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# Integration of miRNA-regulatory networks in hepatic stellate cells identifies TIMP3 as a key factor in chronic liver disease

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## Abstract

**Background & Aims:** Activation of hepatic stellate cells (HSC) is a critical process involved in liver fibrosis. Several miRNAs are implicated in gene regulation during this process but their exact and respective contributions is still incompletely understood. Here we propose an integrative approach of miRNA-regulatory networks to predict new targets.

**Methods**: miRNA regulatory networks in activated HSCs were build using lists of validated miRNAs and the CyTargetLinker tool. The resulting graphs were filtered according to public transcriptomic data and the reduced graphs were analyzed through GO annotation. A miRNA network regulating the expression of TIMP3 was further studied in human liver samples, isolated hepatic cells and mouse model of liver fibrosis.

**Results:** Within the up-regulated miRNAs, we identified a subnetwork of five miRNAs (miR-21-5p, miR-222-3p, miR-221-3p miR-181b-5p and miR-17-5p) that target TIMP3. We demonstrated that TIMP3 expression is inversely associated with inflammatory activity and IL1-ß expression *in vivo*. We further showed that IL1-ß inhibits TIMP3 expression in HSC-derived LX-2 cells. Using data from The Cancer Genome Atlas (TCGA), we showed that, in hepatocellular carcinoma (HCC), TIMP3 expression is associated to survival ($p<0.001$), while miR-221 ($p<0.05$), miR-222 ($p<0.01$) and miR-181b($p<0.01$) are markers for a poor prognosis.

**Conclusions**: Several miRNAs targeting TIMP3 are upregulated in activated HSCs and down-regulation of TIMP3 expression is associated with inflammatory activity in liver fibrosis and poor prognosis in HCC. The regulatory network including specific miRNAs and TIMP3 is therefore central for the evolution of chronic liver disease.

**Key words**: hepatic stellate cells, liver fibrosis, miRNA networks, TIMP3

**Lay summary**

Hepatic stellate cells (HSC) play a critical role in the progression of chronic liver diseases. Our study identified TIMP3 as a gene downregulated in activated HSCs and liver fibrosis, and further showed that TIMP3 is a marker of good prognosis in hepatocellular carcinoma. Measurement of TIMP3 expression could help diagnose the progression of liver disease.

## Introduction

Hepatic fibrosis is a dynamic and reversible process of scarring, which leads to excessive deposit of extracellular matrix (ECM) during most chronic liver diseases (CLDs) whatever the etiology including viral hepatitis, alcoholic liver cirrhosis and non-alcoholic fatty liver disease (NAFLD). Despite recent advances in antifibrotic therapies,[1] CLDs account for 2 million deaths per year.[2] Understanding the complexity of the underlying molecular mechanisms remains an open challenge for developing new efficient therapies.

Hepatic stellate cells (HSCs) store vitamin A in normal liver but they are also critical players in liver fibrosis. Following injury, they undergo an activation process leading to myofibroblast-like phenotype characterized by proliferation, contractility, chemotaxis and fibrogenic properties.[3] The molecular mechanisms that regulate HSC activation are driven by changes in microenvironment involving hepatocytes, inflammatory cells and the extracellular matrix (ECM). Such environment generates extracellular stimuli including growth factors and cytokines, cell-cell and cell-matrix interactions that modulate transcriptional gene expression. Epigenetic and post-transcriptional events are also critically involved and the role of microRNAs is now considered central in the regulation of liver fibrosis and HSC activation.[4]

MicroRNA (miRNAs) are small non coding RNAs interacting with the 3'-untranslated region of mRNAs to promote their degradation and/or repress their translation. 40 miRNAs have been associated with fibrosis in several tissues, including liver, kidney, heart and lung, but more specific tissue signatures were also identified.[5] In the liver, miRNAs and their potential use as biomarkers or therapeutic tools or targets have been widely documented.[6] Regulatory roles of miRNAs have been described in NAFLD,[7] viral hepatitis,[8,9] alcoholic liver diseases,[10] cholestatic liver diseases[11] and hepatocellular carcinomas.[12] Pathways leading to fibrosis in CLDs are tightly regulated by miRNA–dependent mechanisms.[13] However only 49 genes were reported as targets of down and up-regulated miRNAs in activated HSCs when compared to quiescent cells.[4] The present study aimed to develop a whole-genome integrative approach based on miRNAs previously associated with HSC activation in order to identify new targets associated with liver fibrosis.

## Methods

**Human Samples**

Matching non-tumor liver samples were obtained from patients undergoing surgical hepatectomy for hepatocellular carcinoma (HCC). The patients were 7 women (mean age 61.8±8.6 years) and 57 men (mean age 61.6±8.7 years). Etiology included chronic alcoholism, hepatitis B and C (supplementary table S1). Histological stages of fibrosis were graded according to the METAVIR score: F1, portal fibrosis without septa; F2, portal fibrosis with rare septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Necroinflammation activity (A) was graded as A0 (absent), A1 (mild), A2 (moderate) or A3 (severe). Liver samples were from the Biological Resources Center (BRC) at Rennes University Hospital. Access to this material was in agreement with French regulations and satisfied the requirements of the local Ethics Committee.

**Cell culture**

Human hepatocytes and HSCs were isolated from histologically normal specimens of partial hepatectomies from patients undergoing hepatic resection for liver metastases as previously described.[14] HSCs, LX-2 cell line and human Huh7D12 (Huh7) and HepG2 hepatoblastoma cell lines were maintained in Dulbecco's minimal essential medium supplemented with 25 mM D-Glucose, 4 mM L-Glutamine and 10% fetal bovine serum (FBS). LX-2 cells were serum starved for 24H before treatment with IL1-β (3ng/ml) or TGF-β (5 ng/ml) for 24H in serum free medium.

**Mouse model of liver fibrosis**

Seven-week-old female C57Bl/6 mice were treated by intraperitoneal injection of CCl4 (Sigma-Aldrich, St. Louis, MO, USA) diluted at 3% v/v in olive oil. Control mice were treated with the vehicle only (olive oil). For acute treatment, a single dose of 0.3 ml/kg of mouse weight was administered to mice. After 4h, 12h, 24h or 7 days, mice were sacrificed. Livers were collected, weighed and treated as previously described.[15] Experiments performed on animals were approved by the local ethics committee (file number 17-1997) in accredited facilities (LA1610002) from the University of Liege.

**In situ hybridization**

Formalin fixed, paraffin embedded (FFPE) tissue sections were deparaffinized with xylene (2x5min), rehydrated with decreasing concentrations of ethanol (100-60-30% for 2 minutes each), washed with distilled water and then dried for 10min at room temperature. Localization of mRNA expression in tissue samples was performed using the RNAscope® in situ hybridization (ISH) assay according to the manufacturer's instructions (Advanced Cell Diagnostics Inc., Hayward, Ca, USA). We used RNAscope® mouse probes predesigned by the manufacturer for detection of Mm-*Timp3* in the second channel (probe mm-*Timp3*-C2, Cat No. 471311-C2), and of Mm-*Acta2* in the first channel (probe Mm-*Acta2*-C1, Cat No. 319531). C2 and C1 signals were detected in red (FastRed) and green-blue respectively, using the RNAscope® 2.5 HD Duplex detection kit. Slides were counterstained with 25% hematoxylin and mounted with VectaMount™ Mounting Medium (Vector Laboratories, H-5000).

**RNA isolation and gene expression analysis by quantitative real-time PCR**

Total RNA and miRNA were extracted either from cells or tissues using QIAzol Lysis Reagent (Cat No. 56302331) and miRNeasy Mini kit (Cat No. 217004) from Qiagen according to the manufacturer's instructions. Reverse transcription was performed from 0.5 µg total RNA using Superscript II (Invitrogen). Real-time quantitative PCR was performed using the qPCR™ Core Kit for SYBR Green I® (EUROGENTEC™) and the ABI Prism 7700 real-time thermo-cycler (Perkin-Elmer, Foster city, CA, USA). Primer pairs for target genes are described in supplementary table S2. For miRNA 200 ng RNAs were reversed-transcribed using the miRCURY LNA miRNA PCR System (Qiagen). The primers were hsa-miR-21-5p (YP0020430), hsa-miR-222-3p (YP0020455), hsa-miR-221-3p (YP00204532), hsa-miR-181b-5p (YP00204530), hsa-miR-17-5p (YP02119304). and hsa-miR-29b-3p (YP00204679) (Qiagen).

**Proteomic analyses of ECM from human fibrotic tissue samples**

Tissue samples from 18 adjacent non-tumor liver tissues were used for specific extracellular matrix (ECM) extraction. Tissue preparation and ECM protein enrichment were performed according to Dr Naba's protocol.[16] Briefly, tissue samples were mechanically homogenized and extracted sequentially using the CNMCS compartmental protein extraction kit (Millipore,Billerica, MA). Cytosolic, nuclear, membrane and cytoskeletal proteins were discarded and the final insoluble fraction enriched for ECM proteins was treated for spectrometry analyses. The ECM-

enriched samples were solubilized in urea, digested with PNGaseF, Lys-C, and trypsin. The resulting peptides were analyzed by LC-MS/MS on Orbitrap Fusion (Thermo Fisher Scientific, San Jose, CA). Mass spectra were interpreted with MaxQuant/Andromeda and MS/MS spectra were searched against the UniprotKB Homo sapiens (CP_Homo-Sapiens) database with fixed modification: carbamidomethyl(C), variable modifications: oxidation (M), oxidation (K), oxidation (P). Peptide identifications were filtered at 1% false discovery rate. Protein digestion and mass spectrometry analyses were performed by the Proteomics Platform of the CHU de Québec Research Center (Quebec, Qc, Canada).

### Bioinformatic Tools

CyTargetLinker[17] is a cytoscape plug-in for integrating regulatory interactions in network analysis. We used the Regulatory Interaction Networks (RegINs) provided on the CyTargetLinker website. RegINs describes networks containing miRNA regulatory interactions that are derived from online interaction databases. In the present study we selected miRTarBase (7.0) that contains MiRNA-Target Interactions (MTIs) experimentally validated. MTIs are viewed as having strong support when they are validated by western blot, qPCR, or reporter assays. Gene annotations were made using Gene Ontology (GO) or DisGeNet, a discovery platform that contains a comprehensive catalogue of genes and variants associated to human diseases.

### Statistical analyses

Experimental data are presented as box-and-whisker plots showing distribution of values from at least 3 independent experiments. Significant differences between groups were compared by Mann-Whitney U (two groups) test or Kruskal-Wallis (more than two groups) test or Wilcoxon rank-sum test, when samples are apparied. Statistical calculations were performed with the R software, version 3.1.1. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ were considered significant.

## Results

### Building miRNA regulatory networks in activated hepatic stellate cells

Based on the list of miRNA differentially expressed in activated HSCs (supplementary table S3), we searched for all target genes of up- and down-regulated miRNA. Using CyTargetLinker tool, 8243 target genes were identified including 1215 targeted by only up-regulated miRNA, 4862 targeted by only down-regulated miRNAs and 2166 targeted by both up-regulated and down-regulated miRNA. In order to reduce the complexity of the graph, we selected miRNA-target interactions (MTI) that were previously experimentally validated according to the criteria from miRTarBase. A miRNA-target interaction is qualified as *Strong* if experimental evidences including qPCR, western-blot and/or Luciferase reporter assay support MTI. The reduced graph contained 825 target genes including 429 targets of only down-regulated miRNAs and 281 targets of only up-regulated miRNAs (Figure 1). The lists of genes are provided in supplementary table S4 and comparisons between lists are shown as Venn diagrams in supplementary figure S1. Among the resulting 825 genes, only 81 genes were annotated as *liver fibrosis* in the DisGeNET database (http://www.disgenet.org/).

Among the 49 target genes experimentally validated in activated HSCs[4] (supplementary table S3), 37 were also found by CyTargetLinker using strong criteria, suggesting a good accuracy of the integrative approach (Figure 2). Among these genes, 24 and 11 are targets of down- or up-regulated miRNAs, respectively, while 2 are targets of both up- and down-regulated miRNAs. In accordance with the dogma that activated HSCs are responsible for matrix remodeling, down-regulated miRNAs led to up-regulation of matrix components (FN1, COL1A1, COL4A1, TNC) and inhibitor of matrix metalloprotease (TIMP2) while up-regulated miRNAs led to down regulation of matrix metalloproteinase (MMP2, MMP9). Similarly, decrease in SMAD7 and increase in both SMAD4 and TGFBR1 expression supported evidences for induced TGF-β signaling and activity in activated HSCs.

In order to validate new target genes predicted by CyTargetLinker, we used transcriptomic data from genes differentially expressed in quiescent and culture-activated HSCs.[18] For that purpose, we first selected genes showing two-fold changes (>2 and <0.5, for up- and down-regulated genes, respectively) and extracted miRNA-target interactions that matched between prediction and experiment. As shown in Figure 3A and 3B, 34 up-regulated genes and 17 down-regulated genes matched with genes predicted as targets of, respectively, down-regulated and up-regulated miRNAs in activated HSCs. We next focused on these reduced graphs for more in-depth analyzes.

**Down-regulated miRNA networks correlate with extracellular matrix gene overexpression and cell proliferation**

22 of the 34 identified up-regulated genes were associated with two major biological processes: *extracellular structure organization* (GO:0043062) and *mitotic cell cycle process* (GO:1903047) (supplementary table S5). In accordance with the role of miR29 family members as major regulators of fibrosis,[13] mir29 down-regulation was associated with up-regulation of extracellular matrix genes including SPARC, COL5A1, COL3A1, COL1A1, LOX, LOXL2, TGFB2, ITGB1, DAG1 (Figure 3A). Similarly, IGF1 and LPL which are upregulated during HSC activation were recently identified as targets of mir29.[19,20] Two other mir29 target genes, RNASEL and GPR85 that have not been documented in liver fibrosis to date were also identified. RNASEL is an endoribonuclease implicated in the antiviral response and GPR85 is a member of the G protein-coupled receptor (GPCR) family mainly associated with central nervous system disease.

Beside the miR29-dependent regulatory networks, 8 miRNAs were associated with cell cycle genes (CCNE1, CDK6, CDKN3, CCNA2, CCNE1) and microtubule-regulatory genes (STMN1, KIF22 and AURKB) illustrating regulation of cell proliferation. In agreement with this, miR146a-5p that targets KIF22, CDKN3 and CCNA2 inhibits HSC proliferation.[21] Similarly miR155-5p targeting RAD51 attenuates HSC activation.[22] Interestingly, the reduced graph enlightened the critical role of BIRC5 (alias survivin) which is controlled by four miRNAs (miR165-5p, miR-195-5p, miR-150-5p, miR-355-5p). While the increased expression of BIRC5 was widely documented in chronic liver diseases and HCC,[23] its regulation in HSCs remained poorly understood. Our study indicates that its increased expression is partly secondary to modifications affecting the abundance of a small number of miRNA.

**Identification of a miRNA subnetwork targeting TIMP3**

The up-regulated miRNA network was reduced to 9 miRNAs targeting 17 downregulated genes in activated HSCs (Figure 3B, supplementary table S5). Because TGFB1 signal is the driving force of liver fibrosis, it was not surprising to identify down regulation of antagonists of TGFB1 signal including the E3 ubiquitin ligase SMURF1 (responsible for TβRII ubiquitination and degradation in HSC[24]) and BMP-2 (antagonizing TGF-β1/ROCK-dependent fibrotic signaling[25] and osteoblast differentiation[26]). Similarly, TGFBR3 (alias betaglycan) a non-signaling receptor for TGF-β was downregulated during activation of HSCs.[27]

The main intriguing observation was the targeting of the Tissue Inhibitor of Metalloproteinases (TIMP3) by five up-regulated miRNAs including miR-21-5p, miR-222-3p, miR-221-3p, miR-181b-5p and miR-17-5p, thereby suggesting a strong negative regulation of TIMP3 in activated HSCs. To our knowledge, the regulation of TIMP3 by miRNAs was not documented in liver fibrosis and HSC to date. Based on literature meta-analysis we observed that miR-17-5p, miR-21-5p, miR-181b-5p and the homologous miR-221/222-3p have already been implicated in the regulation of TIMP3 in other pathologies including cancer (Table 1).

**TIMP3 expression is down-regulated in liver fibrosis**

In order to explore the regulation of TIMP3 in liver fibrosis, we first measured mRNA levels in adjacent non tumor liver tissues from the whole cohort of 64 patients with HCC. These samples encompass different stages of fibrosis (5 F1, 4 F2, 7 F3, 48 F4) and inflammatory activities (28 A0, 17 A1, 16 A2, 3 A3). We observed a bell-shaped distribution of TIMP3 mRNA levels among fibrosis stages (Figure 4A). Remarkably, TIMP3 mRNA levels were significantly lower in liver fibrosis with highest inflammatory activities (Figure 4B). Because TIMP3 is a secreted protein that binds to extracellular matrix,[28] we used the original approach developed by Naba *et al*[16] to analyze its accumulation in ECM-enriched fraction of liver tissues from selected patients with fibrosis at different stages (5 F1, 3 F2, 4 F3 and 6 F4). As shown in Figure 4C, we observed a decrease (although not reaching statistical signification) in TIMP3 protein amounts associated with the highest fibrosis stages ($p= 0.08225$ between F1 and F4).

To refine our understanding of the regulation of TIMP3 in liver fibrosis, we have investigated its expression in human hepatic cells in culture. TIMP3 was expressed in both *in vitro* activated human HSCs and the LX-2 cell line stimulated by TGF-β while cultured human hepatocytes and hepatocellular carcinoma cell lines (HepG2 and HuH7) expressed lower levels of TIMP3 (Figure 5A). These observations could appear as counterintuitive since we showed that miRNA network up-regulated in activated HSC is associated with TIMP3 down-regulation (Figure 3B). However, HSCs activation is a complex phenomenon encompassing several phases and depending on the microenvironment.[3] In agreement with the phenotypic heterogeneity of activated HSCs, cultured-HSCs transdifferentiate into fully mature myofibroblasts while the expression of profibrotic genes is still responsive to TGF-β in LX-2 cells. More recently, using single cell RNA sequencing approach, Krenkel et al[29] described four sub-populations of activated myofibroblasts

(MFB) in CCl4-fibrotic livers (chronic treatment for three weeks) and further demonstrated that *in vitro* activation of HSC induced heterogeneity over time.

Since TIMP3 expression was found to depend on the inflammatory context (Figure 4B), we investigated its expression in LX-2 cells stimulated by IL1-β, a cytokine produced by activated monocytes during inflammatory response. As shown in Figure 5B, IL1-β diminished both TIMP3 and TGF-β mRNA levels, while MMP9 expression, used as positive control, was induced as previously reported[30]. These observations are therefore consistent with LX-2 cells being in a transient state where gene expression can be modulated in response to both inflammatory cytokines and TGF-β. These responses are obviously disconnected from the complex response to liver injury that occurs in the whole tissue.

We therefore used a CCl4-induced acute liver failure model to investigate Timp3 mRNA levels during the first steps of liver response. Animals were sacrificed at 4-, 12-, 24- and 168-hours after CCl4 injection. At 24h, a decrease in Timp3 expression and a strong increase in IL1-β were observed, which perfectly matches with our *in vitro* studies (Figure 6A). To further explore the regulation of TIMP3 expression in liver fibrosis, we analyzed the expression of miR targeting Timp3 in the same samples. We observed that the expression of miR-17 (p=0.008), miR-181b (P=0.003), miR-222 (p=0.016) and miR-221 (P=0.055) was increased at 24h hour post-injection (Figure 6B). These observations suggest that inflammatory response induced by acute liver injury is associated with downregulation of Timp3 gene expression and upregulation of miRNAs targeting Timp3.

Together our results have shown that five miRNAs upregulated in activated HSC are predicted to target TIMP3, suggesting the existence of a robust mechanism that tightly controls TIMP3 expression. Following liver injury, inflammatory response is associated with HSCs activation and reduced TIMP3 expression as demonstrated by the inverse association of TIMP3 expression with inflammatory activity and IL1-ß expression.

**miR-21, miR-221 and miR-222 are markers of poor prognosis in hepatocellular carcinoma**

Because TIMP3 expression was recently associated with a favorable survival in HCC,[31] we hypothesized that miRNAs targeting TIMP3 might be inversely associated with survival. Taking advantages of recent studies that integrate miRNA or mRNA expression data from independent sets of HCC[32,33] we examined the prognostic value of miR-21, miR-222, mi-R221, miR-181b and

miR-17 expression. Using Kaplan-Meier Plotter tool (http://kmplot.com), we selected RNAseq data corresponding to the 379 HCC samples referred as Liver Hepatocellular Carcinoma (LIHC) in The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/). miR-221, miR-222 and miR-21 expressions are inversely associated with overall survival (Figure 7A), while miR-17 and miR-181b did not show significant association (data not shown). At the opposite, high TIMP3 expression in HCC samples from TCGA database is positively associated with survival. In accordance with the correlation between TIMP3 expression and favorable outcomes, we further showed that downregulation of TIMP3 in HCC is associated with tumor grade and gene signatures from HCC subtypes that include NCIP (The National Cancer Institute Proliferation subtype), SNUR (Seoul National University Recurrence subtype) and RS65, (65-gene risk score subtype) (Figure 7B). To further investigate the correlation between TIMP3 expression and the miRNAs targeting TIMP3 in HCC samples, we selected 290 hepatocellular carcinoma (TCGA samples) corresponding to primary HCC tumors with both miRNA expression data[33] and gene expression data (RNA-seq normalized read counts) in the "international cancer genome consortium" database (https://icgc.org). Next, we extracted miRNA expression data for these samples from Nagy's study[33] that integrated several HCC miRNa data sets including TCGA samples and we elaborated a new expression data set to study correlation between TIMP3 and miRs (supplementary table S6). Spearman's correlation analysis showed that the expression of miR-17 ($\rho$= -0.29, p=3.0 $10^{-7}$), miR-21($\rho$= -0.25, p=1.6 $10^{-5}$) and miR-222 ($\rho$= -0.20, p=5.0 $10^{-4}$) was negatively correlated with that of TIMP3. The correlation of TIMP3 expression with miR-181b and miR-221 was negative but not significant ($\rho$= -0.1, p=0.09). Together these observation support evidence for the implication of miR-17, miR21, miR-181b, miR-221 and miR-222 in regulation of TIMP3 expression in chronic liver diseases.

## Discussion

On the basis of miRNA differentially expressed during activation of HSCs, we developed an integrative approach to identify new target genes and regulatory hubs. A major finding from this study was the miRNA subnetwork that targets TIMP3. Unlike other tissue inhibitors of metalloproteases, TIMP3 has been described as a tumor suppressor since decrease in TIMP3 expression is associated with tumor aggressiveness and bad prognosis.[34] Silencing of TIMP3 has been associated with promoter hypermethylation, loss of heterozygosity or miRNA-dependent regulation. In chronic liver disease, methylation status of TIMP3 is controverted since TIMP3 has

been found scarcely or not methylated in tumor, cirrhosis and chronic hepatitis,[35] while other studies reported association of TIMP3 gene methylation with hepatocellular carcinoma.[36] The regulation of TIMP3 expression by miRNAs has been reported in several pathologies (Table 1). However, there are no studies in liver fibrosis yet. Here, we showed that TIMP3 mRNA can be targeted by 5 miRNAs upregulated in *in vitro* activated HSCs, as compared to quiescent HSCs. In accordance with our study, miR-221-3p, miR-181b-5p and miR-222-3p are up-regulated in cirrhosis,[37] miR-21 shows a 2-fold increase in HCV-dependent human fibrosis[38] and miR-17-5P was identified as a serum biomarker for HCV-positive cirrhosis.[39] Similarly TIMP3 is down-regulated in livers from patients with nonalcoholic steatohepatitis and in HSCs treated with advanced glycation end-products which accumulate in patients with diabetes.[40] Together these observations suggested that TIMP3 expression was down-regulated in liver fibrosis.

Using HCC RNAseq data from TCGA database, we further showed that TIMP3 expression was associated with overall survival while expression of miR-222, miR-221 and miR-21 predicted bad prognosis. The protective role of TIMP3 expression was also supported by its association with HCC subtypes characterized by low grade and low recurrence (Figure 7B).

When searching in MiRTarBase that contains more than $38x10^4$ interactions between 15064 human genes and 2599 miRNAs, only TIMP3 and the tumor suppressor gene PTEN were commonly targeted by the five miRNAs miR-21-5p, miR-81-5p, miR222-3p, miR221-3p and miR17-5p (supplementary figure S2, supplementary table S7). In line with this common regulation, TIMP3 and PTEN were both implicated in miR-221/miR-222-mediated TRAIL resistance[41] and miR-21-5p-mediated doxorubicin resistance.[42] The critical role of PTEN in chronic liver diseases was recently demonstrated *in vivo* with Pten-null mice being more prone to develop progressive liver fibrosis and HSC activation than their wild type littermates.[43] In addition, PTEN negatively correlates with liver fibrosis and activated HSCs in rat liver fibrosis.[44] Conversely, over-expression of PTEN inhibited HSCs activation and induced apoptosis.[45]

TIMP3 deficiency was previously associated with inflammatory cells accumulation[46] and exacerbated liver injury through enhanced inflammation and IL1-ß expression.[47,48] Our observations have consistently shown that IL1-ß and TIMP3 expressions are inversely correlated in the mouse model of CCl4-induced acute liver injury. From a mechanistic point of view, TIMP3 is the major inhibitor of ADAMs enzymes regulating the biodisponibility of cytokines and growth factors activity implicated in inflammatory response. Its decreased expression upon acute response

to liver injury might therefore contribute to the implementation of the inflammatory response required for activated HSCs-dependent tissue repair. As shown by in situ hybridization patterns (supplementary figure S3), diverse cell types expressed Timp3 with complex dynamics in both healthy and injured regions. The expression dynamics of Timp3 and miRNAs in whole liver are therefore not contributed by the sole hepatic stellate cells. Nevertheless, our observations indicate that IL1-ß inhibits TIMP3 expression in LX-2 cells raising the possibility that repression of this gene is part of an early response of HSCs to inflammation. We have also shown that TGF-β induces TIMP3 expression and that IL1-β downregulates TGF-ß expression, explaining how it affects also TIMP3 synthesis. A control of TIMP3 production by TGF-β might therefore contribute to the maintenance of immune homeostasis, in line with the anti-inflammatory role of TGF-β in liver injuries.[49]

Together our results demonstrate that TIMP3 expression can be regulated by miR-21-5p, miR-181b-5p, miR-222-3p, miR-221-3p and miR-17-5p in activated HSC and that TIMP3 expression is inversely correlated to the expression of these miRs, and to the grade and inflammation activity of liver fibrosis. This regulation is associated with IL1-β-dependent immune response and future challenges will be to integrate the dynamic changes in miRNA networks to propose a model for TIMP3 gene regulation during the development of fibrosis.

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**Table 1:** List of miRNAs previously associated with down-regulation of TIMP3 gene expression

| miRNA | reference | Physiopathologic context/effects |
|---|---|---|
| | | |
| miR-21 | present study | Liver fibrosis/associated with activation of hepatic stellate cells |
| miR-221/222 | present study | Liver fibrosis/associated with activation of hepatic stellate cells |
| miR-181 | present study | Liver fibrosis/associated with activation of hepatic stellate cells |
| miR-17 | present study | Liver fibrosis/associated with activation of hepatic stellate cells |
| | | |
| miR-17 | (Yang et al., 2013) | prostate tumor/induced growth and invasion |
| miR-21 miR-222 | (Zhou et al., 2015) | Peripheral nerve injury/inhibition of apoptosis |
| miR-21 | (Chen et al., 2018) | Gastric cancer/ increased chemoresistance |
| miR-21 | (Gutsaeva et al., 2017) | Ischemic retinopathies/ induced angiogenesis |
| miR-21 | (Zhang et al., 2018l) | Cervical Cancer/ increased cell proliferation, migration and invasion |
| miR-21 | (Chen et al., 2017) | Renal Cancer/increased cell proliferation and invasion |
| miR-21 | (Hu et al., 2016) | Spinal cord injury /pro-angiogenesis |
| miR-21 | (Martin del Campo et al., 2015) | Melanoma/increased cell invasion |
| miR-21 | (Nagao et al., 2012) | Pancreatic ductal adenocarcinoma/associated with poor survival |
| miR-21 | (Song et al., 2010) | Breast Cancer/induced cell invasion |
| miR-92 | (Liu et al., 2018) | Atherosclerosis/ cell migration |
| miR-136 | (Jin et al., 2017) | Spinal cord ischemic injury/inhibition of apoptosis |
| miR-142 | (Wu et al., 2018) | Sciatic Nerve injury/inhibition of apoptosis |
| miR-181 | (Heath et al., 2018) | Calcific aortic valve disease/induced ECM degradation |
| miR-181 | (Di Gregoli et al., 2017) | Atherosclerosis/ induced ECM degradation |
| miR-181 | (Zhou et al., 2016) | Gastric cancer/ increase TGF-β-induced EMT |
| miR-181 | (Panda et al., 2012) | Endometrial cancer/ |
| miR-181 | (Wang et al., 2010a) | hepatocarcinogenesis/induced cell survival, migration and invasion |
| miR-191 | (Wang et al., 2018b) | Prostate cancer/increased cell growth |
| miR-191 | (Dong et al., 2015) | endometriosis-associated ovarian cancer (EAOC)/ increased cell proliferation and invasion |

| | | |
|---|---|---|
| miR-191 | (Qin et al., 2014) | Colorectal Cancer/ increased cell invasion |
| miR-206 | (Limana et al., 2011) | HMGB1 injection in Chronically failing hearts/induced ECM degradation |
| miR-221&222 | (Garofalo et al., 2009) | Lung and liver cancer/induced chemoresistance |
| miR-221 | (Diao et al., 2017) | Papillary Thyroid Carcinoma/increased cell proliferation |
| miR-222 | (Xu et al., 2017) | Pulmonary artery hypertension / cell proliferation |
| miR-365 | (Wang et al., 2018a) | Diabetic retinopathies/ increased oxidative stress |
| miR-373 | (Liu et al., 2016) | Esophageal carcinoma/increased cell proliferation, migration and invasion |

**Figure legends**

**Figure 1**. **miRNA-genes regulatory interaction networks**. The list of down-regulated and up-regulated miRNA in activated hepatic stellate cells (supplementary table S3) was used as input for CyTargetLinker application. The resulting graphs illustrate the interactions between miRNA and genes. (A) Full graph including all target genes of down-regulated miRNA (blue) and up-regulated miRNA (red). (B) and (C) Graphs were reduced to interactions only supported by *strong evidences* as defined by CyTargetLinker application either for down-regulated miRNA network (B) or up-regulated miRNA network (C). Target genes annotated by the term *liver fibrosis* in DisGeNet database (Disease id: C0239946) are shown in bold characters

**Figure 2. Comparison of genes targeted by up- and down-regulated miRNA in activated stellate cells**. Genes identified by CyTargetLinker application with *strong evidences* criteria (green circle, n=825) were compared with target genes of up-(UP-HSC, n=17) and down-regulated miRNA (DOWN-HSC, n=35) previously reported in activated HSCs (supplementary table S3).

**Figure 3. miRNA-genes interaction networks of differentially expressed genes between quiescent and cultured HSCs**. Genes differentially expressed during activation of HSCs from De Menicis's et al [18] were used to filter miRNA-genes regulatory interaction networks (Figure 1). Resulting reduced networks are shown: (A) Up-regulated miRNA networks. (B) Down-regulated miRNA networks.

**Figure 4. TIMP3 expression in human liver fibrosis.** (A) and (B) Boxplots showing mRNA levels of TIMP3 in liver biopsy samples with various (A) fibrosis stages (5 F1, 4 F2, 7F7 and 48 F4) and (B) inflammatory activities (28 A0, 17 A1, 16 A2 and 3 A3). Statistical significance was calculated using the Wilcoxon rank-sum test. (C) TIMP3 protein levels in liver fibrosis. Specific extraction of extracellular matrix was performed according to Naba's protocol[16] and protein extracts were analyzed by mass spectrometry from human tissue samples at various fibrosis stages (5F1, 3F2, 4F3, 6F4).

**Figure 5. TIMP3 expression is down-regulated by IL1-β in LX-2 cells.** (A) Comparative analysis of TIMP3 expression in cultured hepatic cells. TIMP3 and ALB mRNA levels were

measured in activated HSC, LX-2 cell line treated or not with TGF-β, primary human hepatocytes (HH), carcinoma cell lines (HuH7 and HepG2) as indicated. Bar plots show normalized levels relative to average TIMP3 levels in HSCs. (B) Box plots showing mRNA levels of TIMP3, TGF-β and MMP9 expression in LX-2 cells treated by IL1-β (three independent experiments).

**Figure 6. Timp3 and miR-17-5p miR-181b-5p miR-21-5p miR-221-3p miR-222-3p expression in CCl4-treated mice.** Mice were treated by a single dose of CC4 (0.3 ml/kg of mouse weight) and were sacrificed 4-, 12-, 24- and 168-hours post-injection (A) Box plots show mRNA levels of Timp3 and IL1-β expression. (B) Box plots show miR-17-5p, miR-21-5p, miR-181b-5p, miR-221-3p and miR-222-3p expression.

**Figure 7. TIMP3 expression is a marker for good prognosis of hepatocellular carcinoma**. (A) Kaplan-Meier survival curves for TIMP3, miR-222, miR-221 and miR-21 expression. Clinical and expression data were extracted from TCGA_LIHC samples (n=364). (B) Box plots show TIMP3 mRNA levels in different HCC grades and subtype from TCGA_LIHC primary HCC samples (n=294): histologic grades (Grades G1, G2 and G3), the Seoul National University recurrence subtype (SNUR, low and high recurrence), the 65-gene risk score subtype (RS65, low and high risk) and The National Cancer Institute proliferation subtype (NCIP, low and high proliferation). Statistical significance was calculated using the Wilcoxon rank-sum test (two groups) or the Kruskal-Wallis non-parametric test (more than two groups). HCC subtypes were extracted from[50] .

**Figure 1**

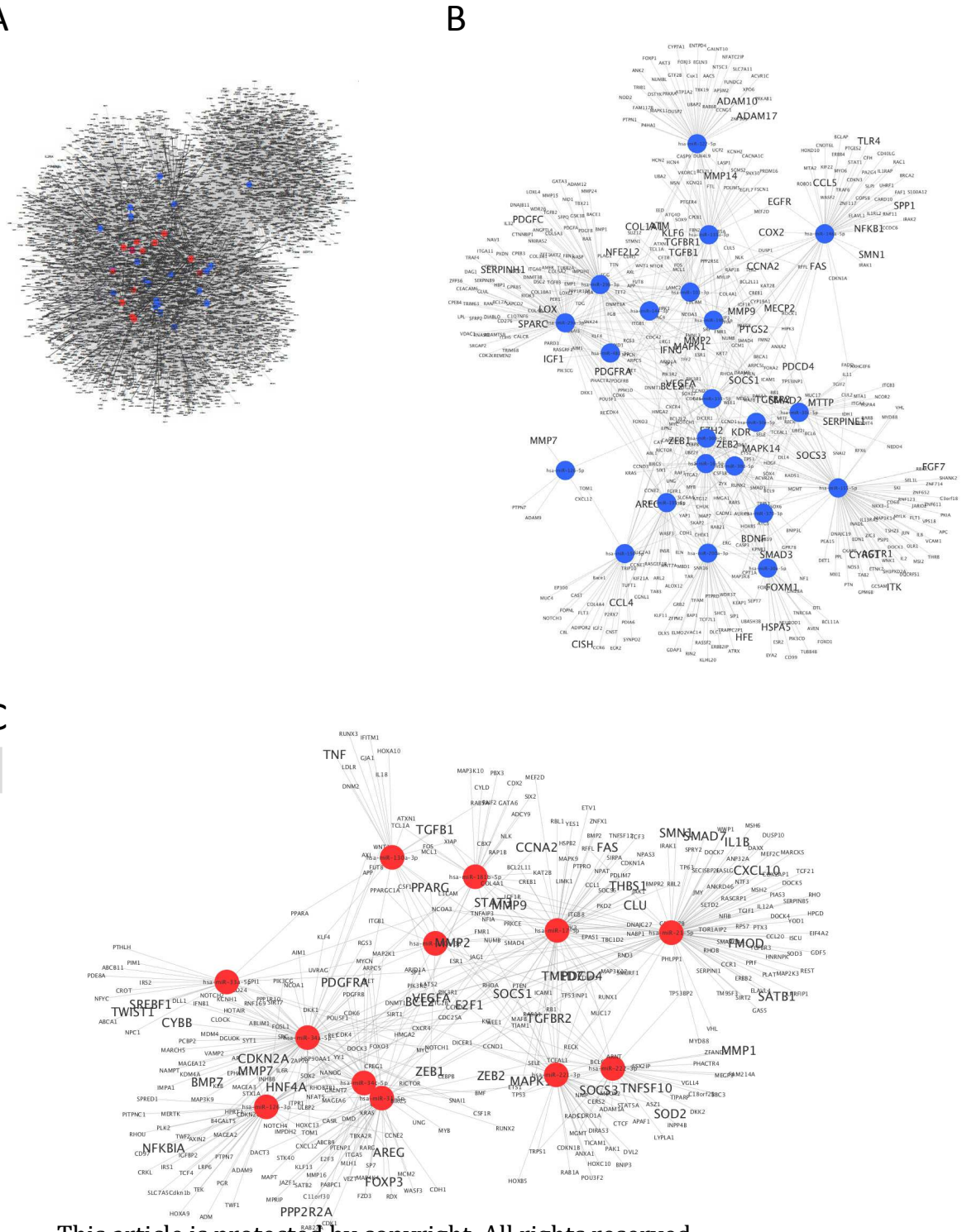
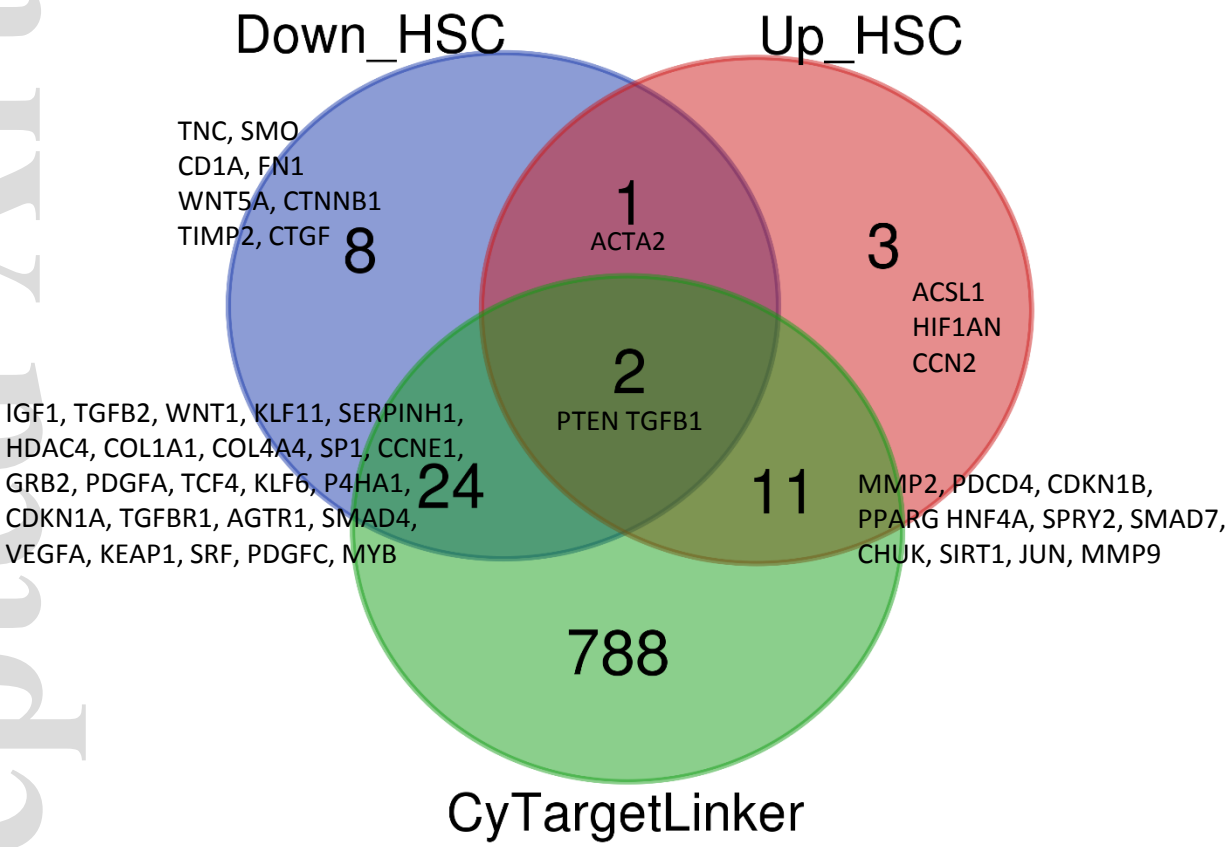


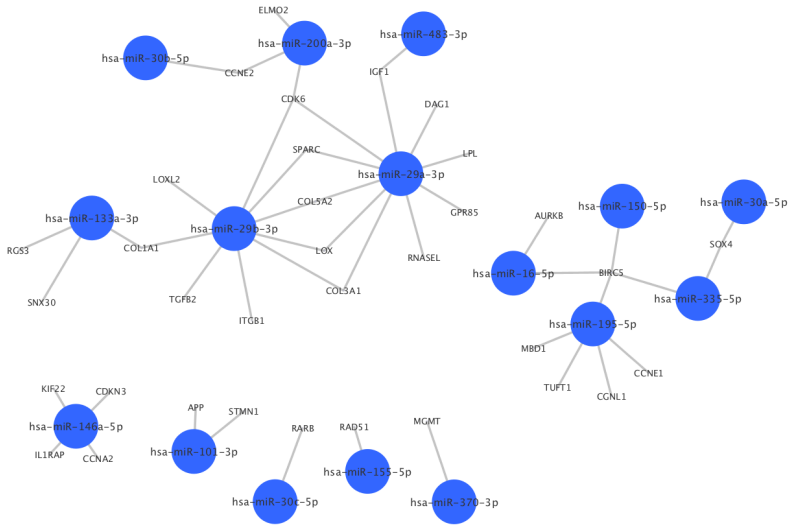
**Figure 2**



# Figure 3



A

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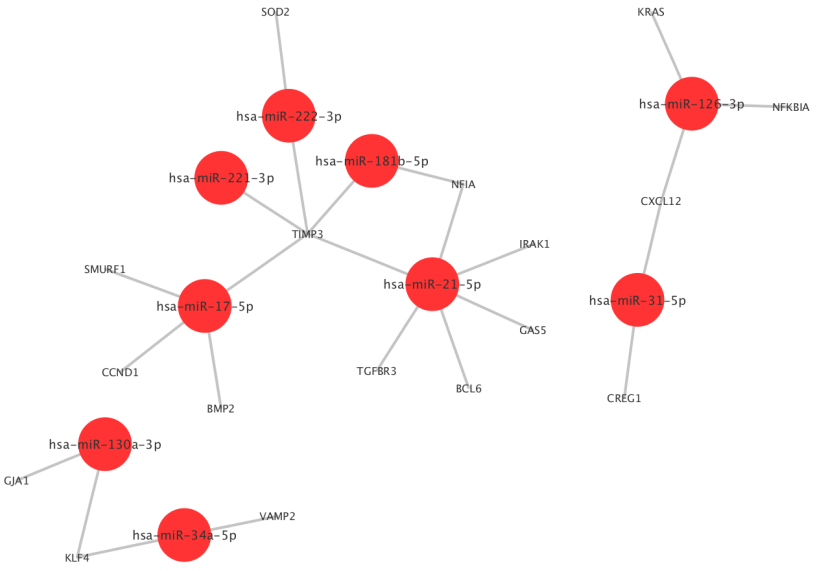
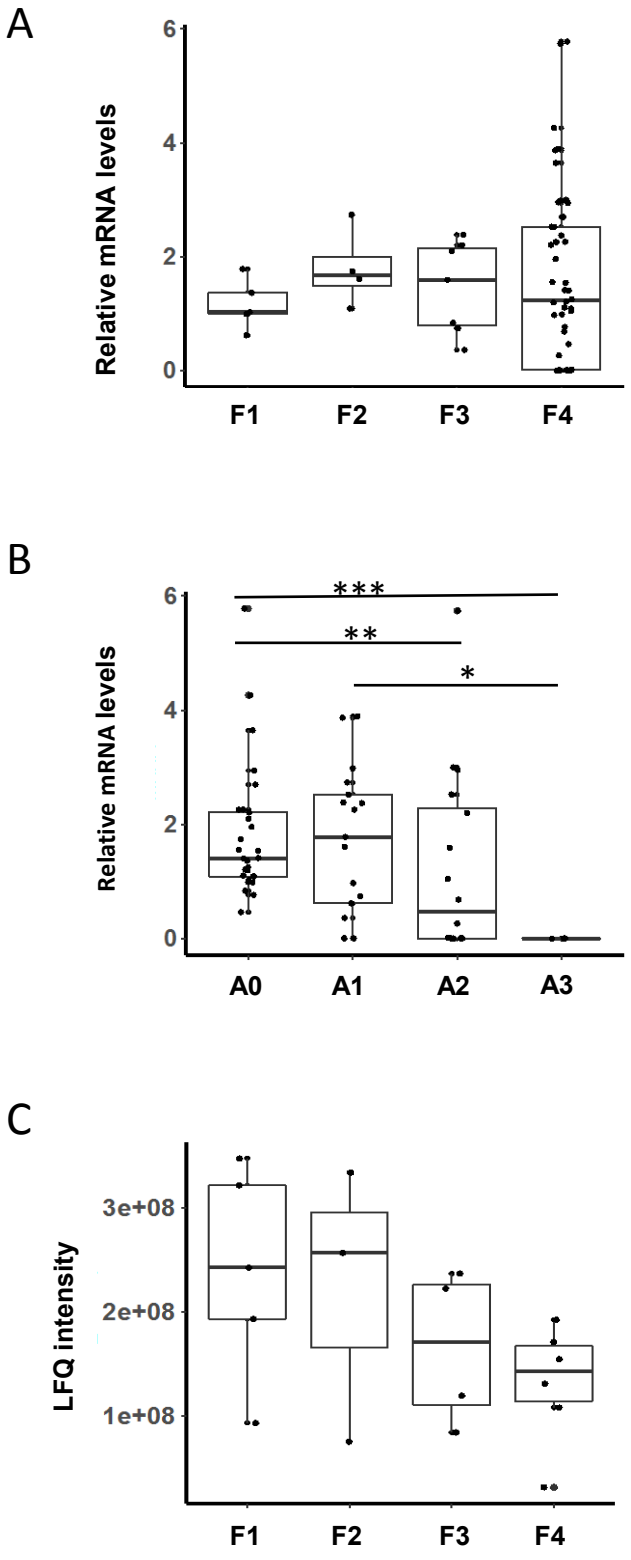
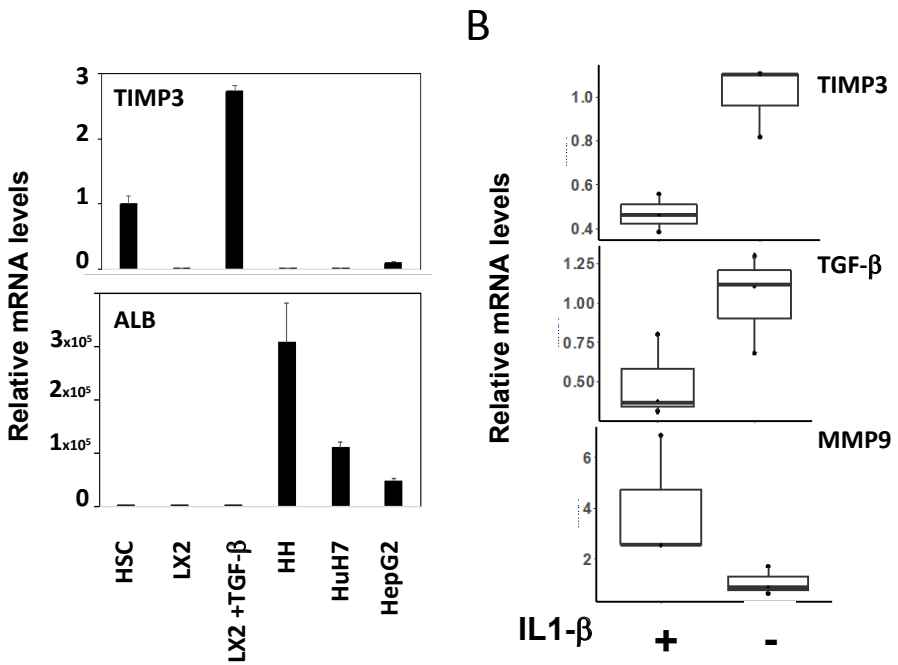


**Figure 4**



# Figure 5



# Figure 6



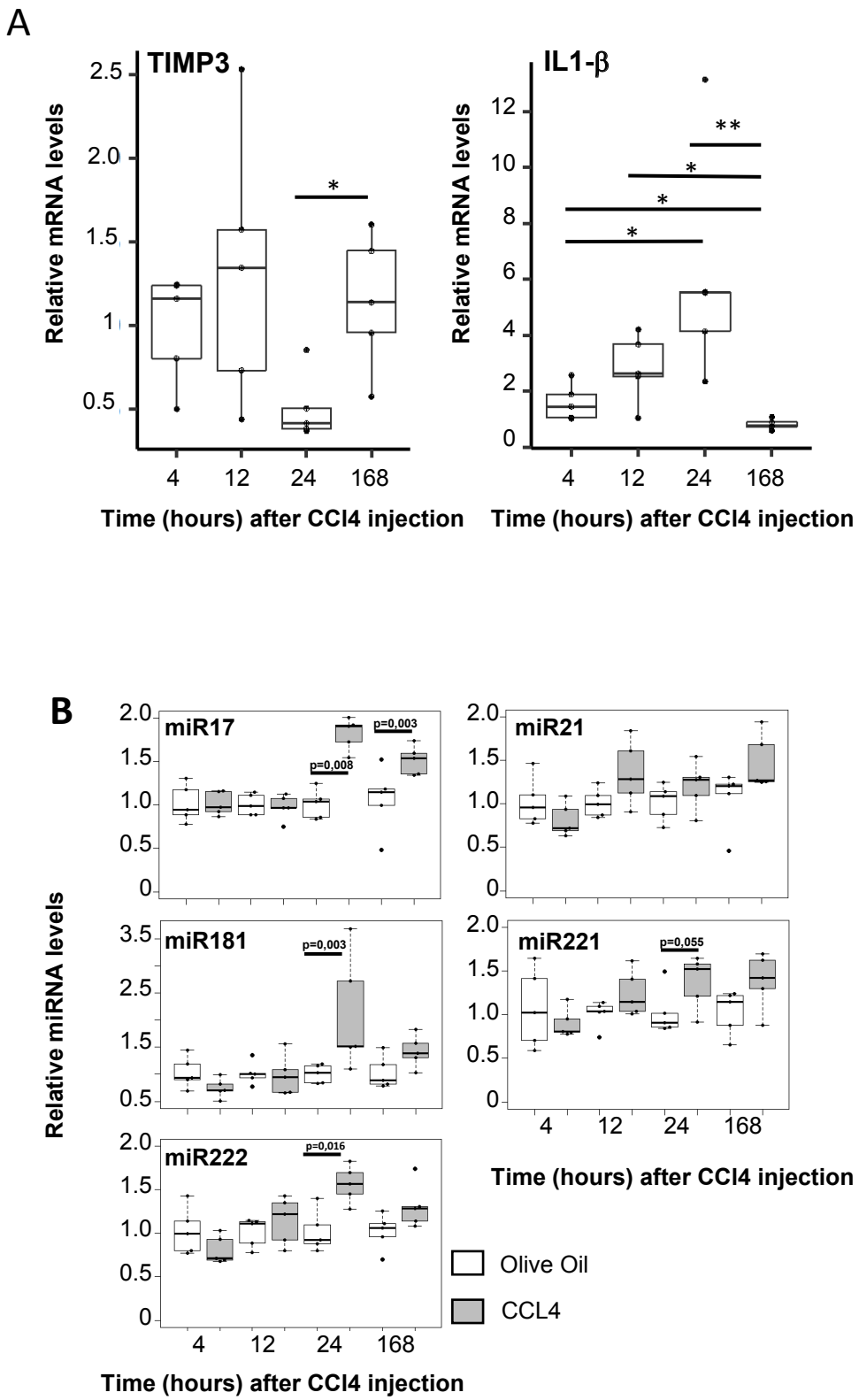


**Figure 7**