green) and desmin (HSC marker: red). Strong staining for PMLC can be seen in desmin-positive septae, but this staining is absent in fibrotic animals treated with PPH and PGE_2.

Figure 5: Effects of PGE_2 on intracellular localization of Rap1 in NIH/3T3 cells. Pictures show fluorescent staining for Rap1 which is a downstream effector of Epac-1. Note the more intense staining along the plasma membranes in PGE2-treated cultures, which is reversed by addition of the Epac-1 inhibitor CE3F4 (original magnification 40x10).

Figure 6: In vitro effects of PDGF-BB and TGFβ on Epac-1 and PKA expression (panel *A*.) and effects of Epac-1 and PKA agonists on PDGF-induced biological responses in fibroblasts (panel *B*.). *A:* Quantitative real-time PCR analysis of Epac-1 and PKA mRNA expression levels after PDGF- or TGFβ-induced activation of primary HSC, normalized to β-actin expression. *B:* The effects of Epac-1 agonist 8-pCPT-2'-O-Me-cAMP and the PKA agonist 6-Bnz-cAMP on PGDF-induced migration and proliferation in primary HSC. Data are presented as the mean (± SEM) of 4 independent experiments.

Tables

Table 1: Patient characteristics of the human livers used in this study

	Normal livers	Cirrhotic livers
	N=7	N=6
Age (min-max) years	41 (10-57)	49 (35-66)
Gender	n=4: F n=2: M n=1: not known	n=3: F n=1: M n=2: not known

Table 2: Plasma ALT and AST levels (AVG ± SEM) in each experimental group at the time of sacrifice.

	AST (U/l)	ALT (U/l)
Healthy	33±9	13±3
CCL_4	572±129 (** relative to healthy)	182±37 (*** relative to healthy)
CCL_4+PGE_2	326±117 (ns relative to CCL_4)	152±34 (ns relative to CCL_4)
CCL_4+NFA	704±118 (ns relative to CCL_4)	62±12 (* relative to CCL_4)

*= p< 0.05; **=p<0.01; ***=p<0.001, ns= non-significant,

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Figures

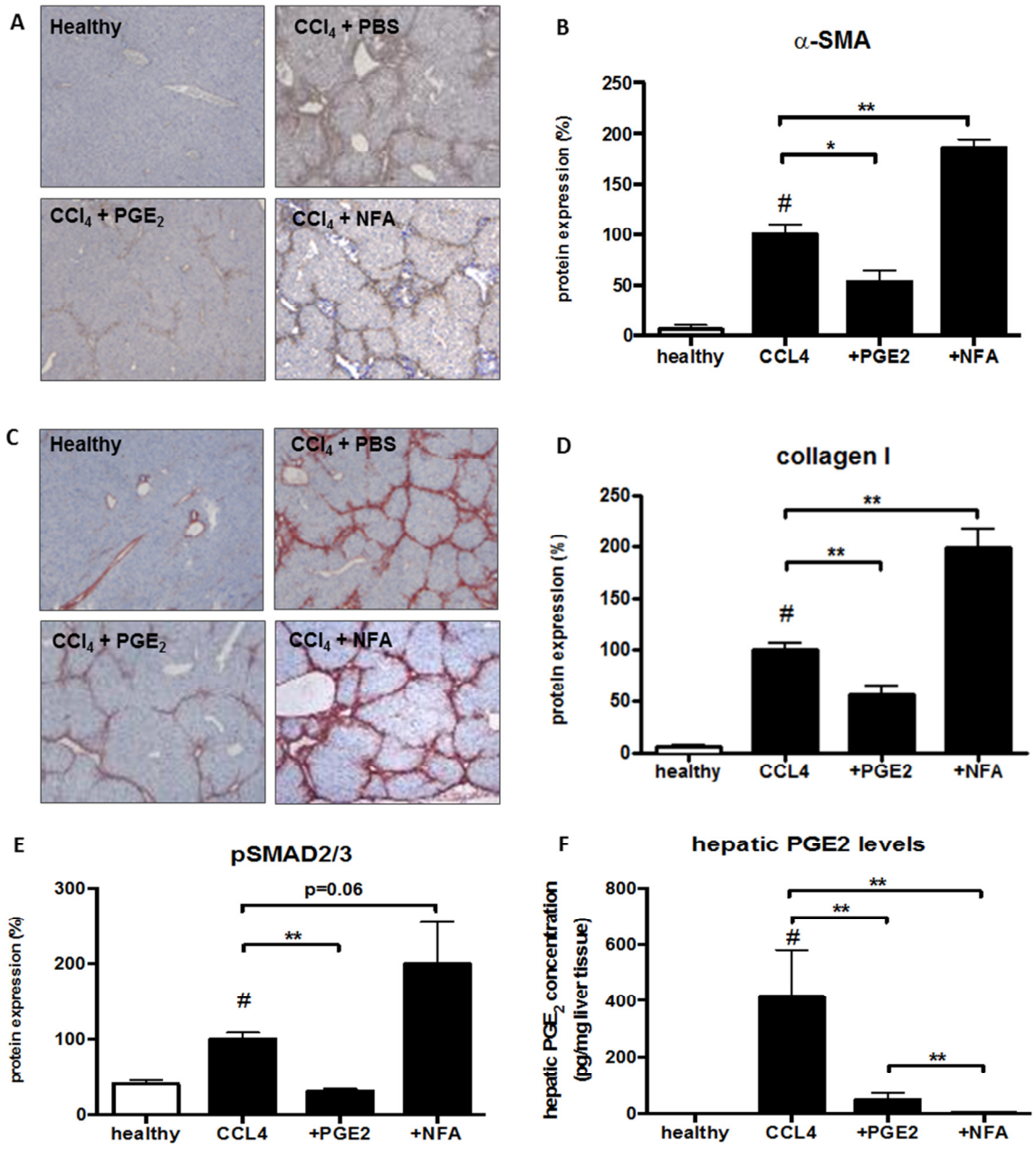

Figure 1 Schippers et al

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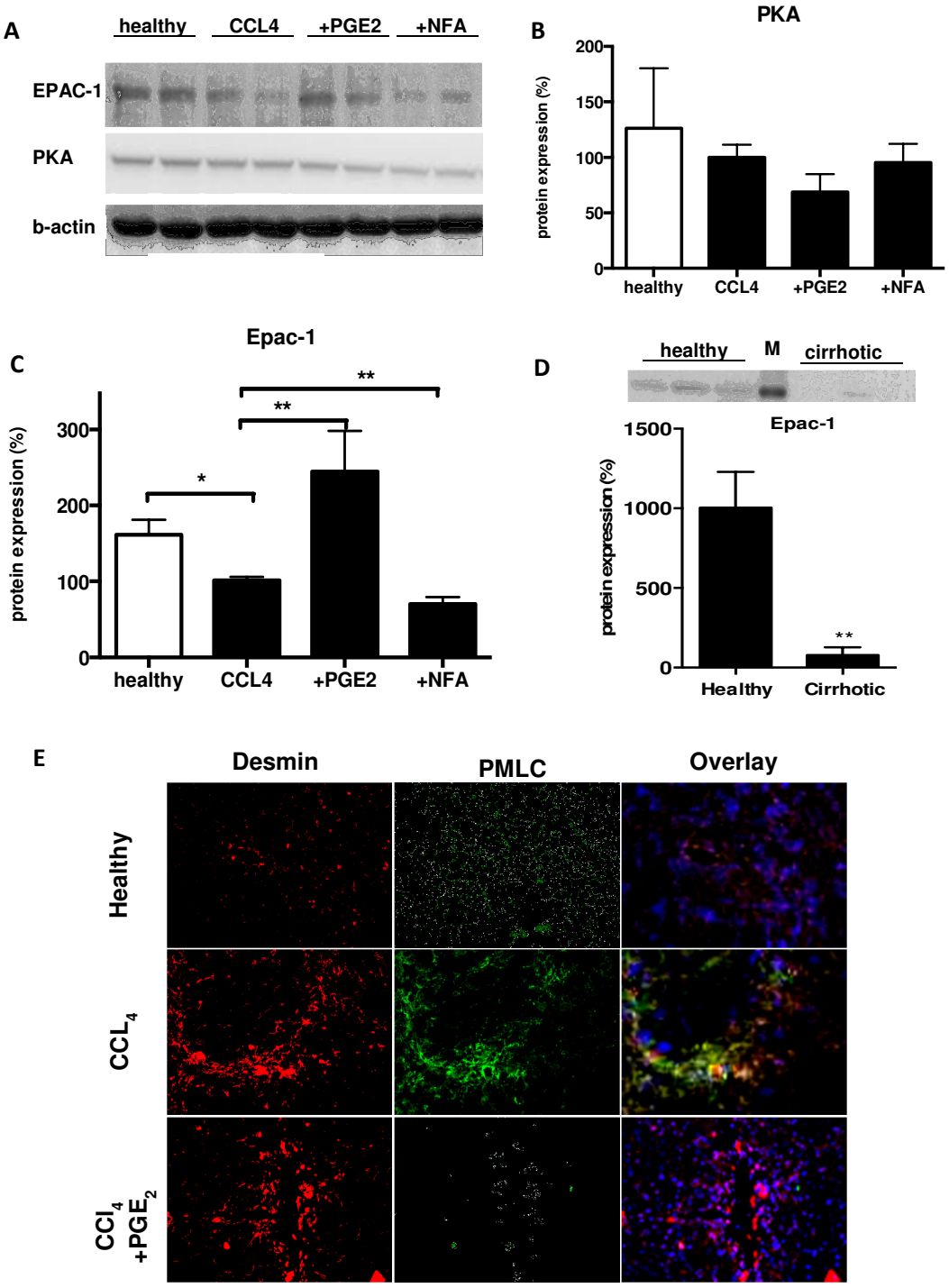

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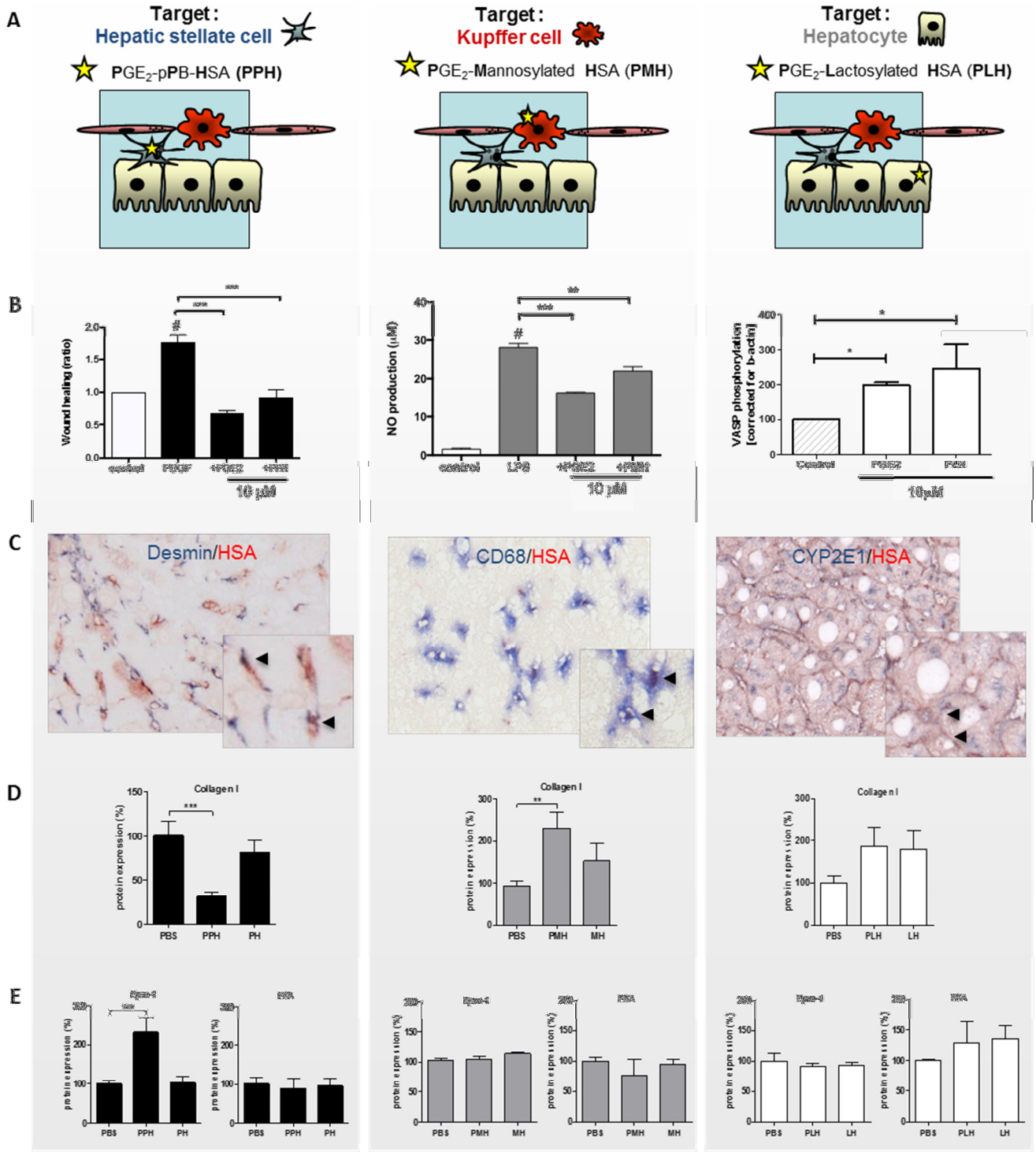

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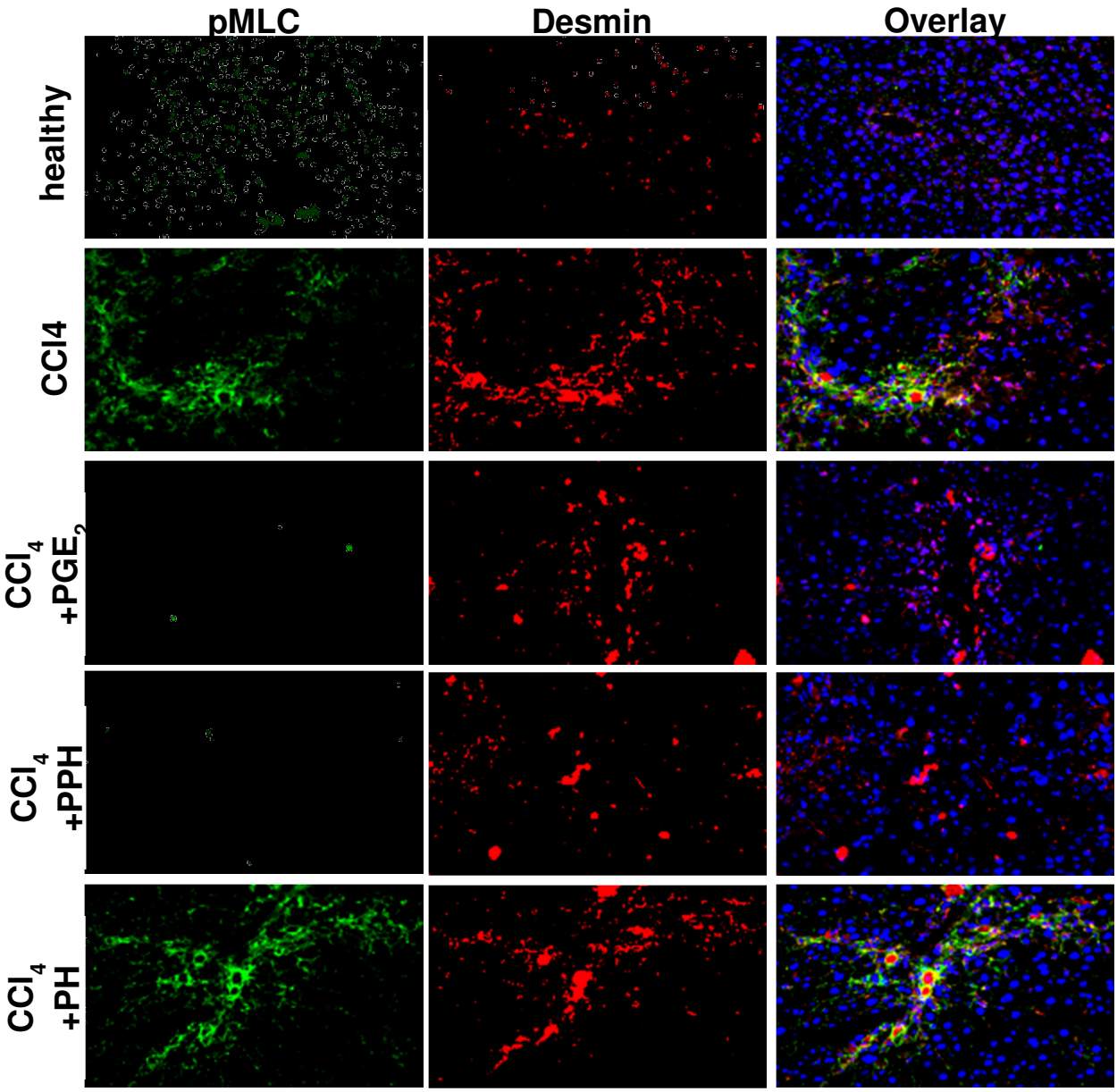

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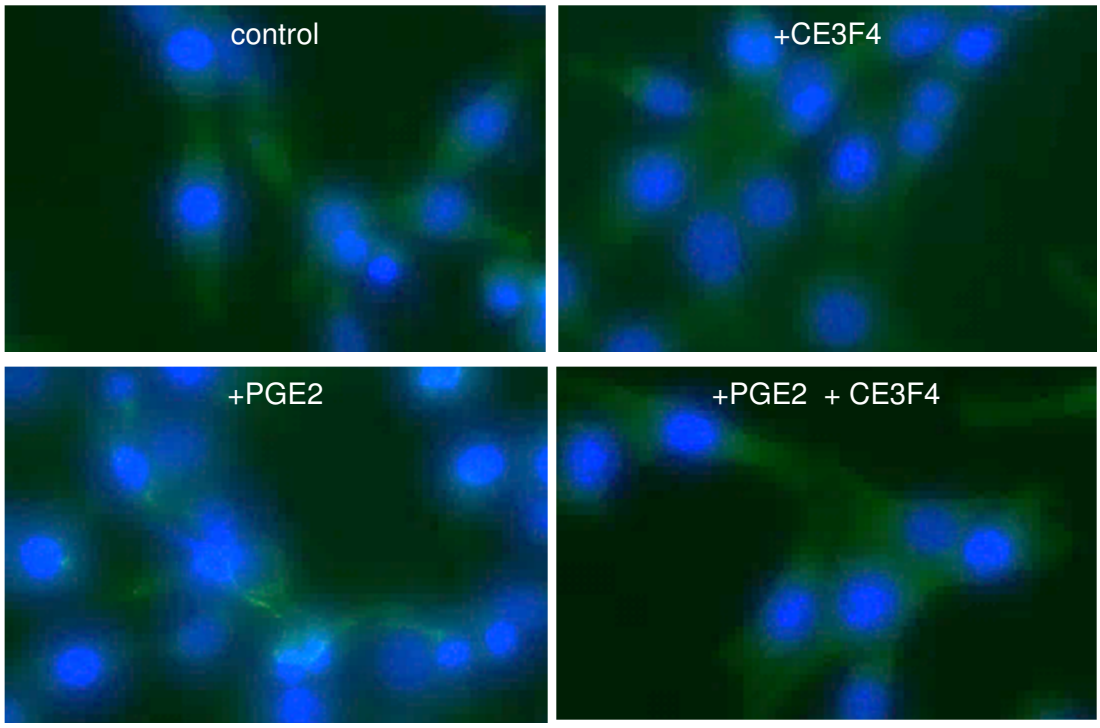

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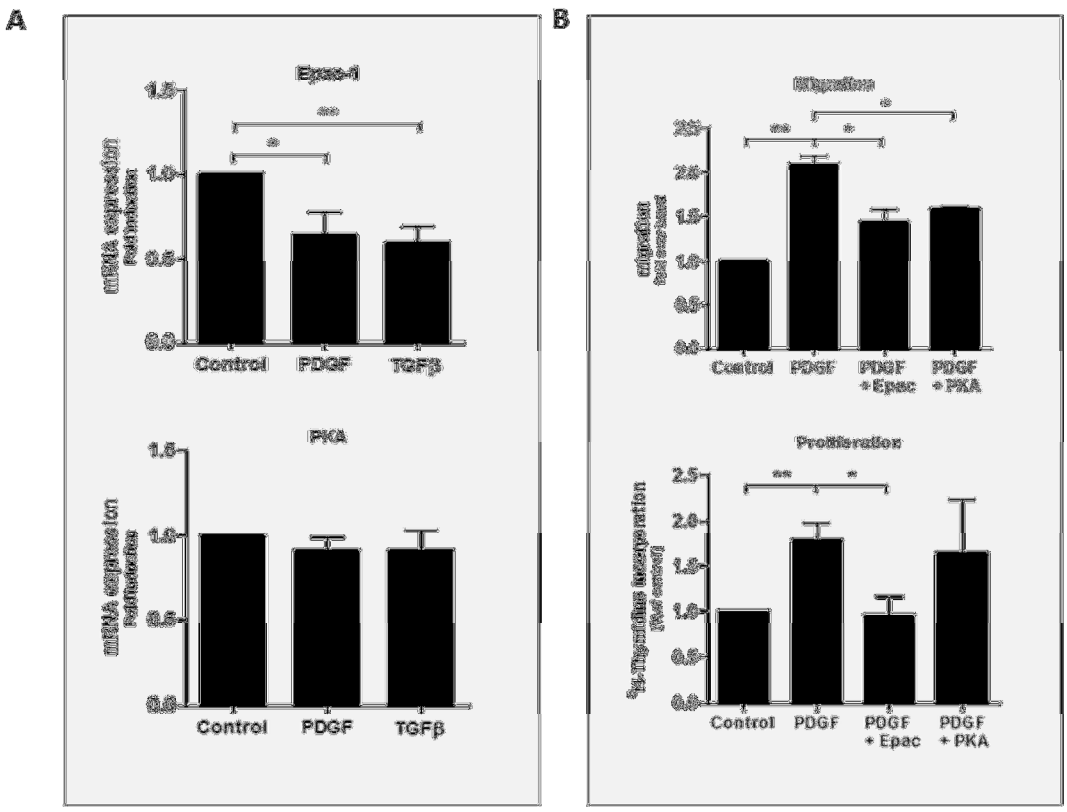

Figure 6. Schippers et al