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PGE_2 induces apoptosis of hepatic stellate cells and attenuates liver fibrosis in mice by downregulating miR-23a-5p and miR-28a-5p

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ABSTRACT

MicroRNAs (miRNAs), small noncoding RNAs modulating messenger RNA (mRNA) and protein expression, have emerged as key regulatory molecules in chronic liver diseases, whose end stage is hepatic fibrosis, a major global health burden. Pharmacological strategies for prevention or treatment of hepatic fibrosis are still limited, what makes it necessary to establish a better understanding of the molecular mechanisms underlying its pathogenesis. In this context, we have recently shown that cyclooxygenase-2 (COX-2) expression in hepatocytes restricts activation of hepatic stellate cells (HSCs), a pivotal event in the initiation and progression of hepatic fibrosis. Here, we evaluated the role of COX-2 in the regulation of a specific set of miRNAs on a mouse model of CCl_4 and bile duct ligation (BDL)-induced liver fibrosis. Our results provide evidence that COX-2 represses miR-23a-5p and miR-28-5p expression in HSC. The decrease of miR-23a-5p and miR-28-5p expression promotes protection against fibrosis by decreasing the levels of pro-fibrogenic markers α-SMA and COL1A1 and increasing apoptosis of HSC. Moreover, we demonstrate that serum levels of miR-28-5p are decreased in patients with chronic liver disease. These results suggest a protective effect exerted by COX-2-derived prostanoids in the process of hepatofibrogenesis.

1. Introduction

Cyclooxygenase-1 (COX-1) and -2 catalyze the first step in prostanoid biosynthesis. COX-1 is constitutively expressed in many tissues, whereas COX-2 is induced by a variety of stimuli [1]. Adult hepatocytes fail to induce COX-2 expression regardless of the pro-inflammatory factors used [2]. However, our group and others demonstrated that partial hepatectomy (PH) induced COX-2 in hepatocytes and contributed to the progression of cell cycle during regeneration [3]. In addition, COX-2 is up-regulated in the livers of patients with chronic viral hepatitis, cirrhosis and hepatocellular carcinoma (HCC), and this upregulation leads to increased production of prostaglandins, mainly PGE_2 [4].

Liver fibrosis is characterized by an excessive accumulation of extracellular matrix (ECM) proteins in response to chronic hepatic injury [5]. Hepatic stellate cells (HSC) have been recognized as the principal cell type responsible for ECM formation during hepatic fibrogenesis and TGF-β represents one key factor stimulating collagen and ECM production in these cells [6]. Induction of α-smooth muscle actin (α-SMA) is the most reliable marker of HSC activation [7]. Moreover, hepatic TGF-β1 is increased in animal models of liver fibrosis and in patients with chronic liver diseases [8] and has been inversely correlated to PGE_2 action over HSCs activation [9].

miRNAs are small non-coding RNAs that negatively regulate their

Abbreviations: COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; ALT, alanine transaminase; TGF-β1, transforming growth factor β1; BAX, Bcl-2-associated X protein; Bcl-x_L, B-cell lymphoma-extra-large; α-SMA, alpha smooth muscle actin; COL1A1, collagen type I alpha 1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PCNA, proliferating cell nuclear antigen; ERK1, extracellular signal-regulated kinase 1; Bmp2, bone morphogenic protein 2; CDH1, E-Cadherin; Alf2, transcription factor 3; p38, mitogen-activated protein kinase 14; Fn1, fibronectin 1; Apaf1, apoptotic peptidase activating factor 1; Tradd, TNFRSF1A-associated *via* death domain; Cav1, caveolin 1

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target genes primarily through RNA destabilization or translational repression. Aberrant miRNA expression is associated with pathologic conditions. In the context of liver diseases, previous studies revealed a role for miRNAs in acute liver injury, viral hepatitis, hepatocarcinogenesis, hepatic fibrosis and NAFLD [10–12]. Up to now, several miRNAs are known to affect different steps of fibrogenesis including HSC activation, proliferation, migration, and ECM deposition. Members of the miR-29 family are downregulated in HSCs in response to TNF-α and TGF-β signaling and suppress the transcription of ECM genes like *Col1a1* [13]. miR-133a and miR-101 are other miRNAs regulated by TGF-β in HSCs, influencing ECM-related gene translation in liver fibrosis [14]. Next to these antifibrotic miRNAs, several pro-fibrotic miRNAs are up-regulated during fibrogenesis: elevated expression of miR-21 enhances HSC-activation and EMT by activating the PTEN/Akt [15] pathway. Moreover, TGF-β induced up-regulation of both miR-199s and miR-200 that indirectly promotes liver fibrosis by increasing the expression of pro-fibrotic genes (*e.g.* collagens, MMPs) [16,17].

Regarding COX-2 and the impact of PGE_2 on the development of fibrosis the data are controversial. Some studies indicate that PGs favor the development of hepatic steatosis, NASH and ultimately fibrosis [18–20], while others provide evidence that PGE_2 suppresses fibrogenesis and NASH progression since COX-2 inhibition potentiates inflammation and liver fibrosis [9,21,22]. Our results indicate that constitutive expression of COX-2 in hepatocytes protects against high fat diet-induced steatosis, inflammation, obesity and insulin resistance [23]. These findings prompted us to screen the role of hepatic-specific COX-2 expression in a murine model of fibrosis induced by CCl_4. In our previous work we have demonstrated that expression of COX-2 in hepatocytes was able to diminish the levels of pro-fibrogenic markers and to reduce the progression of the CCl_4-induced fibrotic process by restricting HSC activation and ECM deposition [24], but the involvement of miRNAs has not been analyzed previously. In this study, we examine a group of miRNAs that were modulated in HSC in response to COX-2 hepatocyte-specific expression. miR-23a and miR-28a were found to be downregulated by PGE_2 in a TGF-β-dependent manner, which could attenuates the activation of HSCs, with growth-suppressive and pro-apoptotic activities. Moreover, we demonstrate that serum levels of miR-28 decreased in patients with chronic liver disease. Based on our findings, we propose that miR-23a and miR-28a are part of a novel signaling pathway that mediates a COX-2-dependent protective role by modulating the activation and apoptosis of HSC during hepatofibrogenesis.

2. Experimental procedures

2.1. Animal experimentation

hCOX-2-Tg mice and their corresponding wild type (Wt) litter-mates were generated by systematic mating of the heterozygous B6D2-Tg (APOE-PTGS2/4)Upme expressing 55 copies of transgene with B6D2F1/OlaHsd Wt mice in our animal house for more than seven generations. The hCOX-2-Tg animals were phenotypically similar to their normal litter-mates and did not exhibit a detectable histological change in the liver at 12-weeks of age. Integration of transgene was systematically checked by PCR analysis of genomic tail DNA. Transgenic mice (hCOX-2-Tg) constitutively express human COX-2 in hepatocytes under the control of the human ApoE promoter and its specific hepatic control region (HCR), a unique regulatory domain that directs ApoE expression in liver [25], lacking macrophage-specific regulatory regions (ME.2 and ME.1) [26]. The animals were maintained in light/dark (12 h light/12 h dark), temperature (22 °C) and humidity-controlled rooms with free access to drinking water. Mice were fed with regular chow diet (RCD; SAFE A04-10 Panlab, Barcelona). To induce fibrosis, CCl_4 (1:4 in olive oil) was intraperitoneally (i.p.) administered to hCOX-2-Tg and Wt mice at a dose of 1.6 ml/kg body weight twice-weekly. Control animals were i.p. injected with olive oil. In another approach to induce fibrosis, some animals were subjected to bile duct ligation (BDL) for 21 days. 9 weeks after CCl_4 treatment or 21 days after BDL, the animals were sacrificed and liver was snap-frozen in liquid nitrogen and stored at − 80 °C for further mRNA analysis, or fixed in 4% buffered formalin to later make paraffin blocks. Plasma was obtained from retro-orbital sinus.

All animal experimentation was controlled following the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) on health monitoring, the European Community Law (86/609/EEC) and the Spanish Law (R.D.1201/2005), and the use of animals in experimental procedures was approved by the Ethics Committee of the Bioethical Commission from CSIC, Spain.

Histochemistry analysis, immunoblot assays, RNA isolation and real-time RT-PCR; hepatic cell isolation and other *in vivo* and *in vitro* experimental procedures are detailed in the Supplementary data section.

2.2. Isolation and culture of hepatic stellate cells (HSC)

The isolation of HSCs from mice livers was performed as described [27] and can be divided into: *In situ* perfusion with pronase (0.4 mg/ml)/collagenase (0.56 mg/ml, increasing 1.5-fold after CCl_4 treatment) solution in William's E medium of mouse liver; subsequent *in vitro* digestion; and density gradient–based separation of HSCs from other hepatic cell populations. Briefly, after the *in situ* digestion, the liver was carefully removed and minced under sterile conditions. The minced liver was further digested *in vitro* with pre-warmed pronase (0.5 mg/ml)/collagenase (0.4 mg/ml)/DNase I (0.1 mg/ml)/HEPES (10 mM) in 1 × HBSS pH 7.4 solution. Then, the liver cell suspension was filtered through a 100 μm cell strainer to eliminate undigested tissue remnants and centrifuged at 1900 rpm for 10 min at 4 °C. The pellet of non-parenchymal cells was resuspended in GBSS (Sigma) and purified by density gradient centrifugation using 14% Nycodenz (Sigma). HSC cells were collected from the diffuse white interphase layer and centrifuged twice in 1 × PBS/0.3% BSA at 1900 rpm for 5 min at 4 °C. Cells were resuspended directly in 700 μl QIAzol Lysis Reagent (Qiagen) for further mRNA analysis or were plated in 6 or 12 multiwell dishes (Corning, New York, USA) with DMEM, 20% FBS and 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin. Cells were cultured to further mRNA and protein analysis. A primary HSC cell line from Wt mice was established by spontaneous immortalization.

2.3. miRNA microarray analysis

miRNAs were extracted from 26 Wt and 21 hCOX-2-Tg isolated HSC samples after oil or CCl_4 treatment using QIAzol Lysis Reagent (Qiagen, Valencia, CA) and purified with a miRNeasy Mini Kit (Qiagen). The quality and integrity of the microRNAs were assessed in an Agilent Bioanalyzer. Three pools (minimum 3 HSC samples per pool) of miRNAs were prepared for each experimental condition. For miRNA array analysis, we used a miRCURY LNA™ Universal RT microRNA PCR, 4 × Mouse & Rat panel I + II (Exiqon, Vedbaek, Denmark). It contains pre-aliquoted PCR primer sets in 384-well PCR plates, allowing the study of 748 mouse and rat microRNAs and 6 reference genes.

For cDNA synthesis, RNA (20 ng) was polyadenylated and reverse transcribed to cDNA using a Universal cDNA Synthesis Kit II (Part from miRCURY LNA™ Universal RT microRNA PCR, Exiqon), according to the manufacturer's instructions. cDNAs were diluted 80 × and were used as the template for quantitative real-time PCR (RT-PCR). ExiLENT SYBR® Green master mix (Exiqon), loaded into pre-aliquoted PCR primer sets in 384-well PCR plates. RT-PCR was performed with a 7900HT Fast-Real Time PCR System (Life Technologies), and the thermocycling parameters were 95 °C for 10 min and 40 cycles of 95 °C for 10 s and 60 °C for 1 min; followed by a dissociation curve consisting of 95 °C for 15 s; 60 °C for 15 s; 95 °C for 15 s. The mean of triplicate probes, analyzed in three pools of each experimental group, was used

for the array, and expressed data were normalized using the global normalization method. The fold change in expression level of each miRNA was determined by comparing the expression levels of miRNAs in COX-2-Tg cells to those in Wt cells. Data analysis was performed using Excel and GenEx2.0 Software. The Heatmaps and clustering of differentially expressed genes were constructed in Excel using conditional formatting.

Microarray data were validated by RT-PCR analysis of individual miRNAs. Analysis of pathways, networks and target genes was performed using miRWalk and "Database for Annotation, Visualization and Integrated Discovery" (DAVID: Functional Annotation Tools) platforms, allowing the identification of several candidate pathways for the miRNA target genes. Cytoskape software was used to illustrate the overlap of the putative miRNAs targets.

2.4. Data analysis

Data are expressed as means ± S.E of at least three independent experiments. Statistical significance was estimated using Student 2-tailed *t*-test to evaluate the differences between treated and untreated mice or cells within a single genotype and between genotypes. Analysis was performed by using the statistical software GraphPad Prism 5. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-23a and miR-28a are specifically repressed by COX-2-derived prostanoids in hepatic stellate cells upon induction of liver fibrosis

To investigate the role of COX-2 activity on fibrosis, we injected CCl_4 in Wt and hCOX-2-Tg mice twice a week for 9 weeks. Liver sections were stained with hematoxylin and eosin (H&E), Masson's trichrome (MTC) and Picro Sirius Red to evaluate the fibrosis stage and to quantify the fibrotic area. We found that hepatocyte COX-2 expression delayed fibrosis progression induced by CCL_4 treatment, through a decrease in α-SMA and COL1A1 expression and significantly reduced the fibrotic area due to a lesser activation of HSC, in agreement with our previous results (Supplementary Fig. 1) [24]. Since we have demonstrated that COX-2 regulates miRNA processing [28], here we sought to analyze whether miRNAs might be implicated in the COX-2-dependent attenuation of fibrosis.

As CCl_4 is the condition leading to fibrosis, we will compare the hCOX-2-Tg *vs*. Wt under CCl_4 condition. Microarray analysis in HSC (the most important source of ECM) revealed that in CCl_4 mice, the expression level of 11 miRNAs was downregulated in hCOX-2-Tg mice *vs*. Wt mice, while the expression of miR-29a was higher ($P \leq 0.05$; fold change cutoff ≥ 1.5) (Fig. 1A). According to previous results, it is well established the powerful anti-fibrotic role of miR-29 in different mouse models of liver fibrosis and in different species including human. Besides, members of the miR-199-family have a pro-fibrotic effect since they are increased in fibrotic liver diseases in both mouse and human [12]. These two miRNAs, along with the two most statistically significant (miR-23a and miR-28a), were chosen to validate the microarray results using real-time qPCR and these data supported the result of the microarray analysis (data not shown).

To determine an HSC-specific role for miR-23a and miR-28 in liver fibrosis, the expression levels of these two miRNAs were examined in primary HSCs isolated from the livers of hCOX-2-Tg mice after 9 weeks of CCl_4 treatment, as well as from Wt mice. The expression of miR-29a and miR-199a was also analyzed. In HSCs from hCOX-2-Tg mice with liver fibrosis, we found a substantial downregulation of miR-23a, miR-28a and miR-199a *vs*. Wt (Fig. 1B), contrary to what happens in hepatocytes (Fig. 1C). We failed to detect significant differences of miR-23 and miR-28 in total liver and Kupffer cells isolated from fibrotic liver of Wt and Tg mice (Fig. 1D, E). As in HSC, serum levels of miR-23a and miR-28a tend to decrease in Tg mice following treatment with CCL_4 (Fig. 1F).

3.2. PGE_2 attenuates TGF-β-dependent increase of miRNA expression in HSC cells

As miR-23a and miR-28a exhibited a consistent downregulation in HSCs from fibrotic hCOX-2-Tg mice and they were not examined in this context in previous analyses, these miRNAs were selected for further evaluation.

First we investigated whether PGE_2 affects TGF-β-induced HSC activation by regulating miR-23a and miR-28a expression. RT-PCR results demonstrated that PGE_2 significantly decreased the TGF-β1-dependent rise of miR-23a and miR-28a in isolated HSC cells from Wt mice, as well as in human and murine HSC cell lines, LX2 and GRX, respectively, treated with PGE_2 after TGF-β1 in comparison with cells activated with TGF-β1. (Fig. 2A–C). Our results validate the upregulation of pro-fibrogenic genes upon TGF-β1 treatment and revealed that PGE_2 counteracted TGF-β1-induction of α-SMA and COL1A1 (Fig. 2D).

To determine whether PGE_2 exerts its effects *via* the TGF-β1 classical signaling pathway, we treated HSC with LY364947, an inhibitor of TβR-I. Interestingly, LY364947 significantly prevented the PGE_2 dependent downregulation of miR-23a and miR-28a (Fig. 2E). These data highlight a potential role for TGF-β1 in the HSC specific regulation of miR-23a and miR-28a by PGE_2 in the process of fibrosis.

3.3. COX-2-Tg mice subjected to bile duct ligation are protected against fibrosis

To confirm our results, we next used a second model of experimental liver fibrosis and analyzed the expression of miR-23a and miR-28a, 21 days after bile duct ligation (BDL) in Wt and hCOX-2-Tg mice. The degree of liver fibrosis was determined by H&E, Picrosirius red and Masson's staining. Histopathological analysis revealed that BDL caused significant bridging fibrosis, but PGE_2 synthesis caused marked reduction in the distribution of collagen fibers in the fibrotic liver of hCOX-2-Tg mice. (Fig. 3A). These results, together with the ALT, AST and bilirubin values, confirm the hepatoprotective role of COX-2 on the BDL-induced liver fibrosis in mice (Fig. 3B). As shown in Fig. 3C, miR-23a and miR-28a were downregulated in Tg *vs*. Wt animals, further supporting the hypothesis that downregulation of miR-23a and miR-28a by PGE_2 constitutes an important feature in murine fibrosis. Fluorescence immunohistochemistry analysis demonstrated that the levels of α-SMA and COL1A1 were higher in BDL mice, compared with the sham-operated, and attenuated in hCOX-2-Tg mice (Fig. 3D–E).

3.4. Relationship between COX-2-regulated miRNAs, TGF-β1 and apoptosis pathways

To further characterize the functional significance of the differentially miRNAs regulated by COX-2, we performed a systematic analysis of their putative gene targets and searched for pathways which were enriched. Putative target genes were identified by using www.microrna.org, a well-established target prediction algorithm for miRNAs. To interpret the biological effect of miR-23a and miR-28a, genes obtained in this analysis were further processed by DAVID 6.7 software for functional pathway enrichment. Various targets of these miRNAs were significantly enriched in different Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using corrected *P* value ≤ 0.05 as a threshold, including the top hit 'pathways in cancer', but also 'focal adhesion', 'apoptosis', 'TGF-β signaling pathway' and other potentially involved in liver fibrosis (Fig. 4A), suggesting that fibrosis-relevant target genes might be regulated by the putative network of PGE_2/TGF-β1-dependent miRNAs in HSCs. A total of 10 genes were selected to illustrate the overlap of the COX-2-regulated miRNA target genes. These 10 genes belonged to the affected pathways including apoptosis and TGF-β signaling pathway (Fig. 4B).

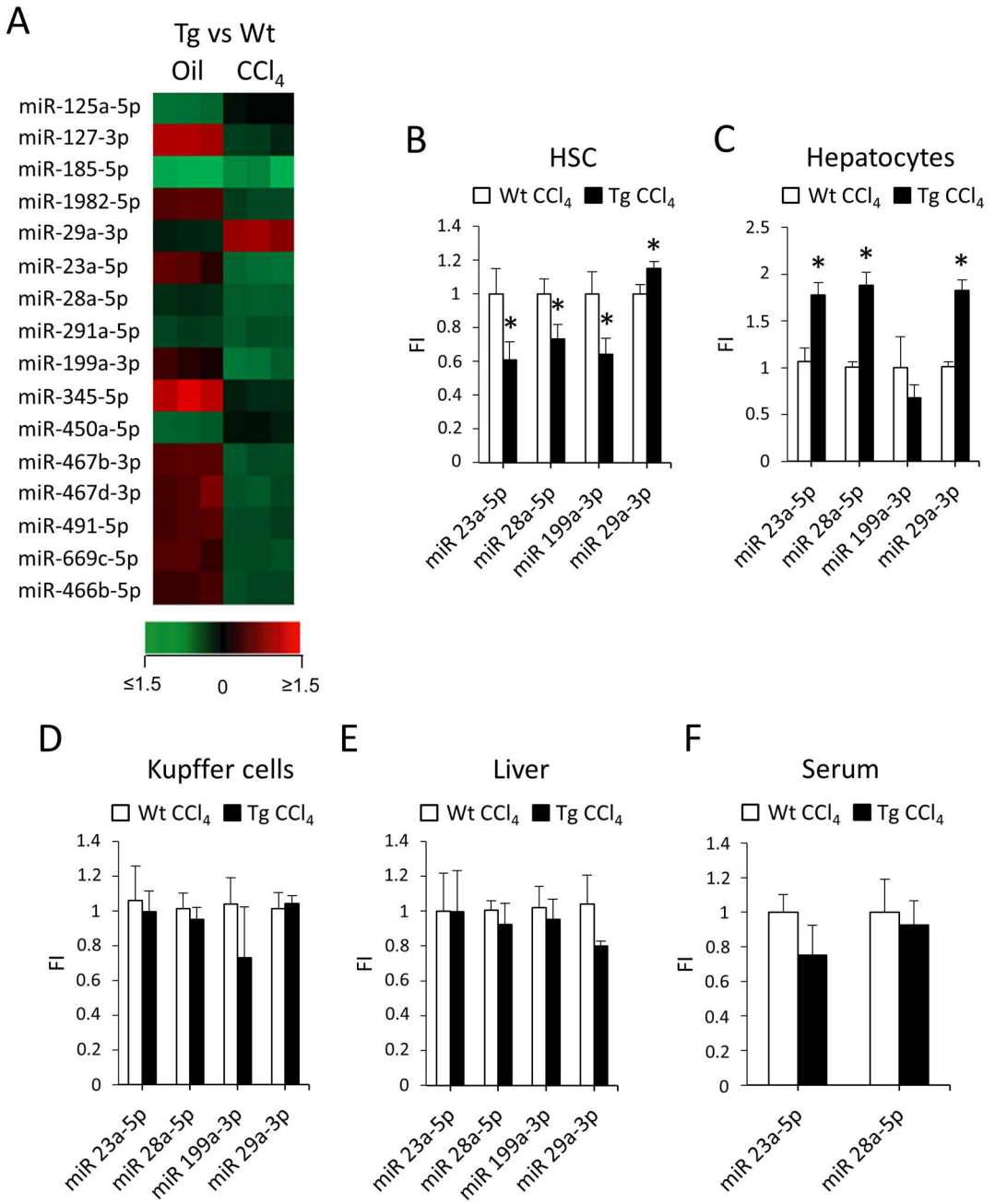

Fig. 1. ***Expression of miR 23a-5p, miR 28a-5p, miR 199a-3p and miR 29a-3p in Wt and hCOX-2-Tg mice.***
(A) Identification of 16 differentially expressed miRNAs in isolated hepatic stellate cells (HSC) from 26 Wt and 21 hCOX-2-Tg after oil or CCl_4 treatment, as detected by quantitative real-time PCR (RT-PCR) array. Three pools (minimum 3 HSC per pool) of miRNAs were prepared for each experimental condition. Values of triplicates are represented in heat map relative to HSC of Wt mice, oil or CCl_4 as appropriate. (B–E) Expression of miRNAs in Wt and hCOX-2-Tg isolated HSC (B), isolated hepatocytes (C), isolated Kupffer cells (D) total liver (E) and serum (F) analyzed by RT-PCR. miRNA expression was normalized to the average of miR 191-5p, miR 103a-3p and RNU5G RNA expression, and expressed as FI (fold induction) *vs.* Wt CCl_4 defined as 1. Data are means ± SE of 14–18 (B); 5–7 (C); 4 (D, E, F) mice per group. *$P < 0.05$ *vs.* Wt CCl_4.

The deregulation of these putative target genes was finally confirmed by RT-PCR in different HSC cell models: HSC isolated from Wt and hCOX-2-Tg livers treated with CCl_4 and Wt HSC treated with PGE_2 and TGF-β1. The results confirmed the upregulation of these potential target genes in HSCs in the presence of PGE_2, upon CCl_4 or TGF-β1 treatment (Fig. 4C). Furthermore, the expression of target genes was downregulated when HSC cells were transfected with miR-23a and miR-28a (Fig. 4D).

3.5. PGE_2 reduced the activation of hepatic stellate cells in vitro

Given the crucial role of COX-2 in suppressing liver fibrosis *in vivo*, we examined whether PGE_2 contributes to modulation of the activation of HSC *in vitro*. HSC were treated with conditioned medium of hepatocytes isolated from Wt and hCOX-2-Tg mice after Oil or CCl_4 treatment. As shown in Fig. 5B, PGE_2 levels were higher in the culture media from Tg hepatocytes. This approach resembles what occurs physiologically in Tg mice. Pro-fibrogenic markers such as α-SMA and COL1A1, were measured by immunofluorescence and as shown in Fig. 5A, HSC-Tg and HSC-Tg-CCl_4 cells showed lesser levels of both markers confirming our previous results [24]. Moreover, pSmad2/3 was decreased in HSC-Tg-CCl_4 cells in comparison with Wt- CCl_4 (Fig. 5C).

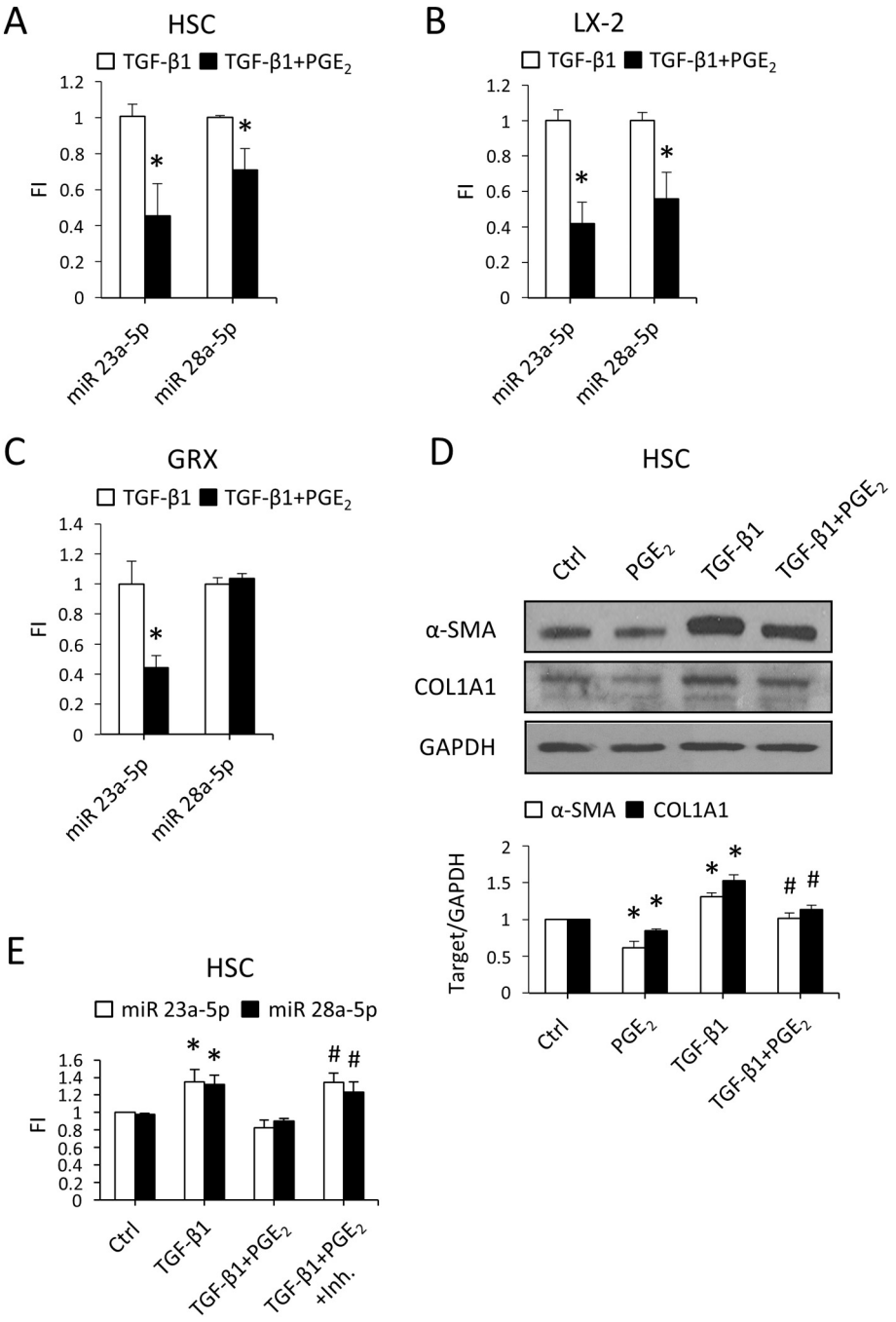

Fig. 2. ***COX-2-dependent PGs regulate the expression of miR 23a-5p and miR 28a-5p in HSC.***
(A, B, C) Validation of the downregulated expression of miR 23a-5p and miR 28a-5p by PGE_2 in primary HSC (A), LX-2 cells (B) and GRX cells (C) analyzed by RT-PCR. Cells were treated with 5 μM PGE_2 (overnight) and 2 ng/ml TGF-β1 (6 h). $^*P < 0.05$ *vs.* TGF-β1 treated cells. (D) Representative Western blot (up) showing expression of α-SMA and COL1A1 protein levels. For densitometric analysis, the relative level of Control (Ctrl) expression was defined as 1, and GAPDH served as a loading control. Results are expressed as band ratio. $^*P < 0.05$ *vs.* Ctrl. $\#P < 0.05$ *vs.* TGF-β1 treated cells. (E) Expression of miR 23a-5p and miR 28a-5p in primary HSC cells, treated with 5 μM PGE_2, 2 ng/ml TGF-β1 and 3 μM TβR-I inhibitor (LY364947). miRNA expression was normalized to the average of miR 191-5p, miR 103a-3p and RNU5G RNA, and expressed as FI *vs.* Ctrl. Data are means ± SE of three independent experiments. $^*P < 0.05$ *vs.* Ctrl. $\#P < 0.05$ *vs.* TGF-β1 + PGE_2 treated cells.

3.6. PGE_2 has a growth-suppressive and pro-apoptotic role in HSC cells

We then investigated the effects of PGE_2 on HSC proliferation and apoptosis. We found that PGE_2 decreases the CCl_4-induced Ki-67 and PCNA expression, markers of cell proliferation (Fig. 6A). Moreover, as determined by the MTT cell viability assay, incubation with conditioned media from Tg mice hepatocytes caused a significant inhibition of HSC cell proliferation (Fig. 6B).

To determine whether the observed suppressive effect of cell growth by PGE_2 was due to an induction of apoptosis, cell death was evaluated by immunostaining of the pro-apoptotic proteins BAX and cleaved caspase-3, showing that expression of COX-2 in hepatocytes led to an increase expression of these pro-apoptotic markers in HSC (Fig. 6C, D and Supplementary Fig. 2). These findings indicate that PGE_2 induces cell death and reduces subsequent proliferative activity specifically in HSCs.

3.7. Ectopic expression of miR-23a and miR-28a induced HSC cell activation

Next, we transiently transfected HSC with a vector expressing miR-23a and miR-28 separately or in combination (Supplementary Fig. 3A–B). As shown in Fig. 7A, the transfection with miR-23a significantly increased α-SMA and COL1A1 protein levels in HSCs, reflecting their pro-fibrotic function. We analyzed a potential additive effect of miR-23a and miR-28a on the expression of fibrotic markers. Co-transfection of miR-23a and miR-28a into HSC did not show a synergistic effect. Furthermore, overexpression of miR-23a and miR-28a counteracted the effect of PGE_2 on the expression of α-SMA and COL1A1 after TGF-β1 treatment of HSCs, further highlighting the role

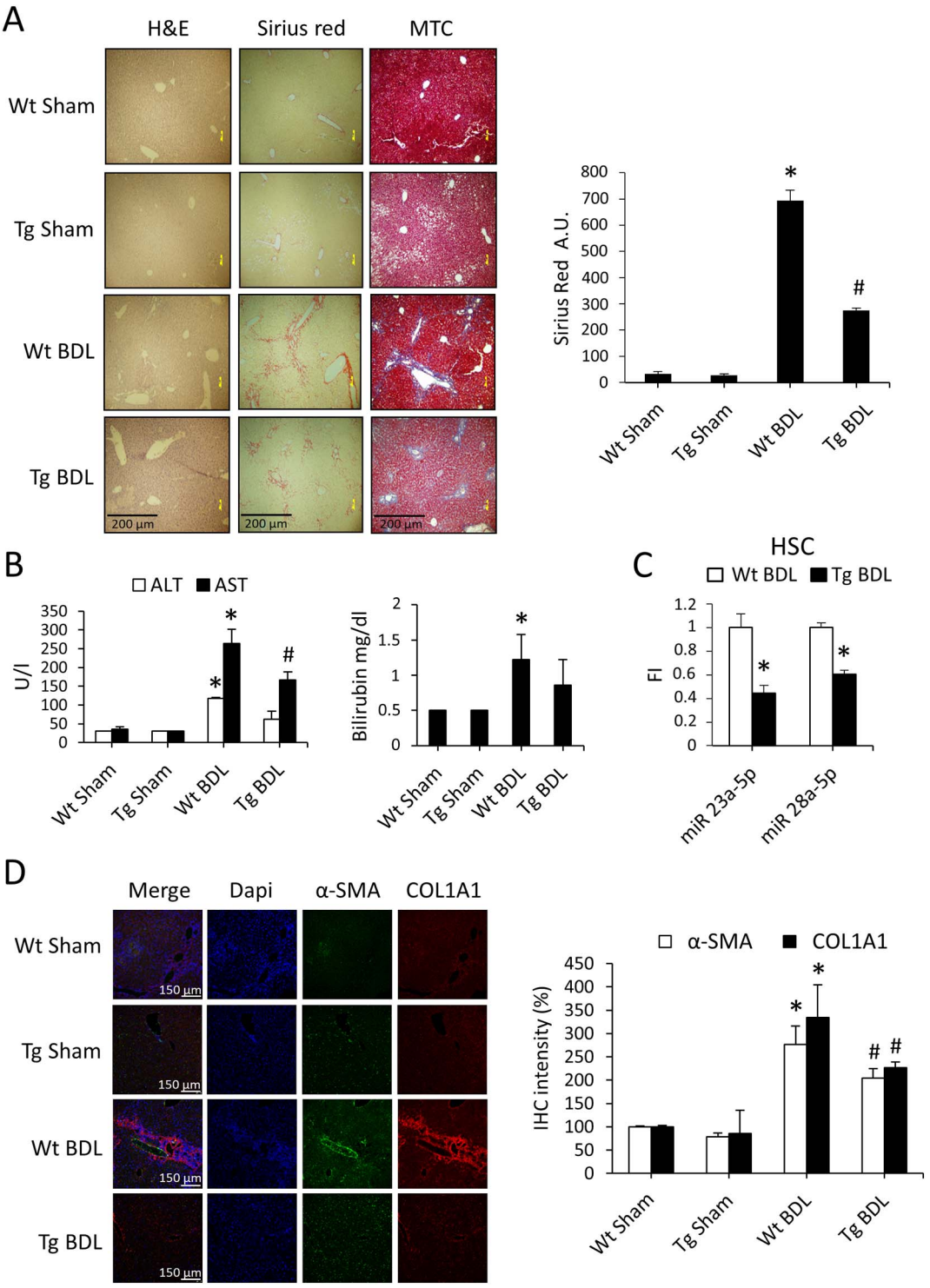

Fig. 3. ***miR 23a-5p is differentially expressed in hCOX-2-Tg mice after BDL.***
(A) Representative images of hematoxylin/eosin (H&E), Picro-Sirius Red and Masson's trichromic (MTC) stained liver paraffin-embedded sections from Wt and hCOX-2-Tg mice after bile duct ligation (BDL). Values of Sirius Red are represented in arbitrary units (A.U.). *$P < 0.05$ *vs*. Wt Sham. #$P < 0.05$ *vs*. Wt BDL. (B) Blood levels of ALT, AST (U/l) and bilirubin (mg/dl) from Wt and hCOX-2-Tg mice after BDL. *$P < 0.05$ *vs*. Wt Sham. (C) Expression of miR 23a-5p and miR 28a-5p in Wt and hCOX-2-Tg after BDL analyzed by RT-PCR. miRNA expression was normalized to the average of miR 191-5p, miR 103a-3p and RNU5G RNA, and expressed as FI (fold induction) *vs*. Wt BDL defined as 1. Data are means ± SE (n = 3–4 mice per group). *$P < 0.05$ *vs*. Wt BDL. (D) Representative fluorescent immunohistochemistry (IHC) for α-SMA and COL1A1 staining (left panel) from Wt and hCOX-2-Tg mice after BDL. Relative values of α-SMA and COL1A1 IHC intensity (%) are at right. *$P < 0.05$ *vs*. Wt Sham. #$P < 0.05$ *vs*. Wt BDL.

of these miRNAs in the regulation of collagen during hepatofibrogenesis (Fig. 7B). Next, we measured ERK1, BMP2 and CDH1 protein levels, target genes implicated in apoptosis, TGF-β signaling and focal adhesion pathways identified in Fig. 4, and as expected these proteins were increased in HSC cells stimulated with PGE_2 after TGF-β1 compared with TGF-β1. Transfection with miR-23a and miR-28a reversed this effect (Fig. 7B). In cells transfected with miR-23a and/or miR-28a, we observed a significant increase in cell viability compared with cells transfected with control miRNA and treated with PGE_2 and TGF-β1, as assessed by MTT cell viability assays (Fig. 7C). Moreover, the

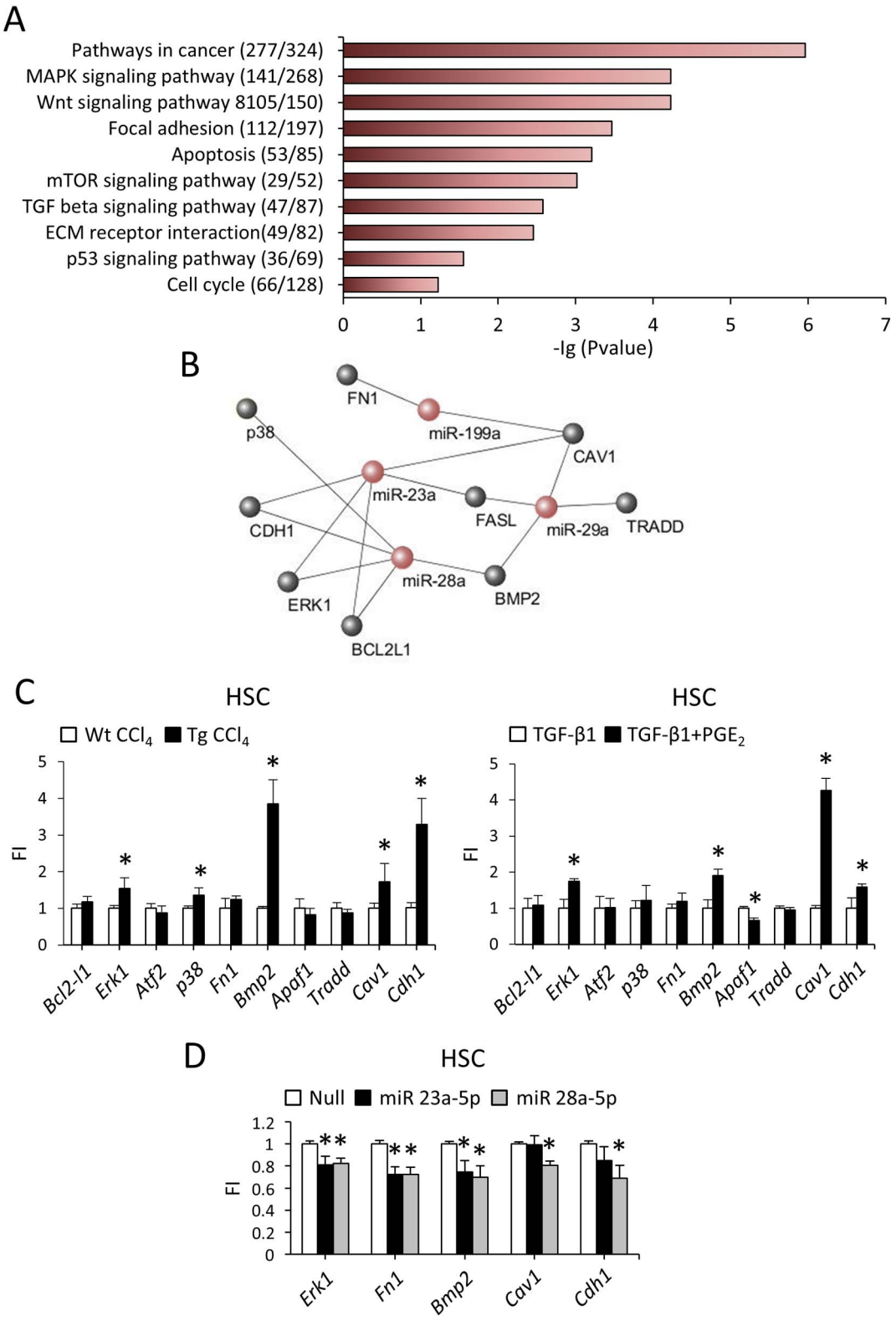

Fig. 4. ***Functional enrichment of miRNA putative gene targets***.

(A) KEGG pathway enrichment. The top most significantly enriched specific terms were plotted against the negative log10 of the *P* value. Number of target genes and total genes for each term are given in parentheses. There is enrichment of terms related to cell death, actin-filament based processes, proliferation and fibrosis. (B). Interactions within the differentially regulated miRNAs and their gene targets were illustrated using Cytoscape. (C) Expression of *Bcl2-l1*, *Erk1*, *Atf2*, *p38*, *Fn1*, *Bmp2*, *Apaf1*, *Tradd*, *Cav1* and *Cdh1* RNA in isolated HSC from Wt and hCOX-2-Tg mice treated with CCl_4 (left), and in primary HSC treated with 5 μM PGE_2 and 2 ng/ml TGF-β1 (right), analyzed by RT-PCR. Values are FI *vs*. Wt CCl4 or TGF-β1 defined as 1. $^*P < 0.05$ *vs*. Wt CCl4 (left) or *vs*. TGF-β1 treated cells (right). (D) Expression of *Erk1*, *Fn1*, *Bmp2*, *Cav1* and *Cdh1* RNA in transfected HSC cells, analyzed by RT-PCR. Primary HSC were transfected with pEGP-miR Null, miR 23a-5p or miR 28a-5p for 48 h, using Lipofectamine 2000. Values are normalized to 36b4 RNA and expressed as FI *vs*. Null. Data are means ± SE of 3–5/14–18 mice per group (C left) or ± SE of three independent experiments (C right, D). $^*P < 0.05$ *vs*. Null.

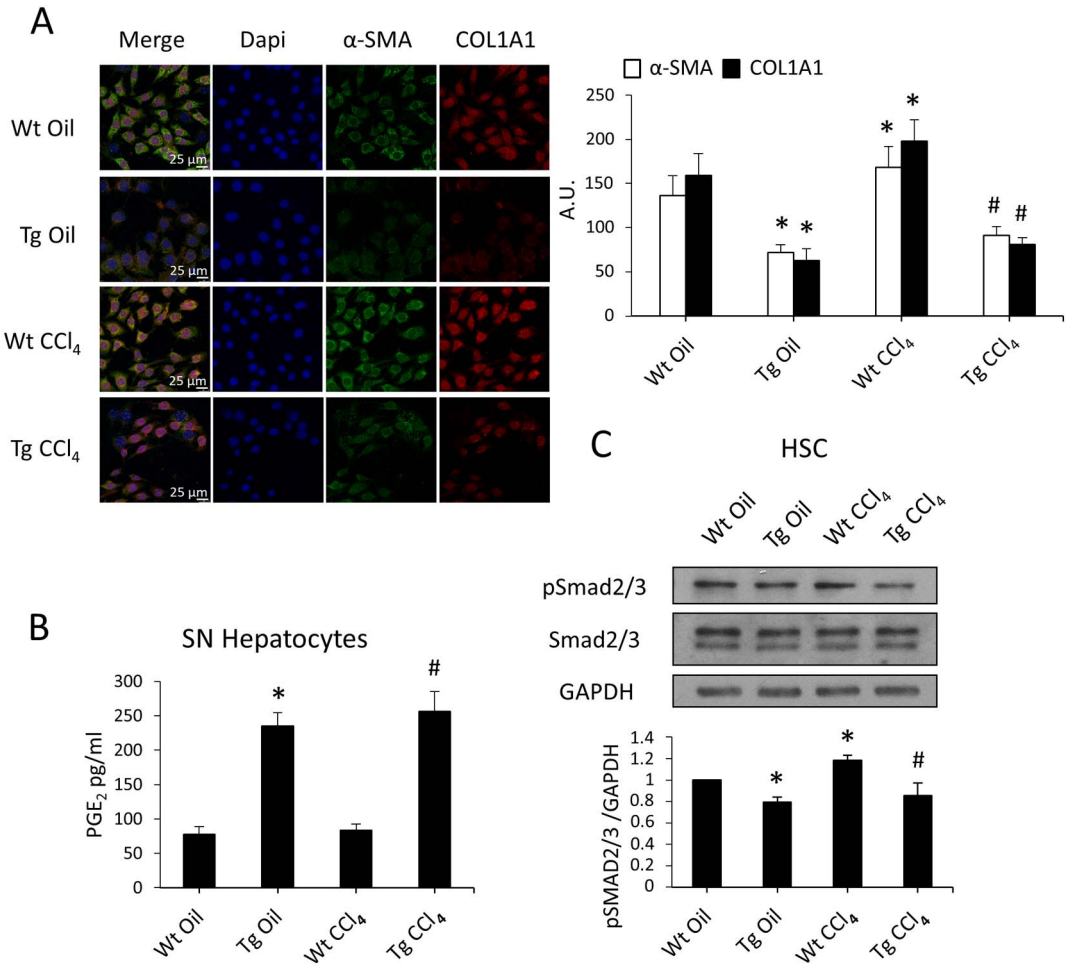

Fig. 5. ***Conditioned medium from hCOX-2-Tg mice isolated hepatocytes protects against HSC activation.***
Primary HSC were treated with conditioned medium from Wt and hCOX-2-Tg mice isolated hepatocytes after oil or CCl_4 treatment. (A) Representative images of α-SMA and COL1A1 immunofluorescence. Cells were analyzed by confocal microscopy and the fluorescence intensity was digitalized and measured. Values of the average fluorescence intensity are expressed up right, in arbitrary units (A.U.). (B) Measured levels of PGE_2 in conditioned medium from Wt and hCOX-2-Tg mice isolated hepatocytes after 24 h of culture. (C) Representative Western blot (up) showing expression of Smad 2/3 protein levels. For densitometric analysis, the relative level of Wt oil expression was defined as 1, and GAPDH served as a loading control. Data are expressed as band ratio. Data are represented as means ± SE of three independent experiments. *$P < 0.05$ *vs.* Wt oil. #$P < 0.05$ *vs.* Wt CCl_4.

overexpression of these miRNAs reduced the apoptosis, as measured by cleaved caspase-3 and Bcl-xL protein levels, opposite to the effect of PGE_2 (Fig. 7D).

3.8. Serum levels of miR-23a and miR-28a are differentially regulated in patients with liver fibrosis

We analyze miRNAs expression in serum of 25 non-diabetic patients with a clinical diagnosis of fibrosis (grade I-III), who underwent a liver biopsy for diagnostic purposes, and compared them to the sera of 13 healthy controls (normal liver, NL). There was a reduction in the expression of miR-28a with the progression of hepatic fibrosis in relation to the increase levels of PGE_2 (Supplementary Fig. 4A–B) and a correlation between serum PGE_2 and miR 28-5p expression (Supplementary Fig. 4D). Analysis of miRNAs expression in available samples of liver tissue from these patients shows that miR-28a expression does not vary with fibrosis progression, whereas miR-23a expression tends to decrease (Supplementary Fig. 4C), suggesting there might be a similar regulation and function of these miRNAs in human fibrogenesis and in murine experimental fibrotic process.

4. Discussion

The current report proposes a novel miRNA-dependent mechanism by which COX-2 modulates liver fibrosis. Hepatocyte COX-2 expression represses miR-23a-5p and miR-28a-5p expression in HSC and exerts a protective effect in the process of liver fibrosis through the activation of apoptosis and the decrease in pro-fibrogenic markers.

Liver injury leading to fibrosis occurs in response to a variety of insults including alcohol, viral hepatitis, steatosis and insulin resistance. Fibrosis is the consequence of an overactive wound healing process in response to the injury. Initially, this process is driven by an inflammatory response and results in a controlled deposition of extracellular matrix; however, if the underlying insult persists, there is an excessive deposition of extracellular matrix including cross-linking of collagen. The critical step in the development of scar is activation of hepatic stellate cells (HSCs), which become the primary source of extracellular matrix [29]. Our previous data indicates that constitutive hepatocyte COX-2 expression ameliorates liver fibrosis development in mice by reducing inflammation, and by modulating activation of hepatic stellate cells [24].

miRNAs play essential roles in virtually all cellular and biological processes including liver development, differentiation and homeostasis. Altered expression levels of miRNAs were observed in patients with liver diseases *e.g.* liver steatosis, cirrhosis and hepatocellular carcinoma [10,11,30]. Expression pattern of miRNAs are organ- and cell-specific. As we have observed, miRNAs are not homogeneously distributed in the liver, but are selectively expressed and regulated in distinct hepatic cell

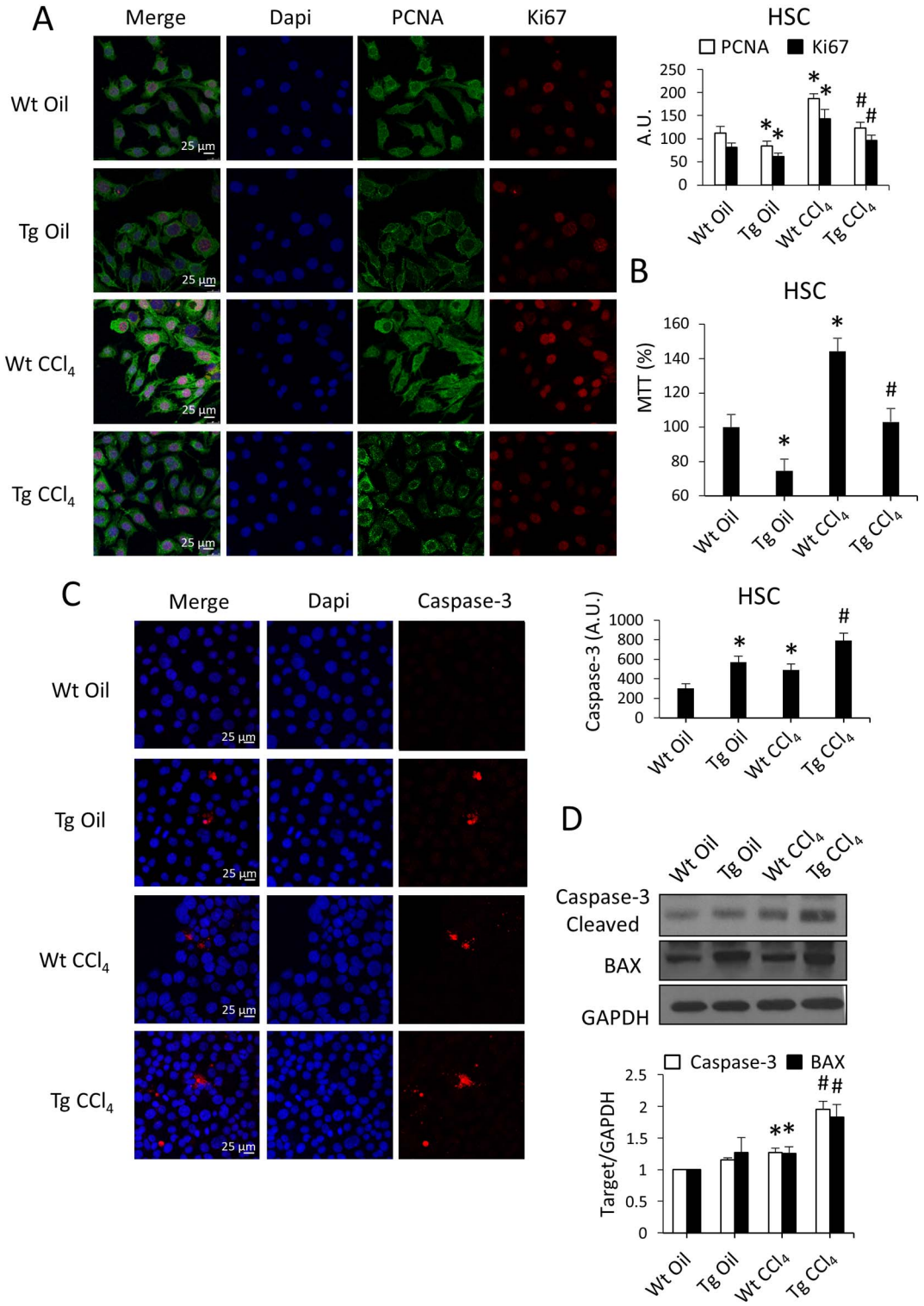

Fig. 6. ***Conditioned medium from hCOX-2-Tg mice isolated hepatocytes induces apoptosis and decreases the proliferation of HSC.***
Primary HSC were treated with conditioned medium from Wt and hCOX-2-Tg mice isolated hepatocytes after oil or CCl_4 treatment. (A) Representative images of PCNA and Ki67 immunofluorescence. Cells were analyzed by confocal microscopy and the fluorescence intensity was digitalized and measured. Values of the average fluorescence intensity are expressed in arbitrary units (A.U.). (B) MTT experiment represented in percentage (Wt oil is 100). (C) Representative images of caspase-3 immunofluorescence. Values of the average fluorescence intensity are expressed in arbitrary units (A.U.). (D) Representative Western blot showing expression of cleaved caspase-3 and BAX protein levels. For densitometric analysis, the relative level of Wt oil expression was defined as 1, and GAPDH served as a loading control. Data are expressed as band ratio. Data are means ± SE of three independent experiments. $^{*}P < 0.05$ *vs.* Wt oil. $\#P < 0.05$ *vs.* Wt CCl_4.

types. Our results show opposite regulation of miR-23a and miR-28a in HSCs compared to hepatocytes, which is in agreement with previous reports indicating that miR-29 was found to be down-regulated in HSCs during hepatofibrogenesis, while its expression was up-regulated in hepatocytes and cholangiocytes during experimental biliary atresia, reinforcing the concept of cell-specific functions of miRNAs in liver pathophysiology [31]. In addition, our results showing an antifibrotic role of miR-29a are widely supported by the literature. Wang. et al.,

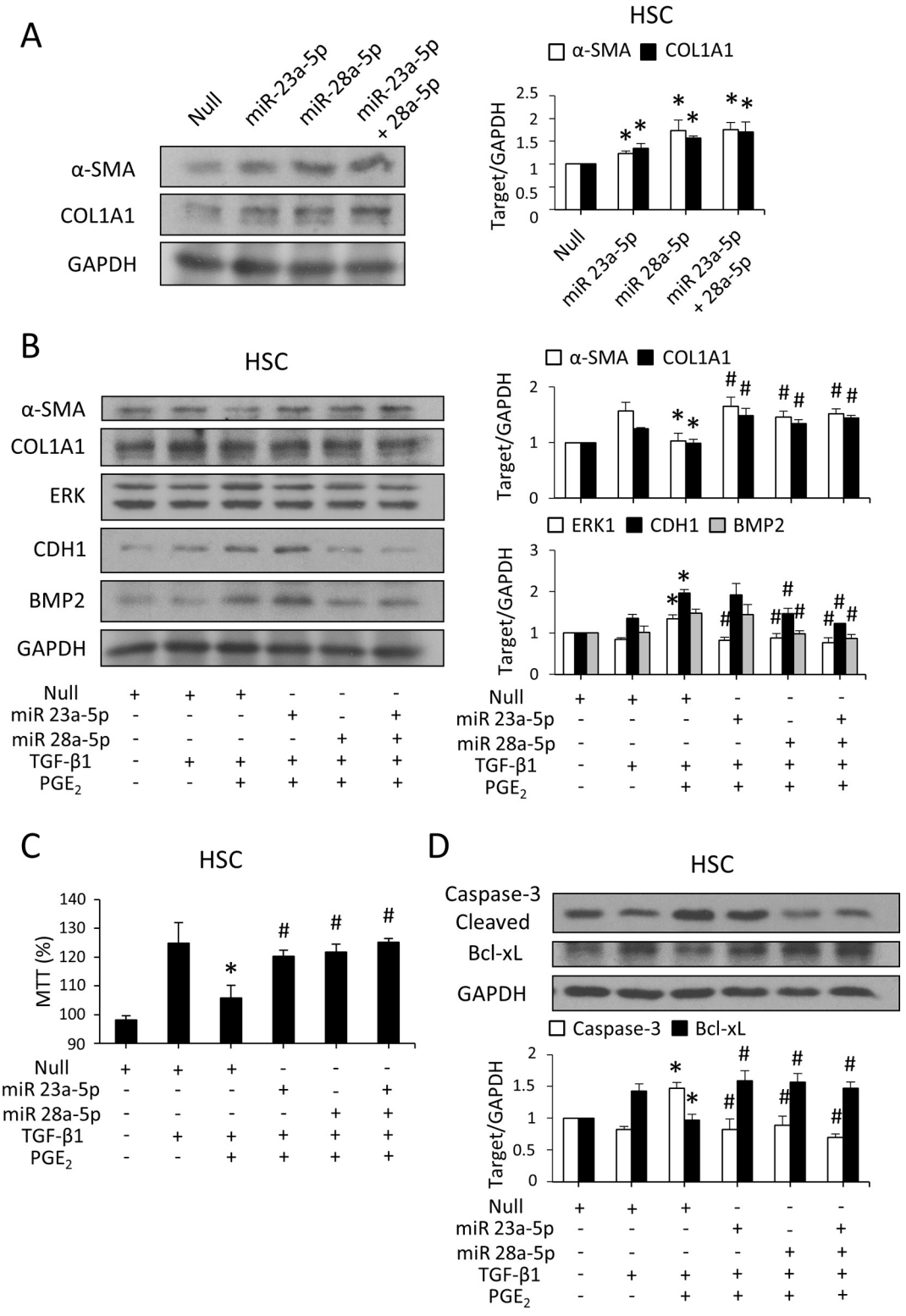

Fig. 7. ***miR 23a-5p and miR 28a-5p induce fibrosis and decrease apoptosis in transfected HSC.***
Primary HSC were transfected with pEGP-miR Null, miR 23a-5p or miR 28a-5p for 48 h, using Lipofectamine 2000. (A) Representative Western blot of α-SMA and COL1A1. *$P < 0.05$ *vs.* Null. (B, C, D) Cells were treated with 5 μM PGE_2 and 2 ng/ml TGF-β1, in addition to transfection. (B) Representative Western blot of α-SMA, COL1A1, ERK, CDH1 and BMP2. (C) MTT experiment relative to Null (100). (D) Representative Western blot of caspase 3 cleaved and Bcl-xL. For densitometric analysis, the relative level of Null was defined as 1, and GAPDH served as a loading control. Data are expressed as band ratio. Data are means ± SE of three independent experiments. *$P < 0.05$ *vs.* TGF-β1 + Null; # $P < 0.05$ *vs.* TGF-β1 + PGE_2 + Null.

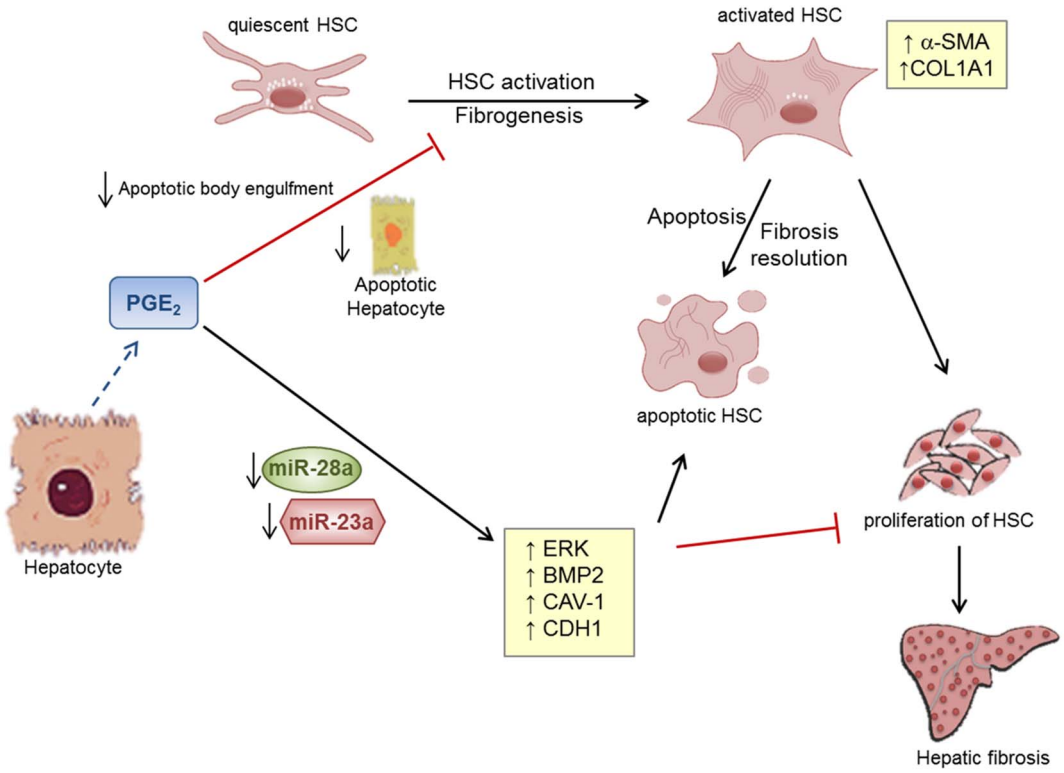

Fig. 8. ***Synthesis of PGE_2 in hepatocytes protects against fibrosis.***
In the presence of PGE_2, the apoptosis of the hepatocytes decreases and, therefore, the apoptotic body engulfment by the HSCs, thus reducing its activation. Moreover, PGE_2 downregulates miR-23a and miR-28a expression in HSCs, resulting in an increase of ERK, BMP2, CAV1 and CDH1 levels. This leads to an increment in apoptosis together with an inhibition of HSC proliferation. As a consequence, a decrease of α-SMA and COL1A1 is shown in HSC.

demonstrated that miR-29 attenuated HSC activation and induced their apoptosis *via* inhibition of PI3K/AKT pathway in CCl_4-induced fibrosis mouse [32]. Moreover, systemic injection of miR-29a expressing adeno-associated virus in mice demonstrated reversal of histologic and biochemical evidence of hepatic fibrosis despite continued exposure to CCl_4 [33].

The role of COX-2 dependent PGs in liver fibrosis is controversial. Some studies indicate that PGs favor the development of hepatic steatosis, NASH and ultimately fibrosis [18–20], while others provide evidence that PGE_2 suppresses fibrogenesis [9,21,22]. The discrepancy on the effect of COX-2 function can partly be attributed to the application of different experimental models. COX-2 inhibitors could also influence gene expression *via* COX-2-independent pathways. To overcome the limitations of pharmacological inhibition of COX-2, we used a Tg mice model with enhanced hepatocyte-specific hCOX-2 expression. We found that in Tg mice, the severity of hepatic fibrosis developed after CCl_4 was lesser than in Wt mice, as demonstrated by reduced degree of fibrosis. Moreover, our result indicate that PGE_2 inhibits HSC activation induced by TGF-β. It is known [34] that PGE_2 abrogated the stimulatory effects of TGF-β on collagen and fibronectin production in lung fibroblasts. The stimulation of endogenous PGE_2 production by TGF-β may serve as negative feedback mechanism to limit the increase in extracellular matrix protein production induced by this cytokine. We validate the upregulation of pro-fibrogenic genes upon TGF-β1 treatment and revealed that PGE_2 counteracted TGF-β1-induction of α-SMA and COL1A1 in isolated HSC. Our results also show that miR-23a and miR-28a are downstream effectors participating in COX-2 function during fibrogenesis and that inhibition of TβR-I significantly prevented the PGE_2 dependent downregulation of miR-23a and miR-28a.

We analyzed the enriched signaling pathways among the predicted targets of the miRNAs regulated by COX-2 in HSC cells and focused in apoptosis and TGF-β signaling, based on our previous results indicating that hepatocyte COX-2 expression ameliorates NASH and liver fibrosis development in mice by reducing inflammation, oxidative stress and apoptosis and by modulating activation of HSC [24]. Some of these miRNA target genes were validated by RT-PCR and *Erk1*, *Bmp2*, *Cav-1* and *Cdh1* exhibited increased expression in isolated HSC from COX-2-Tg liver treated with CCl_4 as well as in HSC incubated with TGF-β and PGE_2. All these genes have antifibrotic and pro-apoptotic functions [35] and their protein levels were increased in HSC cells stimulated with PGE_2, after TGF-β1treatment. Moreover, transfection with miR-23a and miR-28a reversed this effect and reduced the apoptosis of HSC cells, opposite to the effect of PGE_2. These results demonstrate that PGE_2 downregulates miR-23a and miR-28a expression in HSCs, resulting in an increase of ERK, BMP2, CAV1 and CDH1 levels. This leads to an increment in apoptosis together with an inhibition of HSC proliferation. As a consequence, a decrease of α-SMA and COL1A1 is shown in HSC.

In line with our data, it has been described that COL1A2 is downregulated in fibroblast-derived extracellular matrix *via* Ras-dependent activation of the MEK/ERK signaling pathway and that Sp1 is an important mediator of this feedback inhibition [36]. Bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, have anti-fibrotic functions in multiple organs. BMP2 opposes the fibrogenic function of TGF-β in pancreatic stellate cells through the Smad1 signaling pathway, inhibiting TGF-β-induced α-SMA and COL1A1 [37] and inducing apoptosis in mouse hybridoma MH60 cells through the activation of TGFβ-activated kinase (TAK1) and subsequent phosphorylation of p38 stress-activated protein kinase [38]. Regarding caveolin-1 (CAV-1), our results agree with *in vitro* data for the fibromir miR-199a-5p, which is regulated by TGF-β signaling in response to lung injury. Specifically, miR-199a targets CAV-1, a potent negative regulator of TGF-β signaling cascade [39]. By other hand, CAV-1 overexpression resulted in inhibition of α-SMA expression and cell proliferation by blocking the progression through the G1 phase in murine fibroblast [40]. Finally, it has been shown that an increase of E-cadherin expression promotes HSC apoptosis [41] and correlates with the inhibition of LX2 and HSC-T6

cell proliferation after icaritin treatment, a known apoptosis inducer of HSC [42].

Aberrant apoptosis is a feature of chronic liver diseases and is associated with worsening stages of fibrosis. After toxic exposure, hepatocytes undergo apoptosis and hepatic stellate cells migrate to the site of injury to engulf the apoptotic bodies. This engulfment promotes activation of the hepatic stellate cells to hepatic myofibroblasts, and in their activated state these cells promote deposition of extracellular matrix and scar formation in the liver. However, apoptosis is also the main mechanism promoting the resolution of fibrosis [29].

When liver fibrosis resolves, the activated HSCs are either returning to a quiescent state or undergoing apoptosis, which causes a decrease in the number of activated HSCs (Fig. 8).

4.1. Conclusions

Based on our results, we propose that hepatocyte COX-2 expression attenuates liver fibrosis in part through the production of PGE_2 which exerts its hepatoprotective role in at least two different ways: by decreasing hepatocyte apoptosis [24], thus reducing HSC activation; and by downregulation of miR-23a and miR-28a in HSC, decreasing proliferation and increasing its apoptosis, leading to fibrosis resolution (Fig. 8).

Moreover, in this study we detected significantly reduced serum levels of miR-28a in patients with liver fibrosis, and high serum PGE_2 levels were associated with the progression to more advanced fibrosis stages. We hypothesize this fact as a response in order to exert a protective role against liver fibrosis and contribute to its resolution.

Pharmacological strategies to prevent or treat liver fibrosis are still limited. The prospect of treating patients with liver disease with a pharmacological agent to cause the regression of fibrosis is exciting, but further investigation is required into the long-term-efficacy. The link between HSC specific downregulation of miR-23a and miR-28a by PGE_2 and their relation to ECM and apoptotic genes, as well as the prevention of HSCs activation exerted by PGE_2 highlight a potential role for these miRNAs in HSC and may provide further pharmacological targets.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2017.11.001.

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Transparency document

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