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The orphan nuclear receptor COUP-TFII coordinates hypoxia-independent proangiogenic responses in hepatic stellate cells

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Manuscript Draft

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Title: The orphan nuclear receptor COUP-TFII coordinates hypoxia-independent proangiogenic responses in hepatic stellate cells

Article Type: Original Article

Keywords: Hepatic wound healing; angiogenesis; HIF; NF-kB; fibrosis; COUP-TFII; hypoxia; Notch; hepatic stellate cells.

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Corresponding Author's Institution: University of Florence

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Abstract: Background & Aims: Hepatic stellate cells (HSC) transdifferentiation into collagen-producing myofibroblasts is a key event in hepatic fibrogenesis, but the transcriptional network that controls the acquisition of the activated phenotype is still poorly understood. In this study, we explored whether the nuclear receptor Chicken Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TFII) is involved in HSC activation and in the multifunctional role of these cells during the response to liver injury.
Methods: COUP-TFII expression was evaluated in normal and cirrhotic livers by immunohistochemistry and western blot. The role of COUP-TFII in HSC was assessed by gain and loss of function transfection experiments and by generation of mice with COUP-TFII deletion in HSC. Molecular changes were determined by gene expression microarray and RT-qPCR.
Results: We showed that COUP-TFII is highly expressed in human fibrotic liver and in mouse models of hepatic injury. COUP-TFII expression rapidly increased upon HSC activation and it was associated with the regulation of genes involved in cell motility, proliferation and angiogenesis. Inactivation of COUP-TFII impairs proliferation and invasiveness in activated HSC and COUP-TFII deletion in mice abrogate HSC activation and angiogenesis. Finally, co-culture experiments with HSC and liver sinusoidal endothelial cells (SEC) showed that COUP-TFII expression in HSC influenced SEC migration and tubulogenesis via a hypoxia-independent and NF-□B-dependent mechanism.
Conclusion: This study elucidates a novel transcriptional pathway in HSC that is involved in the acquisition of the proangiogenic phenotype and regulates the paracrine signals between HSC and sinusoidal endothelial cells during hepatic wound healing.

Response to Reviewers: Response to reviewer comment
Point-by-point reply letter
Reviewer 1.

1. We agree with the reviewer about the significance of the molecular mechanisms by which COUP-TFII regulates target genes. Molecular mechanism of COUP-TFII actions is complex, this receptor can recognize different response direct repeats (DR) separated by a different number of nucleotides competing for occupancy of the binding sites with other nuclear receptors such as PPARs, RARs, VDR, and GR. In addition COUP-TFII is able to activate transcription through protein-protein interaction with DNA-bound factors. Some proangiogenic gene such as VEGF-C have a COUP-TFII response element in their promoters (DR-1) (Yamazaki T et al. Gene to Cell 2009). On the contrary most cytokines modulated by COUP-TFII have not a specific COUP-TFII response element in their promoters. We hypnotized that COUP-TFII can modulate these genes by an indirect transactivation as accessory regulator. We have demonstrated in ChIP assay that antibodies raised against human COUP-TFII immuniprecipitated DNA fragment corresponding to different NF-kB response elements. This data have been added in the result section and in Supplemental Figure S11

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3. We agree that CCl4 metabolism and hepatocyte toxicity are a central point to validate our result. We tested serum ALT, hepatic content of Malondialdehyde and CYP2E expression. The levels of these parameters were similar both in control and COUP-TFII KO mice. We added this results in Supplemental Figure S9. We are grateful to the reviewer for this suggestion.

Reviewer 2.

1. We agree with the reviewer that Lrat-Cre mice is a better model of stellate cells specific knock-out but it is not available. Anyway GFAP-Cre is still commonly used for stellate cells specific deletion (Mochizuki A, et al. J Immunol 2014). In order to confirm HSC specific targeting we performed a detailed analysis of COUP-TFII expression is isolated different cell populations from control and CCl4 treated mice. We found that COUP-TFII expression was absent in COUP-TFIIfl/fl-GFAP Cre+ , on the contrary both Hepatocytes and Sinusoidal endothelial cells expressed COUP-TFII at a lower level than activated stellate cells and it remained unchanged in all groups with or without CCl4 treatment. We were not able to demonstrate COUP-TFII expression in Kupffer cells. These data are added in Supplementary Figure S8.

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genes were induced in human HSC by transfection of COUP-TFIIwt expression plasmid whereas transfection of the dominant negative construct significantly inhibited α-SMA and Col1α2 steady state levels. These data are included in Supplementary Figure S6 and added in the Result section.
4. Laser Doppler flowmetry that has been shown a suitable method for estimation of hepatic microcirculation was performed to confirm the proangiogenic role of COUP-TFII. We demonstrated that hepatic microcirculation was significantly reduced in CCl4-treated COUP-TFIIfl/fl-GFAP Cre+ mice compared to COUP-TFIIfl/fl-GFAP Cre-. These data were described in the Results section and in the Supplementary Figure S10.
5. We agree with the reviewer about the increased scientific impact of the paper by including a second fibrosis model, so we introduced experiments in bile duct ligated mice. Similar to CCL4 treated mice, BDL-COUP-TFIIfl/fl-GFAP Cre+ mice have a significant reduction of fibrosis compared to BDL-COUP-TFIIfl/fl-GFAP Cre- animals. These experiments are included in Figure 5.

Minor. We realized that details in Figure 5 are difficult to appreciate due to the necessarily low size of the images in a crowded panel. However, making figures in colour will further reduce the contrast, making even more difficult to see positive staining. Therefore, greyscale images were processed by background subtraction algorithm and contrast-stretched to improve clarity. All images were processed uniformly. We hope that positive signal is now easier to see.

Dear Dr. Vijay Shah

Thank you very much for your letter of June 27th 2016 that included the useful comments of the reviewers about the revision of our paper entitled "The orphan nuclear receptor COUP-TFII coordinates hypoxia-independent proangiogenic responses in hepatic stellate cells" **JHEPAT-D-16-01100.**

In the present version of the manuscript we addressed all the points raised by Reviewers as specified in the enclosed point-by point reply letter.

In particular,

1. We included data showing that COUP-TFII receptor is modulated by Notch signaling. Notch is an important regulator of angiogenesis by its target genes Hes and Hey transcription factors. We showed that Notch ligand Jag-1 strongly repressed COUP-TFII expression and transcriptional activity and this effect was mediated by Hey-1. We pointed out that Hey1 silencing restored COUP-TFII expression in Jag-1 treated HSC. In addition we investigated the effect of COUP-TFII on Notch target genes showing that COUP-TFII repressed Hes-1 and Hey1 expression in activate HSC. These data are very intriguing in the context of previous observation indicating that Notch activation in HSC inhibits NF-kB (Oakley F et al. J Biol Chem 2003)
2. We addressed the reviewer 1 request by performing ChIP assay to better define the molecular mechanism by which COUP-TFII regulate target genes. In particular we demonstrate that COUP-TFII binds the NF-kB response elements on IL-8 promoter suggesting that this orphan receptor might function as positive accessory regulator through protein-protein interaction with DNA-bound factors.
3. We reinforced the evidence that COUP-TFII is express in α-SMA expressing cells in the septa

of cirrhotic liver by a more specific confocal microscopy images.

4. In order to confirm the proangiogenic role of COUP-TFII we performed Laser Doppler flowmetry that has been shown a suitable method for estimation of hepatic microcirculation (Hung KC et al. Oncology letters 2013). We demonstrated that hepatic microcirculation was significantly reduced in CCl4-treated COUP-TFII$^{fl/fl}$-GFAP Cre^{+} mice compared to COUP-TFIIfl/fl-GFAP Cre-.
5. A second fibrosis model was included. We introduced experiments in mice after bile duct ligation. Similar to CCL4 treated mice, BDL-ligated COUP-TFIIfl/fl-GFAP Cre+ mice have a significant reduction of fibrosis compared to control mice.
6. Several figures have been added in supplemental material according reviewers' requests and variations in the text have been underlined.

Please find attached the point to point replay to the specific questions raised by the reviewers.

Given the new experimental evidences added, we hope that our paper in now suitable for publication in Journal of Hepatology.

Sincerely

Andrea Galli, MD, PhD

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Point-by-point reply letter

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***Detailed Response to Reviewers**

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*Manuscript
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The orphan nuclear receptor COUP-TFII coordinates hypoxia-independent proangiogenic responses in hepatic stellate cells

Short title: COUP-TFII in hepatic stellate cells

Elisabetta Ceni[1,2,*], Tommaso Mello[1,2,*], Simone Polvani[1,2], Mireille Vasseur-Cognet[3], Mirko Tarocchi[1,2], Sara Tempesti[1], Duccio Cavalieri[5], Luca Beltrame[6], Marroncini Giada[1], Massimo Pinzani[7], Stefano Milani[1,2], Andrea Galli[1,2, #]

[1]Gastroenterology Research Unit, Department of Experimental and Clinical Biochemical Sciences, University of Florence, Florence Italy

[2]Center of Excellence for Research, Transfer and High Education, DENOthe, University of Florence, Italy

[3] INSERM, U1016, Institut Cochin, Paris, France

[4]FiorGen Foundation, Florence, Italy

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[6]Translational Genomics Unit, Department of Oncology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy

[7]UCL Institute for Liver and Digestive Health, University College London, Royal Free Hospital Campus U3, Rowland Hill Street, London NW3 2PF, UK

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Keywords:

Hepatic wound healing; angiogenesis; HIF; NF-kB; fibrosis; COUP-TFII; hypoxia; Notch; hepatic stellate cells.

Abbreviations used in this paper: AP-1, activator protein 1; BINGO, biological network gene ontology; CCl_4, Carbon tetrachloride, COUP-TFII, Chicken Ovoalbumin Upstream Promoter-Transcription factor II; ECM, extracellular matrix; GFAP, glial fibrillary acidic protein; HIF, hypoxia-inducible factor; HSC hepatic stellate cells; PPARγ, Peroxisome Proliferator-activated Receptor γ; IL-8, interleukin 8; RELA, V-rel reticuloendotheliosis viral oncogene homolog A; SEC, sinusoidal endothelial cells, NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; siRNA, small interfering RNA; α-SMA, α smooth muscle actin; VEGF-C, vascular endothelial growth factor C.

World Count: 6000

Number of figures: 8

Conflicts of Interest

The authors disclosure no conflicts

Transcript Profiling: ArrayExpress accession E-MTAB-1795

Authors contributions:

EC*: study concept and design; acquisition of data, analysis and interpretation of data.

TM*: study concept and design; animal experiments; analysis and interpretation of data.

SP: cloning of vector plasmids and transfection experiments, acquisition and interpretation of data, statistical analysis, critical review of the manuscript.

MVC: transgenic mice generation, intellectual and conceptual input, critical review of the manuscript.

MT: mice breeding, data acquisition, critical review of the manuscript.

ST: cell culture and data acquisition.

DC: acquisition of data, statistical analysis, conceptual input, critical review of the manuscript.

LB: microarray analysis, statistical analysis

MG: human sample collection and acquisition of data

MP: intellectual and conceptual input, critical review of the manuscript

SM: intellectual and conceptual input, critical review of the manuscript

AG: study concept and design, study supervision, conceptual & intellectual input, drafting of the manuscript.

*EC and TM equally contributed to the study

Abstract

Background & Aims: Hepatic stellate cells (HSC) transdifferentiation into collagen-producing myofibroblasts is a key event in hepatic fibrogenesis, but the transcriptional network that controls the acquisition of the activated phenotype is still poorly understood. In this study, we explored whether the nuclear receptor Chicken Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TFII) is involved in HSC activation and in the multifunctional role of these cells during the response to liver injury.

Methods: COUP-TFII expression was evaluated in normal and cirrhotic livers by immunohistochemistry and western blot. The role of COUP-TFII in HSC was assessed by gain and loss of function transfection experiments and by generation of mice with COUP-TFII deletion in HSC. Molecular changes were determined by gene expression microarray and RT-qPCR.

Results: We showed that COUP-TFII is highly expressed in human fibrotic liver and in mouse models of hepatic injury. COUP-TFII expression rapidly increased upon HSC activation and it was associated with the regulation of genes involved in cell motility, proliferation and angiogenesis. Inactivation of COUP-TFII impairs proliferation and invasiveness in activated HSC and COUP-TFII deletion in mice abrogate HSC activation and angiogenesis. Finally, co-culture experiments with HSC and liver sinusoidal endothelial cells (SEC) showed that COUP-TFII expression in HSC influenced SEC migration and tubulogenesis via a hypoxia-independent and NF-κB-dependent mechanism.

Conclusion: This study elucidates a novel transcriptional pathway in HSC that is involved in the acquisition of the proangiogenic phenotype and regulates the paracrine signals between HSC and sinusoidal endothelial cells during hepatic wound healing.

Lay Summary: In this study we identified an important regulator of HSC pathobiology. We showed that the orphan receptor COUP-TFII is an important player of hepatic neoangiogenesis. COUP-TFII expression in HSC controls the cross-talk between HSC and endothelial cells coordinating vascular remodeling during liver injury.

Introduction

An exuberant wound healing response to chronic liver injury culminates in excessive and altered deposition of extracellular matrix (ECM) components that is temporally and spatially linked with vascular remodelling of hepatic sinusoids, angiogenesis and recruitment of inflammatory cells (1). Hepatic stellate cells (HSC) play an important role in the physiological homeostasis of ECM in the liver and are the precursors of activated myofibroblast-like cells responsible for the development of liver fibrosis. Indeed, in healthy liver, HSC are specialized pericytes characterized by cytoplasmic lipid droplets rich in retinyl esters and located in the subendothelial space of hepatic sinusoids in direct communication with hepatocytes and endothelial cells (2). Following liver injury HSC undergo an activation process from the resting-fat storing phenotype towards a myofibroblast-like phenotype, characterized by increased cell proliferation, enhanced expression of proinflammatory and proangiogenic cytokines and deranged synthesis of ECM components (3).

Both the initial phase of HSC activation and the persistence of the activated phenotype involve subtle variations of regulatory intracellular mechanisms, which are orchestrated by an elaborated network of transcription factors coordinating a complex reprogramming of gene expression (4). Interestingly, quiescent HSC express adipocytic markers and the transcriptional program required for maintaining their fat-storing phenotype has striking similarities with that of adipocyte differentiation (5). In particular, the peroxisome proliferator-activated receptor γ (PPARγ) and liver X receptor α (LXRα), the master regulators of adipocyte differentiation (6), are expressed in HSC and their expression and activity is reduced during *in vivo and in vitro* HSC activation (7-8).

Although anti-adipogenic pathways as TNF-α and nectin-Wnt promote HSC activation (9), the transcriptional mechanisms linking the loss of adipogenic features with the acquisition of profibrogenic and proangiogenic phenotype in HSC remain unclear.

Chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) are members of the nuclear receptor superfamily. Since no physiologic ligands have been so far discovered they are regarded as "orphan" (10). Three mammalian orthologues have been identified, COUP-TFI (Nr2f1) and COUP-TFII (Arp-1, Nr2f2,) and the more distantly related COUP-TFIII (Nr2f6). These transcription factors are involved in several important biological processes, such as organogenesis, cell fate determination, and energetic metabolism homeostasis (11). COUP-TFs are generally considered to be repressors of transcription for other nuclear hormone receptors but can also function as direct positive regulators for many different genes (12).

COUP-TFI and COUP-TFII show an exceptional homology, suggesting that they may serve redundant functions, nonetheless the spatially and temporally different expression in the developing embryos and in adult animals indicates that these two receptors are involved in different biological processes. COUP-TFII is highly expressed in mesenchymal cells and plays a crucial role in mesenchymal-endothelial interaction determining vein identity and angiogenesis during vascular development (13-14). Recent evidence have shown that COUP-TFII is also expressed in adipose tissue and it represses adipogenesis by antagonizing PPARγ, thus contributing to the antiadipogenic effect of the Wnt pathway (15).

In reason of this converging information, the present study was designed to determine the role of COUP-TFII in hepatic wound healing and HSC biology. We report that COUP-TFII is overexpressed in fibrotic liver and it is up-regulated during HSC activation. Our functional analysis reveals that COUP-TFII transcriptional activity in HSC modulates a hypoxia-independent proangiogenic program involved in the paracrine crosstalk between HSC and sinusoidal endothelial cells (SEC).

Methods

Full methods are available in Supplementary material.

Human Tissue

Normal and cirrhotic liver samples used for histochemical studies were obtained from surgical waste sections obtained from patients undergoing liver resection under institutional review board-approved protocols. In particular, cirrhotic liver tissue (METAVIR 4) was obtained from 11 patients with HCV-related cirrhosis undergoing orthotopic liver transplantation. The use of this material is conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Human Research Review Committee of the University of Florence.

Conditional COUP-TFII-deficient mice

To reduce COUP-TFII levels in HSC, COUP-TFII$^{fl/fl}$ mice, described in detail previously (16) were crossed with mice expressing Cre ricombinase under control of the gliar fibrillary acidic protein (GFAP) promoter (B6.Cg-TgGfap-cre 73.12Mvs/Jmice). Both strains of mice were backcrossed to C57BL/6J (Harlan; Monza, Italy) for at least 12 generation. COUP-TFII$^{fl/fl}$ -GFAP Cre^{-} and COUP-TFII$^{fl/fl}$ -GFAP Cre^{+} littermates were used for these studies. Experiments were performed in accordance with the institutional ethical guidelines of the University of Florence.

DNA vectors and transfection of cultured human HSC

Quiescent HSC were transfected with reporter and expression plasmids at the density of 6 x 10^5 cells/well by TransIT-2020 transfection reagent (Mirus Bio LLC, Medison WI) following the manufacturer's instructions. Forty-eight hours after transfection cells were harvested for luciferase and CAT assay as previoulsly described (8), or used for proliferation and invasion assays. siRNA delivery and knockdown of COUP-TFII was obtained by TransIT-TKO Transfection Reagent

(Mirus Bio LLC, Medison WI) following the manufacturer's instructions. Specific and validate siRNA were purchased from QIAGEN (QIAGEN S.p.A, Milan Italy). Mycoplasm contamination of cell cultures were excluded by MycoProbe (Mycoplasm detection kit, R&D Systems Mineapolis, MN USA)

Statistical Analysis

Results are expressed as mean ± SD for three experiments done in triplicate or quadruplicate. Group means were compared by analysis of variance (ANOVA), followed by the Student-Newman-Keuls test if the former was significant. A *P* value < .05 was considered statistically significant.

Results

COUP-TFII expression is increased in cirrhotic liver.

In normal human liver COUP-TFII is expressed in the nucleus of non-parenchymal elements sparsely distributed in the sinusoids as well as in stromal cells of portal tracts (Fig. 1A). In cirrhotic livers the number of COUP-TFII expressing cells appears strikingly increased. Most of these cells are localized within the fibrotic septa and enlarged portal areas, although an increased expression in perisinusoidal cells in the liver lobule is also visible. COUP-TFII expressing cells are also positive for the mesenchymal cell marker vimentin and for the activated stellate-cells/myofibroblas marker alpha-smooth muscle actin (α-SMA) (Fig. 1A inset, Supplementary Fig. S1A, S1C); on the contrary COUP-TFII expression does not colocalized with the macrophage marker CD68 (Supplementary Fig. 1B, 1D) suggesting that COUP-TFII positive cells are activated HSC/myofibroblasts.

We next sought to recapitulate these finding in the context of a mouse model of liver fibrosis by chronic administration of CCl_4. Expression of COUP-TFII was sparsely distributed in the liver lobule of control mice (Supplementary Fig. S2A) whereas, in cirrhotic liver, expression of this receptor was significantly increased in α-SMA positive cells localized within fibrotic septa (Supplementary Fig. S2C, S2E). Although, the expression of GFAP, a marker of quiescent HSC, is strongly reduced in the parenchyma of the cirrhotic liver, COUP-TFII/GFAP double positive cells could be observed mainly in fibrotic septa, suggesting a progressive expression of this receptor in the process of HSC activation (Supplementary Fig. S2D, S2F).

COUP-TFII gene expression increases during HSC activation *in vivo* and *in vitro*

To further validate this observation, COUP-TFII expression in liver injury was monitored *in vivo* after a single intraperitoneal injection of CCl_4. In control mice, confocal microscopy analysis showed that COUP-TFII expressing-cells distributed in the sinusoids are desmin-positive (Fig. 1B). Forty eight hours after CCl_4 injection there is a significant increase in COUP-TFII positive cells,

particularly around portal areas where tissue damage is more pronounced. After 72 hours, the increase in COUP-TFII expression is paralleled by a marked increase of desmin and α-SMA (Fig. 1B), thus suggesting that COUP-TFII expressing cells may be HSC undergoing activation.

Next, we characterized the expression profile of COUP-TFII during HSC transdifferentiation *in vitro*. Freshly-isolated human HSC do not express COUP-TFII mRNA and protein as shown by immunohistochemistry (Fig. 1B, lower row) and Western blot (Supplementary Fig. S3A and S3B). However, COUP-TFII expression gradually increases after 24 hours, peaking between 48 and 72 hours from plating and remains highly expressed throughout HSC activation [documented by loss of the Oil-Red O stained lipid inclusions (Fig. 1B, lower row) and by the increased of α-SMA expression (Supplementary Fig. S3B)]. In contrast, COUP-TFI is weakly expressed in quiescent cells and its expression remains low at each time point (Supplementary Fig. S3A and S3B). COUP-TFII transcriptional activity was further evaluated by transfection with a COUP-TFII reporter plasmid (NHE_4-TK-LUC) (17). Reporter expression rapidly increased with the duration of culture reaching more than 10-fold increase between 48 and 72 hours after plating. Although at lower levels, COUP-TFII transcriptional activity remains significantly high after 72 hours of culture and during successive passages on plastic (Supplementary Fig. S3C).

COUP-TF II regulates proliferation and invasiveness of activated HSC

Considering that the expansion of HSC pool in injured liver is a fundamental feature of hepatic fibrosis and that COUP-TFII modulates genes involved in cell growth and motility (18), we evaluated the role of COUP-TFII on HSC proliferation and invasiveness by gain and loss of function experiments using a dominant negative COUP-TFII ($COUP\text{-}TFII_{C134S}$) (Supplementary Fig.S4) or COUP-TFII silencing by siRNA. Transfection of wild type COUP-TFII ($COUP\text{-}TFII_{wt}$) induced a significant increase in DNA synthesis and cell growth (Fig. 2A, 2C). On the contrary, transfection of $COUP\text{-}TFII_{C134S}$ reduced basal and serum-stimulated [^{3}H]TdR incorporation and cell

number. Conversely, silencing of COUP-TFII prevented both basal and serum induced HSC proliferation, confirming the role of the nuclear receptor in the regulation of HSC growth. (Fig. 2B-2D). COUP-TFII transcriptional activity was monitored by co-transfection with the NHE_4-TK-Luc reporter in cells maintained in the same experimental conditions. Luciferase activity was induced by expression of COUP-$TFII_{wt}$ whereas it was inhibited by both COUP-$TFII_{C134S}$ expression (Fig. 2E) and COUP-TFII silencing (Fig. 2F). The role of COUP-TFII on the ability of HSC invasiveness was assessed using Boyden chambers. COUP-$TFII_{wt}$ overexpression induced a significant stimulation of HSC invasiveness, whereas transfection of COUP-$TFII_{C134S}$ or COUP-TFII siRNA abrogated HSC invasiveness in presence or absence of transfected COUP-$TFII_{wt}$ (Fig. 3A, 3B).

COUP-TF II targets proinflammatory and proangiogenic genes in human HSC

To identify novel transcription target genes of COUP-TFII that could account for the activated phenotype of human HSC, an exploratory microarray analysis was performed with mRNA extracted from cultured HSC transfected with COUP-$TFII_{wt}$ or control plasmid. A functional analysis with gene ontology categories showed a significant enrichment of fibrogenic-related functions in COUP-TFII overexpressing cells (i.e., response to wounding, immune response, motility, proliferation and angiogenesis) (Fig. 4 and Supplementary Table 1). Validation of selected targets of interest obtained from microarray data was performed by RT-qPCR with mRNA extracted from COUP-TFII-overexpressing and COUP-TFII-silenced HSC. This analysis confirmed that COUP-TFII regulates proangiogenic and proinflammatory genes such as cathepsin S, MMP-9, VEGF-C, IL-8, MCP-1, IP-10, ICAM-1 and ANGPTL4 (Supplementary Fig. S5). In addition, we showed that HSC profibrogenic markers, α-SMA and collagen 1α2 (Col1α2) were modulated by COUP-TFII expression (Supplementary Fig. 6). It is well known that the expression of inflammatory and angiogenic factors mainly relies on the balance between the activation of nuclear factor -κB (NF-κB) and activator protein 1 (AP-1) and repression of PPARγ transcriptional activity (19). Reporter

assay experiments showed that both NF-κB and AP-1 were highly activated in COUP-TFII transfected HSC, whereas COUP-TFII overexpression completely abrogated ligand dependent and independent PPARγ transcriptional activity (Suppplementary Fig. S7).

COUP-TFII deletion in HSC prevents inflammation and neo-angiogenesis in mice

In order to assess the role of COUP-TFII during liver injury *in vivo*, COUP-TFII$^{fl/fl}$ -GFAP Cre^{-} and COUP-TFII$^{fl/fl}$ -GFAP Cre^{+} (Supplementary Fig. 8A) mice were treated with a single dose of CCl_4. CCl_4 metabolism and hepatocyte toxicity were not different between the two strains after intoxication as shown by ALT activity, MDA concentration and CYP2E1 levels (Supplementary Fig. 9). HSC freshly isolated from CCl_4-treated COUP-TFII$^{fl/fl}$ -GFAP Cre^{+} mice did not express COUP-TFII, on the contrary high COUP-TFII expression was documented in HSC isolated from COUP-TFII$^{fl/fl}$ -GFAP Cre^{-} at 72 hours after intoxication (Supplementary Fig. 8B, 8C). Upon culture on plastic, HSC isolated from COUP-TFII$^{fl/fl}$-GFAP Cre^{-} had high COUP-TFII transcriptional activity and underwent myofibroblastic transdifferentiation as demonstrated by increased expression of α-SMA, progressive reduction of PPARγ and a significant increase of cell proliferation (Fig. 5A-5C) . On the contrary, HSC from COUP-TFII$^{fl/fl}$ -GFAP Cre^{+} maintained minimal α-SMA expression and high level of PPARγ after six day of culture (Fig. 5A).

A significant reduction of α-SMA expression was demonstrated in liver section from COUP-TFII$^{fl/fl}$ -GFAP Cre^{+} compared with COUP-TFII$^{fl/fl}$-GFAP Cre^{-} at 72 hours after CCl_4 intoxication (Fig. 5D). Furthermore, COUP-TFII$^{fl/fl}$-GFAP Cre^{+} mice showed a significant reduction of both the macrophage cell marker F4/80 and the endothelial cell marker CD31. These data were strengthened by RT-qPCR showing that the expression of α-SMA are dependent on COUP-TFII presence in HSC isolated from COUP-TFII$^{fl/fl}$ -GFAP Cre^{-} and COUP-TFII$^{fl/fl}$ -GFAP Cre^{+} mice (Fig. 5F). In addition, gene expression of proinflammatory and proangiogenic mediators were decreased in HSC

isolated from COUP-TFII$^{fl/fl}$ -GFAP Cre^{+} (Fig. 5F). Taken together these results confirm that COUP-TFII is involved in HSC regulation of inflammatory and angiogenic responses during liver injury. The proangiogenic role of COUP-TFII was also confirmed by the significant reduction of hepatic microcirculation in CCl_4-treated COUP-TFII$^{fl/fl}$ -GFAP Cre^{+}mice (Supplementary Fig. 10). We next evaluated whether COUP-TFII deletion in HSC affect development of liver fibrosis after chronic treatment with CCl_4 and in BDL mice. As shown in Figure 5E, Sirius red staining in the liver was significantly reduced in COUP-TFII$^{fl/fl}$ -GFAP Cre^{+} mice compared with COUP-TFII$^{fl/fl}$ -GFAP Cre^{-} mice. Similarly, COUP-TFII deletion in HSC significantly affected cholestatic fibrosis (Fig. 5E).

COUP-TFII expression in HSC promotes endothelial cells migration and tube formation in a hypoxia-independent manner.

Liver sinusoidal endothelial cells (SEC) migration is a key step in liver angiogenesis especially in the cirrhotic microenvironment (20). In order to ascertain whether expression of COUP-TFII in HSC mediated hepatic angiogenesis, transfected HSC were co-cultured with SEC in a transwell system. Coculture with COUP-TFII$_{wt}$-overexpressing HSC significantly enhanced SEC chemiotaxis, whereas the presence of COUP-TFII$_{C134S}$-transfected HSC inhibited SEC migration (Fig. 6A). Furthermore, conditioned medium from COUP-TFII$_{wt}$-transfected HSC strongly induces SEC tubulogenesis, and this effect was abrogated by cotransfection with COUP-TFII$_{C134S}$ (Fig. 6B). These data suggest that COUP-TFII might play a central role in the modulation of the angiogenic responses during liver fibrosis via an active cross-talk between SEC and HSC for sinusoidal remodelling.

We next determined whether COUP-TFII contributes to the hypoxia-induced proangiogenic phenotype of HSC (21). COUP-TFII expression and transcriptional activity was not modulated in the presence of hypoxic conditions (Fig. 7A). On the other hand, COUP-TFII expression did not

affected either the expression of the both hypoxia-inducible factors HIF-1α and HIF-2α or their reporter activity in HSC (Fig. 7B). In addition, silencing of HIF-1β (ARNT), the constitutive binding partner of HIF-α isoforms, abrogated hypoxia-induced HIF transcriptional activity both in presence and absence of transfected COUP-TFII (Fig. 7C), but did not influence either COUP-TFII expression or its transcriptional activity (Fig. 7D, 7E). Accordingly, tubulogenesis of SEC treated with the conditioned medium of COUP-TFII-overexpressing HSC was not affected by ARNT silencing independently of hypoxia conditions (Fig. 7F). These results indicate that COUP-TFII might contribute to liver angiogenesis in both normoxic and hypoxic conditions regardless of HIF activity.

NF-kB regulates COUP-TFII-mediated angiogenic response in HSC

Considering that NF-kB is involved in HIF-independent angiogenesis (22), we tested whether NF-kB activity is involved in the COUP-TFII-induced angiogenesis. To directly examine the involvement of NF-kB we silenced RELA, the p65 subunit of NF-kB, in COUP-TFII overexpressing HSC and examined the ability of the conditioned media of these cells to influence SEC angiogenesis. As reported in Fig. 8A, RELA silencing in HSC blocked SEC tubulogenesis induced by conditioned medium from COUP-TFII-overexpressing HSC. Furthermore, to confirm the role of NF-kB in COUP-TFII proangiogenic function we evaluated the effect of RELA silencing on IL-8 and VEGF-C, two COUP-TFII-regulated gene that are known to preserve the angiogenic response independently of HIF pathway (24).

A significant increase of IL-8 protein was measured in conditioned media of COUP-TFII-overexpressing HSC (Fig. 8C and Supplementary Fig S11A). Media preincubation with a neutralizing antibody against IL-8 strongly attenuated LEC migration thus supporting the role of IL-8 in the COUP-TFII-dependent proangiogenic crosstalk between SEC and HSC (Supplementary Fig. S11B). RELA silencing significantly impaired the COUP-TFII-induced stimulation of IL-8

steady state mRNA levels and protein synthesis in transfected HSC (Fig. 8B, 8C). Similarly RELA silencing inhibited COUP-TFII-induced expression of VEGF-C in HSC (Fig. 8D, 8E), confirming the dependence on NF-kB of the COUP-TFII-proangiogenic function. In consideration that COUP-TFII can function as positive accessory regulator for many different genes and activate transcription through protein-protein interaction with DNA-bound factors (11), we performed chromatin immunoprecipitation using COUP-TFII-expressing HSC. Antibodies raised against COUP-TFII could efficiently immunoprecipitate DNA fragments of IL-8 promoter corresponding two NF-kB response elements (Supplementary Fig. S11C).

Notch signalling modulates COUP-TFII expression in HSC

Notch pathway is the master regulator of COUP-TFII expression during vasculogenesis and Notch inhibition promotes angiogenesis causing pathogenic activation of liver stromal cells (13, 25). We tested the effect of Notch activation on COUP-TFII in HSC. Soluble recombinant protein of Notch ligands Jag-1 and Dll4 were directly added in the culture media of HSC. Jag-1 but not Dll4 strongly repressed COUP-TFII expression and transcriptional activity (Supplementary Fig. S12A, S12B). We found that Hey-1, among many Notch downstream effectors tested was exclusively induced by Jag-1 (Supplementary Fig. S12C, S12D). Hey-1 silencing by siRNA induced COUP-TFII expression reversing Jag-1 mediated COUP-TFII down-regulation (Supplementary Fig. S12E). Finally, we demonstrated that increased COUP-TFII expression by transfection selectively modulated Hes-1 and Hey1 expression (Supplementary Fig S12F).

Discussion

The activation of HSC has been proposed as a key event in chronic liver diseases and these cells are currently considered the crossroads of fibrosis, inflammatory response, and angiogenesis during hepatic wound healing (3). The results of the current study indicate that the nuclear receptor COUP-TFII plays a paramount role in the HSC acquisition of proangiogenic phenotype and in the orchestration of myofibroblast-endothelial interaction during liver injury. COUP-TFII is highly expressed in human and rodent cirrhotic liver, in particular in α-SMA positive HSC/myofibroblasts. COUP-TFII is rapidly up regulated in HSC upon activation and its inhibition leads to the inhibition of HSC transdifferentiation delaying the PPARγ down-regulation and the acquisition of myofibroblast markers. It has been recently reported that COUP-TFII is a negative regulator in adipogenesis and that ectopic expression of COUP-TFII in preadipocytes blocks fat-storing and prevents the induction of adipogenic transcription factors such as PPARγ and LXRα (24). Furthermore, COUP-TFII has been shown to be a direct target of Wnt/β-catenin signalling pathway in preadipocytes and HSC and it mediates chromatin hypoacetylation and the consequent PPARγ repression (16, 9). Based on this background and on the results of the present study it is possible to speculate that COUP-TFII plays a primary role in early HSC activation via a Wnt-dependent control of PPARγ repression. In addition, COUP-TFII is a downstream target of Hedgehog and Notch pathways (26), that were shown to control the HSC phenotype by regulating metabolism and inflammatory response (27, 28). Accordingly, it is conceivable to propose that COUP-TFII acts as a gatekeeper in promoting HSC transdifferentiation and that the cross-regulation between COUP-TFII and PPARγ provides the molecular basis underlying the remarkable cell plasticity of HSC.

COUP-TFII expression in HSC modulates an array of genes involved in different aspects of the wound healing process. In particular, COUP-TFII controls the expression of a wide range of chemokines that function in specific autocrine and paracrine axes to coordinate cellular interactions and promote migration and proliferation of immune and angiogenic cells (29). Livers from mice with COUP-TFII deletion in HSC show a concomitant reduced expression of mesenchymal and endothelial markers in association with a significant inhibition of HSC expression of proinflammatory and proangiogenic mediators. Furthermore, inhibition of COUP-TFII blunts HSC proliferation and invasiveness. Interestingly, cancer cells invasiveness is associated with COUP-TFII expression and up-regulation of gelatinolitic and fibrinolitic activity (30-31). Similarly, COUP-TFII expression in HSC positively regulates uPA and MMP-9 which play e central role in hepatic wound healing (32).

The most intriguing function of COUP-TFII emerging from the results of the present study is its role in sinusoidal remodelling and neoangiogenesis. Neoangiogenesis and the establishment of an abnormal angioarchitecture are typical of the pathological progression of chronic liver disease to cirrhosis (21). Interestingly, we showed that COUP-TFII regulates the HSC/SEC cross-talk that is considered of primary importance not just in fibrogenesis but also in liver regeneration and tumor angiogenesis (33). In a co-culture system COUP-TFII-overexpressing HSC release potent angiogenic molecules and promote SEC migration and tubulogenesis. Accordingly, accumulating evidence support the importance of COUP-TFII in angiogenic pathways. COUP-TFII is necessary during normal developmental angiogenesis and lymphoangiogensis, as evinced by the impaired vessel formation and embryonic lethality in COUP-TFII knockout mice (34). Quin et al. have also demonstrated that COUP-TFII is essential for cancer angiogenesis through regulation of pericyte-derived paracrine signals that target endothelium (14). Furthermore, COUP-TFII regulates tumor lymphoangiogenesis by expression of VEGF-C (31) that is one of the main angiogenic factors produced by COUP-TFII-expressing HSC and it recognizes VEGFR-2 and VEGFR-3 receptors that

are expressed in SEC (35). In consideration that angiogenic and antiadipogenic pathways converge in different animal models of chronic metabolic diseases (36), we drew the hypothesis that COUP-TFII functions as a transcriptional hub integrating different inputs to establish a specific reparative niche where the paracrine interplay between HSC and SEC regulates the wound healing process.

The transcriptional mechanisms by which COUP-TFII regulates angiogenic response in the liver are peculiar. Indeed it is well established that hepatic neoangiogenesis induced by hypoxia is a common feature of fibrosis and occurs through activation of HIF transcription factors (21, 37). However, the results of the present study demonstrated that COUP-TFII expression and transcriptional activity is completely independent on hypoxia. In addition, inactivation of COUP-TFII has no effect on HIF isoforms expression and transcriptional activity. Thus, these findings allow to introduce the new concept that modulation of angiogenic factors and neovascularization during liver injury may involve hypoxia- and HIF-1α-independent mechanisms. In colon cancer, xenografts knockout of HIF-1α does not block angiogenesis and a NF-kB-dependent expression of IL-8 and angiogenin is involved in hypoxia-independent angiogenesis (22). Here we demonstrated that COUP-TFII positively controls NF-kB transcriptional activity in HSC and that NF-kB silencing abrogates COUP-TFII-regulated expression of IL-8 and VEGF-C, interrupting the pro-angiogenic cross-interaction between HSC and SEC. Although HIF and NF-kB have a significant and profound crosstalk, it has recently became apparent that they act independently in response of angiocrine non-hypoxic stimuli such as bacterial LPS, TNFα, and reactive oxygen species (38). Both hypoxia/HIF-dependent and COUP-TFII (hypoxia/HIF-independent)-mediated pathways can influence NF-kB, but it remains to be seen in which context one function can prevail over the other during the progression of chronic liver injury. It is possible that these pathways cooperate to provide redundant signals for NF-kB activation in promoting sinusoidal remodelling. Alternatively, in consideration that COUP-TFII-dependent angiogenesis is tightly coupled to HSC activation, we can presume that this pathway may be predominant in the early phase of healing response in non

hypoxic regions and promote the early recruitment of SEC that are required for liver regeneration (35, 39). Finally, we demonstrated that Notch pathway that has a central role during angiogenesis and inflammation by repressing NF-kB (28, 40), is an important mediator of COUP-TFII expression in HSC, confirming the complex network involved in the regulation of NF-kB activity. Further studies will be necessary to elucidate the molecular mechanisms involved in the COUP-TFII regulation of NF-kB transcriptional activity and whether COUP-TFII/NF-kB interactions are important for the perpetuation and resolution of liver fibrosis.

In conclusion, our study uncovers COUP-TFII as a critical new leading player in hepatic wound healing process controlling multiple profibrogenic pathways. The evidence that COUP-TFII deletion has a little impact in adult physiological functions and that its transcriptional activity could be potentially regulated by ligands (11, 41), indicate this nuclear receptor a promising target for antifibrotic interventions and stimulate future investigations to identify COUP-TFII specific antagonists to be tested for the therapy of chronic liver diseases.

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Figure Legends

Figure 1: COUP-TFII expression during liver injury and HSC activation.
(A) COUP-TFII in normal (lower row) and cirrhotic (upper row) human liver. First and second column shown immunohistochemistry of COUP-TFII (DAB, brown colour). Original magnification 50x and 200x respectively. Counterstain: methyl green. Third column shows double-immunohistochemistry of COUP-TFII (DAB, brown) and α-SMA (AP Magenta, red). Inset original magnification 200x. Fourth column shows confocal microscopy double-immunofluorescence of COUP-TFII (green) and vimentin (red) expression. Scale bar 50μm. (B). Confocal microscopy double-immunoflurescence of COUP-TFII (red) and α-SMA (green) (upper row) and COUP-TFII (red) and Desmin (green) (middle row) in a mouse model of acute liver damage by CCl_4 injection. Scale bar 50 μm. Third row shows immunocytochemistry of COUP-TFII (DAB, black) expression during plastic-induced activation of HSC. Counter stain: Oil Red O. Original magnification 400x.

Figure 2. COUP-TFII is involved in HSC proliferation and invasiveness. Quiescent human HSC were transfected with COUP-TFII expression plasmids or with specific siRNA. Twenty four hours after transfection culture medium was replaced with fresh medium with or without 10% FBS and cells were cultured for a further 24 hours. DNA synthesis and cell growth were evaluated by [3H]TdR incorporation (A, B) and cell counting (C, D) respectively. Western Blots panels show COUP-TFII expression in transfected HSC, β2-microglobulin (β2-M) was used as loading control. (E-F) Quiescent human HSC were cotransfected with NHE4-TK-LUC reporter, pSV2CAT and COUP-TFII expression plasmids as indicated. Twenty four hours after transfection fresh medium was replace with or without 10% FBS. After 24 hours of incubation cells were harvested for

luciferase and CAT activity. B) COUP-TFII specific siRNA or control siRNA (siControl) were delivered in NHE4-TK-LUC transfected cells. Data are represented as average ± SD, * P<0.05 or higher degree of significance versus empty vector or control siRNA transfected cells in presence or absence of FBS respectively.

Figure 3. Quiescent human HSC were transfected with COUP-TFII expression plasmids (A) or with specific siRNA (B). Forty eight hours after transfection cells were trypsinized and used for the invasion assay. Lower panels: original magnification 100X. Data are represented as average ± SD, *P<0.05 or higher degree of significance versus empty vector or control siRNA transfected cells. **P< 0.04 versus COUP-TFII$_{wt}$ transfected cells.

Figure 4. COUP-TFII targets proinflammatory and proangiogenic genes in human HSC. Heat map of microarray analysis in COUP-TFII transfected HSC. Gene Ontology (GO) over-representation analysis was performed with the Biological Network Gene Ontology (BINGO) plug-in for the Cytoscape visualization software.

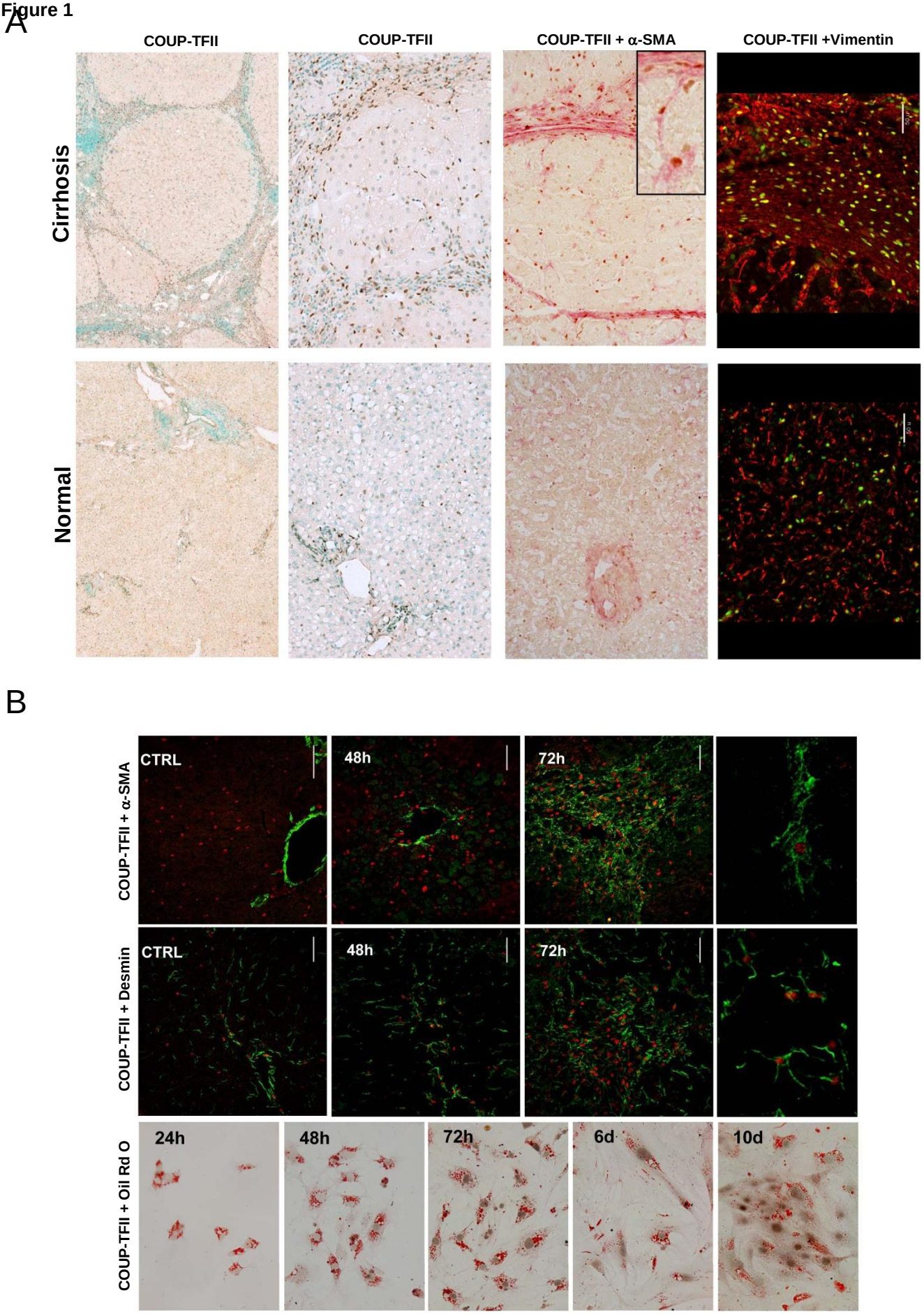
Figure 5. Impact of COUP-TFII deletion in HSC after CCl_4 treatment. (A) Levels of α-SMA, PPARγ and COUP-TFII in HSC freshly isolated from transgenic mice. (B) [3H]TdR incorporation was monitored at the different time points. Data are represented as average ± SD, * P< 0.01 versus HSC from COUP-TFII-GFAP Cre$^+$ mice. (C) COUP-TFII transcriptional activity was monitored in HSC isolated from transgenic mice and cultured on plastic. Cells were transfected with the NHE_4-TK-LUC reporter and pSV_2CAT as an internal control for transfection efficiency at different time points. Data are represented as average ± SD, * P< 0.01 versus HSC from COUP-TFII-GFAP Cre$^+$ mice. (D) Confocal images showing immunostaining for α-SMA, CD31 and F4/80. Scale bar 20 μm. Graphs on the right represent a percentage of area occupied by the signal measured by video-image analysis. For each sample a total of 5 non-consecutive hepatic sections were measured. Data are represented as average ± SD, * P< 0.05 or higher degree of significance versus COUP-TFII-GFAP Cre$^-$ mice. (E) Sirius red staining in liver section from COUP-TFII-GFAP Cre$^+$ and COUP-TFII-GFAP Cre$^-$ mice treated with CCl_4 for 4 weeks or after bile duct ligation. (F) Seventy two hours after CCl_4 injection HSC were isolated from transgenic mice. Total RNA was extracted and RT-PCR was performed with specific primers. Data are from three independent experiments and indicated as average ± SD. * P < 0.05 or higher degree of significance versus control.

Figure 6. COUP-TFII expression in HSC regulates proangiogenic crosstalk with SEC. (A) SEC migration was measured using transwell coculture system. Transwell filters with adherent SEC monolayer were applied on transfected HSC culture 72 hours after transfection. Data are represented as average ± SD, * P<0.05 or higher degree of significance versus empty vector transfected cells. ** P< 0.03 versus COUP-TFII$_{wt}$ transfected cells. (B) SEC tubulogenesis was evaluated after 6 hours incubation in conditioned media obtained from transfected HSC. Data are represented as average ± SD, ** P< 0.02 versus COUP-TFII$_{wt}$ cells.

Figure 7. **COUP-TFII expression in HSC regulates angiogenesis with a hypoxia and HIF independent manner**. (A) Twenty four hours after transfection with NHE$_4$-TK-Luc reporter, HSC were incubated in strictly controlled hypoxic condition for 24 hours and then harvested for luciferase and CAT activity. *Inset* shows COUP-TFII expression in cultured HSC after 24 hours incubation in hypoxic conditions. (B) HSC were co-transfected with HIF-TK-Luc reporter, COUP-TFII$_{wt}$ or COUP-TFII$_{C134S}$ and then incubated in normoxia or hypoxia condition (3% O_2). *Insect* shows HIF isoforms expression in COUP-TFII$_{wt}$ and COUP-TFII$_{C134S}$ transfected HSC. (C) Inhibition of HIF transcriptional activity in COUP-TFII$_{wt}$ or empty vector transfected HSC was obtained by silencing ARNT. Data are represented as average ± SD, * P < 0.05 or higher degree of significance versus empty vector transfected cells or versus siCon transfected cells. (D) ARNT silencing does not modulate NHE$_4$-TK-Luc reporter activity. * P<0.01 versus empty vector transfected cells. (E) Western Blot shows ARNT silencing in COUP-TFII$_{wt}$ transfected HSC. (F) Culture medium of transfected HSC exposed to hypoxia (3% O_2) for 24 hours was collected and use for SEC tube formation assay. Data are represented as average ± SD, * P<0.01 versus empty vector/siCon transfected cells.

Figure 8. NF-kB is involved in COUP-TFII regulation of proangiogenic response. Inhibition of NF-kB in transfected HSC was obtained by silencing RELA. (A) SEC tubulogenesis was evaluated after 6 hours incubation in conditioned media obtained from transfected HSC. * P<0.02 versus empty vector/siCon transfected cells. Data are represented as average ± SD, ** P< 0.05 versus COUP-TFII$_{wt}$/siCon transfected cells. (B, D) Twenty four hours after transfection total RNA was extracted from transfected HSC and RT-PCR was performed. Data are represented as average ± SD *P < 0.01 versus empty vector/siCon transfected cells, ** P < 0.03 versus COUP-TFII transfected cells or higher degree of significance. (C and F) *De novo* synthesis of IL-8 and VEGF-C were determined in HSC supernatants by ELISA. Data are represented as average ± SD, * P < 0.01 versus empty vector/siCon transfected cells, ** P< 0.05 versus COUP-TFII/siCon transfected cells.

Figure 1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COUP-TFII + α-SMA
COUP-TFII +Vimentin
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Normal
B
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COUP-TFII + Desmin
COUP-TFII + Oil Rd O
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72h
CTRL
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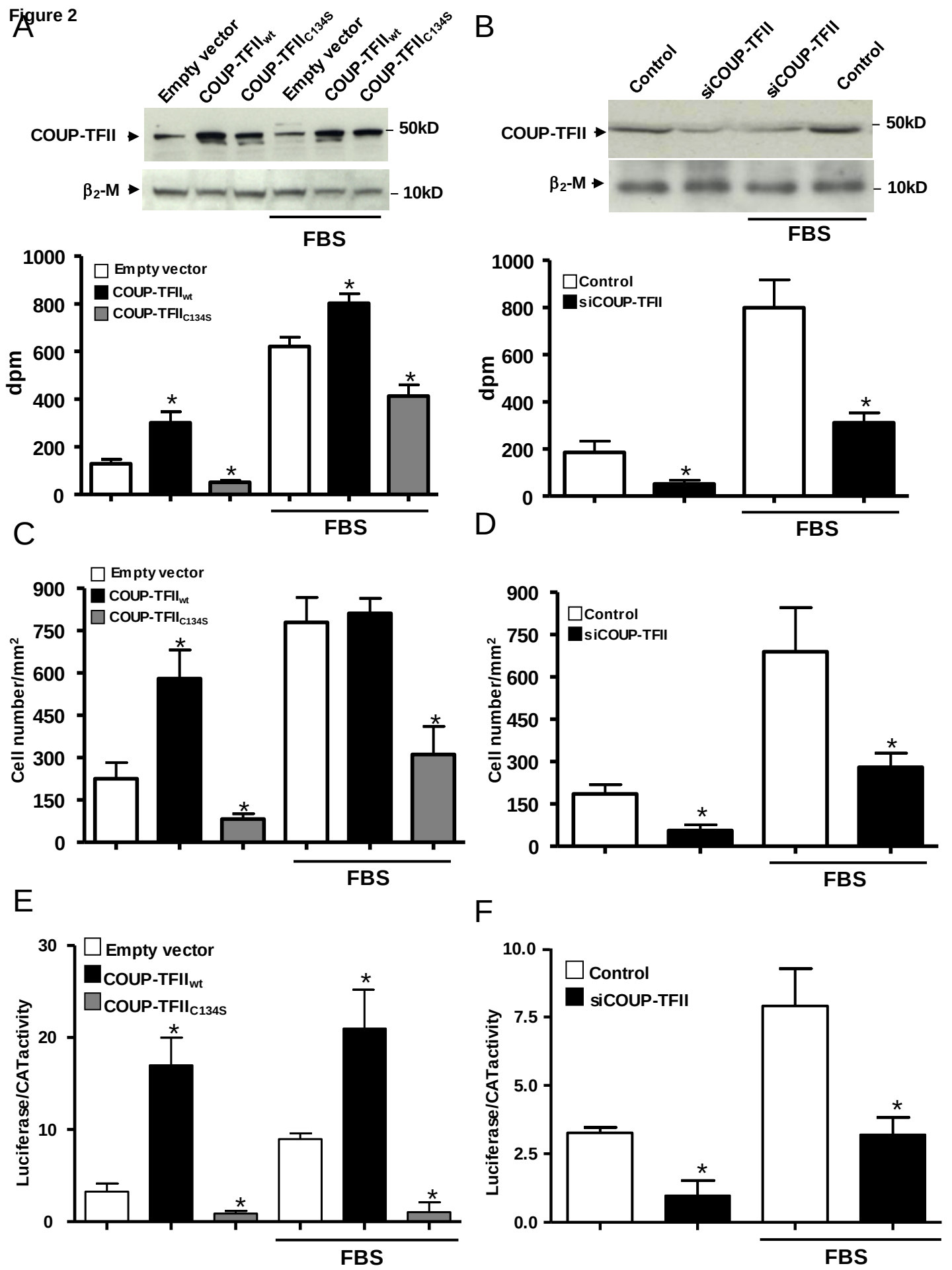
72h
24h
48h
72h
6d
10d

Figure 2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β_2-M
10kD
FBS
B
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Control
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β_2-M
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dpm
Control
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FBS
C
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COUP-TFII$_{C134S}$
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D
Cell number/mm^2
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FBS
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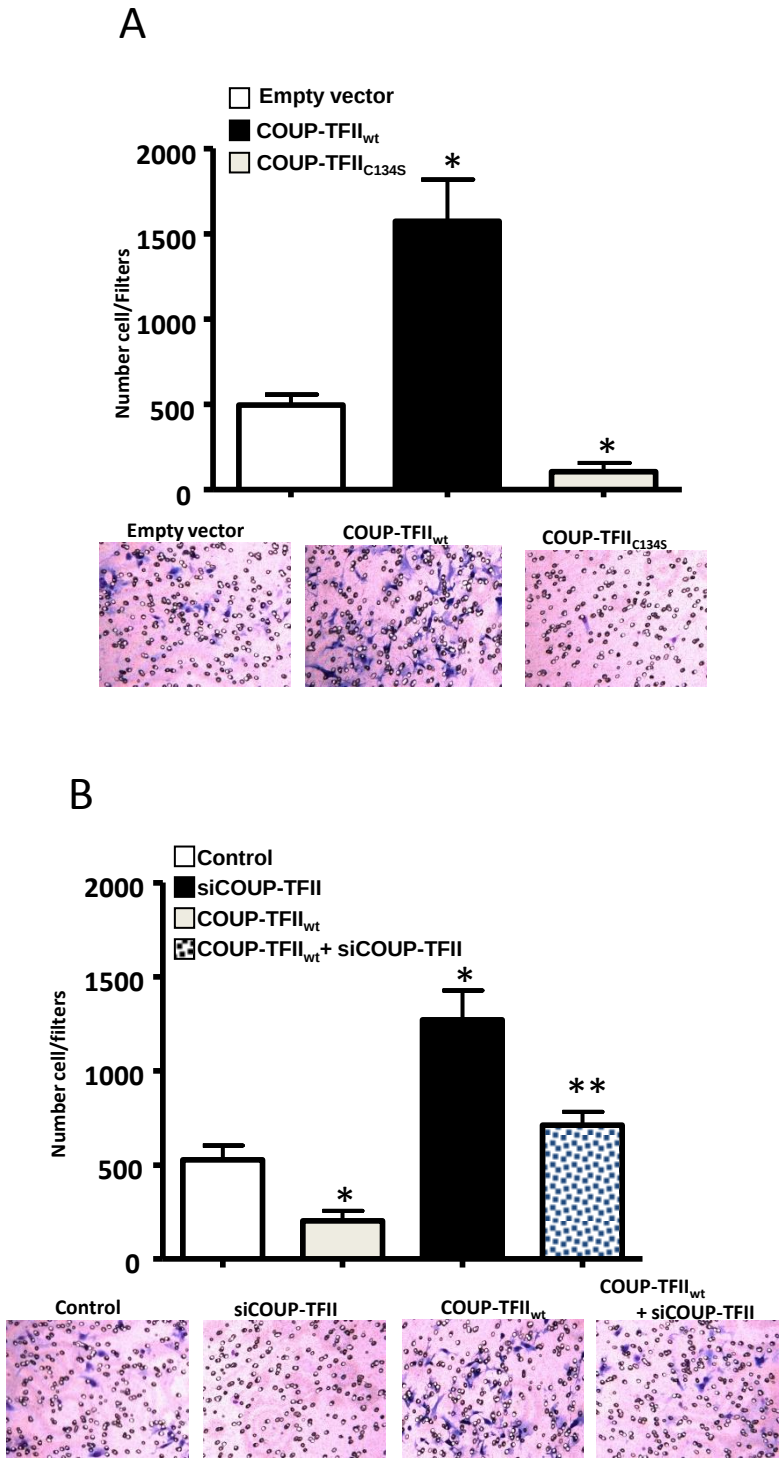
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*
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COUP-TFII$_{C134S}$
B
Control
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COUP-TFII$_{wt}$
COUP-TFII$_{wt}$+ siCOUP-TFII
2000
1500
1000
500
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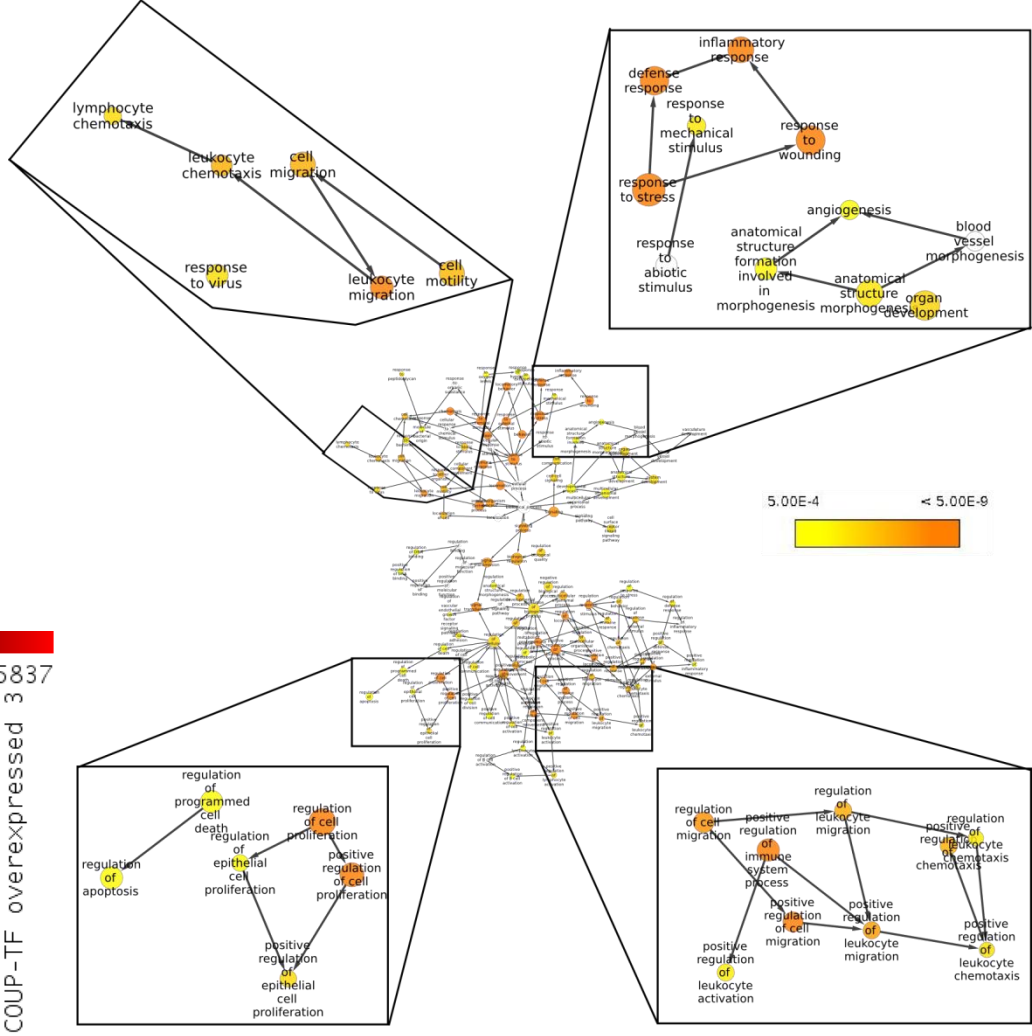
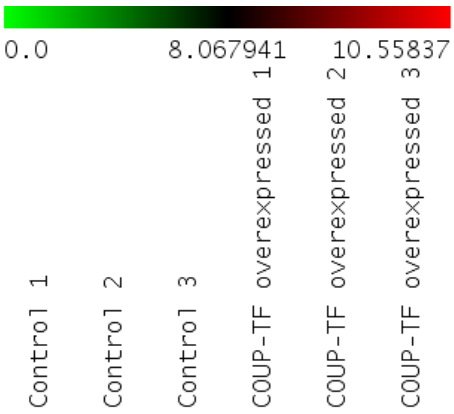
COUP-TFII$_{wt}$
+ siCOUP-TFII

Figure 4





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cathepsin S
fibroblast growth factor 9 (glia-activating factor)
chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
chemokine (C-X-C motif) ligand 2
chemokine (C-X-C motif) ligand 3
intercellular adhesion molecule 1
interleukin 6 (interferon, beta 2)
interleukin 7 receptor
interleukin 8
chemokine (C-X-C motif) ligand 10
integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
laminin, beta 1
matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
angiopoietin-like 4
plasminogen activator, urokinase
chemokine (C-X-C motif) ligand 16
chemokine (C-C motif) ligand 2
chemokine (C-C motif) ligand 5
chemokine (C-C motif) ligand 7
chemokine (C-C motif) ligand 20
chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
chemokine (C-X-C motif) ligand 11
chemokine (C-X-C motif) ligand 5
bone morphogenetic protein 2
tumor necrosis factor, alpha-induced protein 6
vascular endothelial growth factor C
FOS-like antigen 1
interleukin 32
sprouty homolog 1, antagonist of FGF signaling (Drosophila)
CCAAT/enhancer binding protein (C/EBP), delta
tumor necrosis factor (ligand) superfamily, member 13b
basic leucine zipper transcription factor, ATF-like 2
retinol dehydrogenase 10 (all-trans)
fibroblast growth factor 1 (acidic)
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
growth arrest-specific 1
hyaluronan synthase 2
interleukin-1 receptor-associated kinase 2
integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
midkine (neurite growth-promoting factor 2)
phospholipid scramblase 1
podocalyxin-like
XIAP associated factor 1
asporin
secreted frizzled-related protein 1
superoxide dismutase 2, mitochondrial
stanniocalcin 1
toll-like receptor 3
hemicentin 1
regulator of G-protein signaling 5
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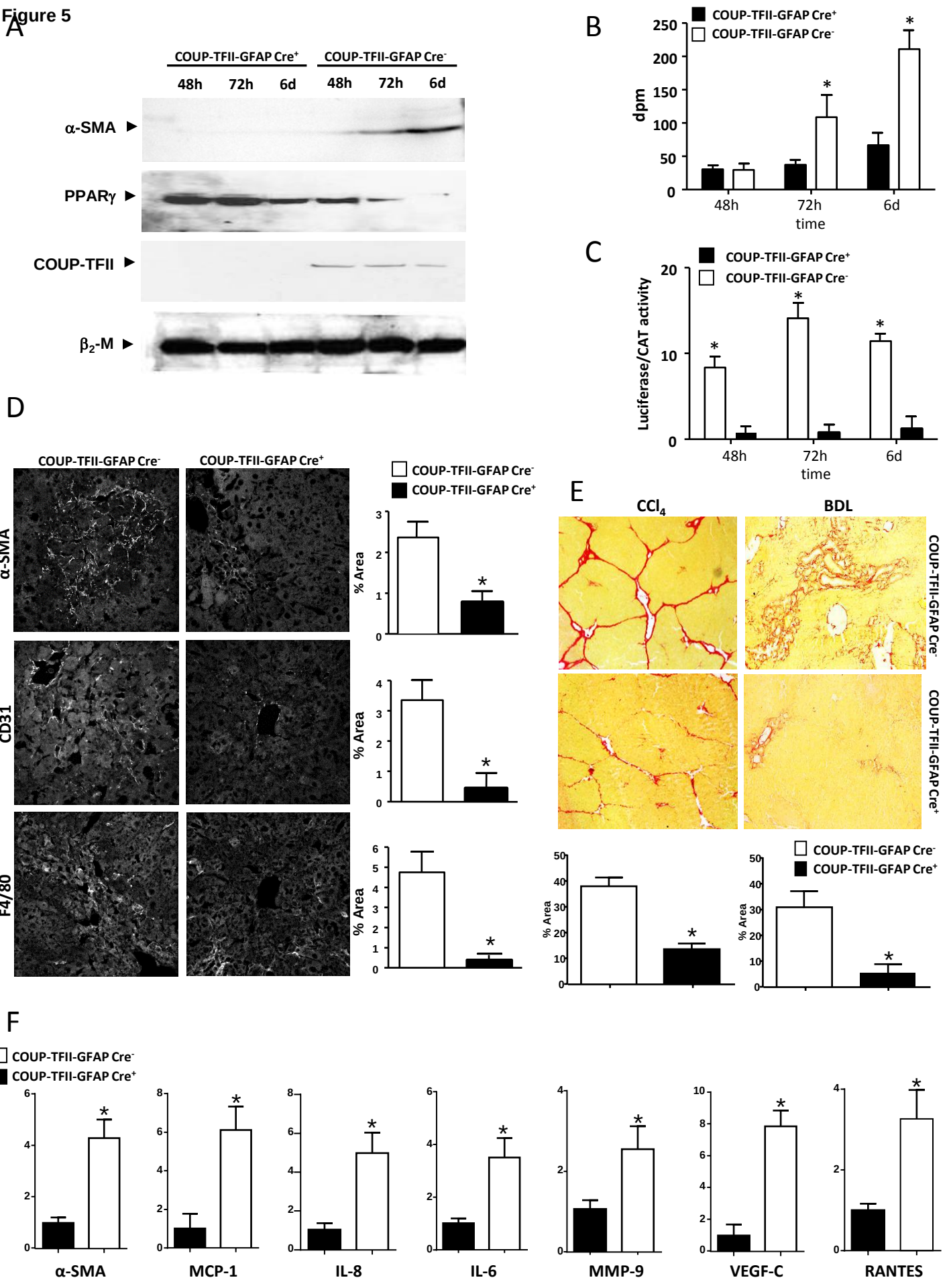
Rho-related BTB domain containing 1

Figure 5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72h
6d
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PPARγ
COUP-TFII
β2-M
B
COUP-TFII-GFAP Cre+
COUP-TFII-GFAP Cre-
dpm
250
200
150
100
50
0
48h
72h
6d
time
C
COUP-TFII-GFAP Cre+
COUP-TFII-GFAP Cre-
Luciferase/CAT activity
20
10
0
48h
72h
6d
time
D
COUP-TFII-GFAP Cre-
COUP-TFII-GFAP Cre+
α-SMA
CD31
F4/80
% Area
E
CCl4
BDL
COUP-TFII-GFAP Cre-
COUP-TFII-GFAP Cre+
% Area
F
COUP-TFII-GFAP Cre-
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IL-6
MMP-9
VEGF-C
RANTES

Figure 6

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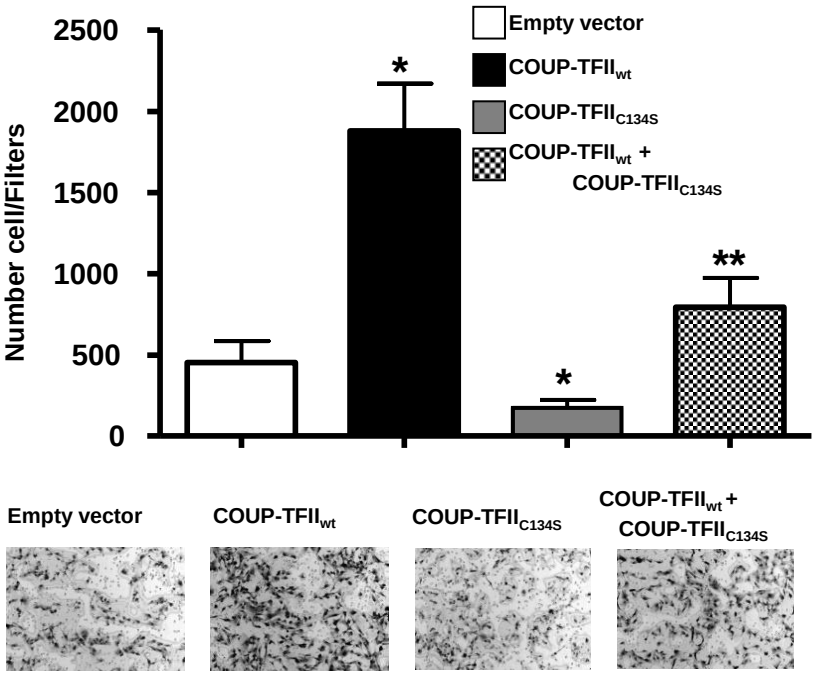
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COUP-TFII$_{wt}$ + COUP-TFII$_{C134S}$
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*
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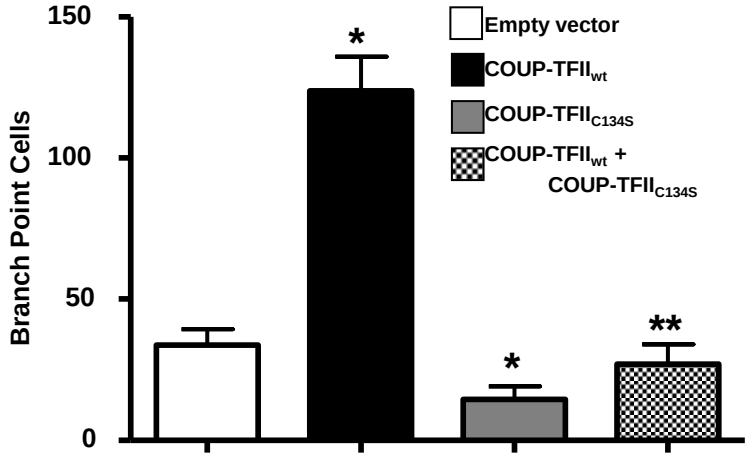
COUP-TFII$_{C134S}$
COUP-TFII$_{wt}$ + COUP-TFII$_{C134S}$

B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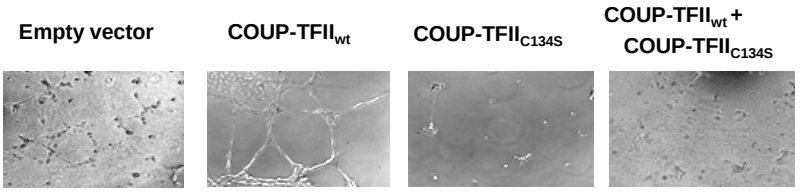
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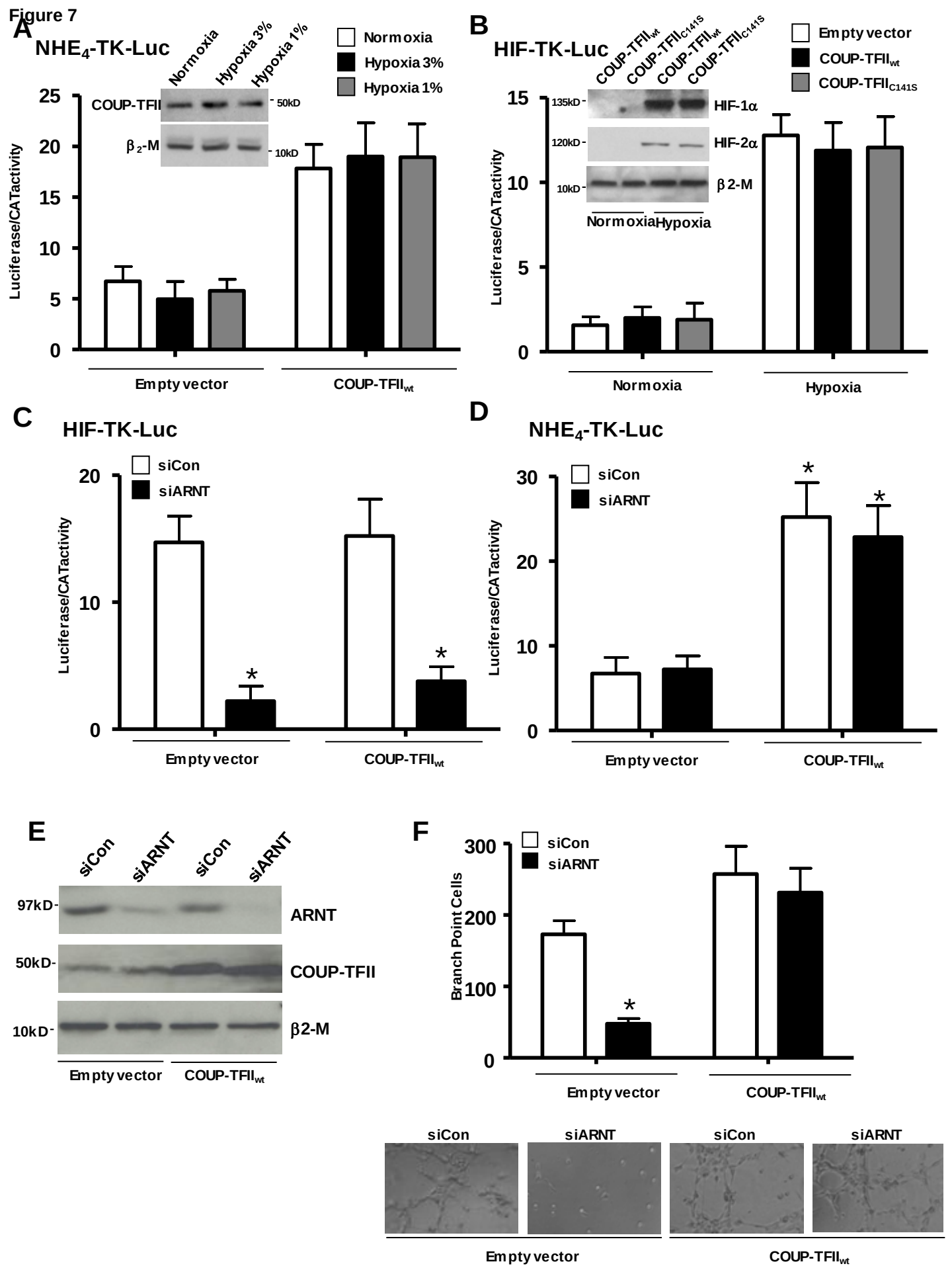
Figure 7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COUP-TFIIC141S
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Hypoxia
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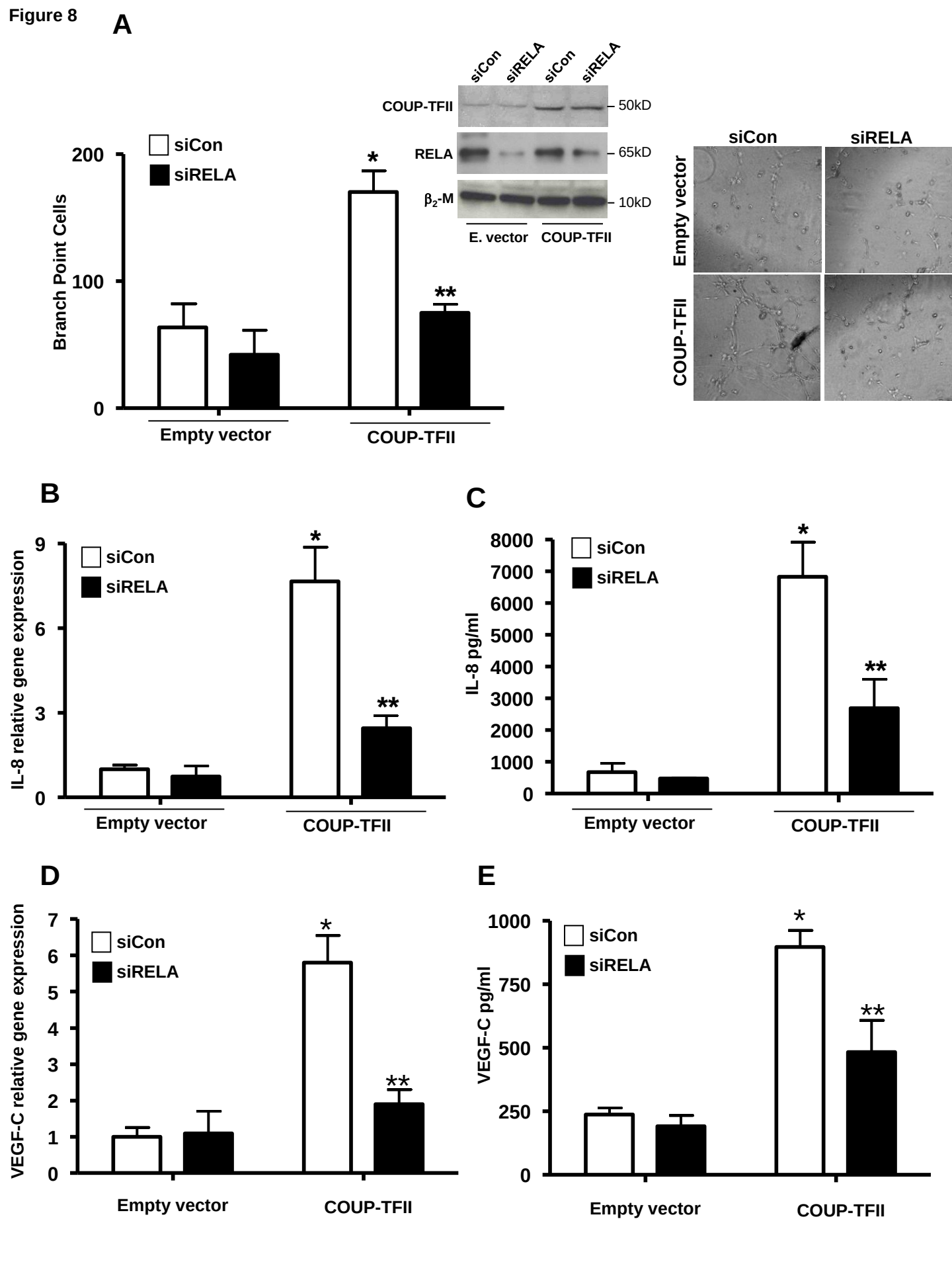
Figure 8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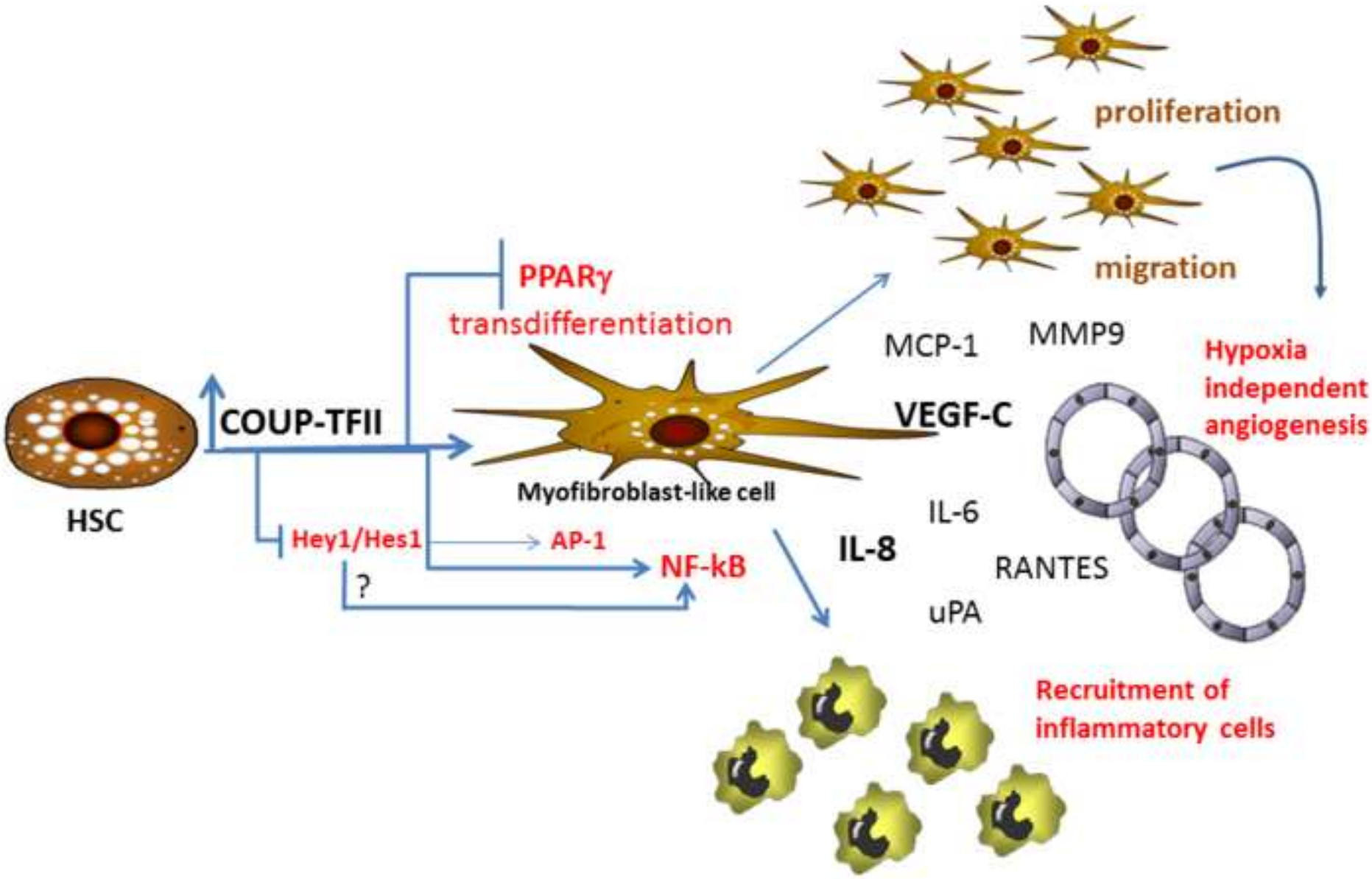
*Graphical Abstract



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