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Aryl Hydrocarbon Receptor-Dependent Induction of Liver Fibrosis by Dioxin

Stéphane Pierre, Aline Chevallier, Fatima Teixeira-Cler, Ariane Ambolet-Camoit, Linh-Chi Bui, Anne-Sophie Bats, Jean-Christophe Fournet, Pedro Fernandez-Salguero, Martine Aggerbeck, Sophie Lotersztajn, et al.

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Aryl hydrocarbon Receptor-dependent induction of liver fibrosis by dioxin

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Abstract

The contribution of environmental pollutants to liver fibrosis is an important and poorly explored issue. *In vitro* studies suggest that the environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin(TCDD) and other Aryl hydrocarbon Receptor(AhR) ligands induce several genes that are known to be up-regulated during liver fibrosis. Our aim was to determine whether exposure to such pollutants can lead to liver fibrosis and to characterize the mechanisms of action. Mice were treated for 2, 14 or 42 days, once a week with 25µg/kg of TCDD. Gene and protein expression, *in vitro* and *in vivo*, as well as liver histology were investigated for each treatment. Treatment of mice with TCDD for 2-weeks modified the hepatic expression of markers of fibrosis such as collagen-1α1 and α-smooth-muscle-actin. This is not observed in AhR KO mice. Following 6-weeks of treatment, histological features of murine hepatic fibrosis became apparent. In parallel, the levels of inflammatory cytokines(interleukin-1-beta, tumor-necrosis-factor-α) and of markers of activated fibroblasts(Fibroblast-Specific-Protein1) were found to be up-regulated. Interestingly, we also found increased expression of genes of the TGFβ pathway and a concomitant decrease of miR-200a levels. Since the transcription factors of the Snail family were shown to be involved in liver fibrosis, we studied their regulation by TCDD. Two members of the Snail family were increased, whereas their negative targets, the epithelial marker E-cadherin and Claudin1, were decreased. Further, the expression of mesenchymal markers were increased. Finally, we confirmed that Snai2 is a direct transcriptional target of TCDD in the human hepatocarcinoma cell line, HepG2. The AhR ligand, TCDD, induces hepatic fibrosis by directly regulating profibrotic pathways.

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Keywords: dioxin; mice; AhR; liver diseases; inflammation

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Abbreviations: Aryl hydrocarbon Receptor (AhR); TetraChloroDibenzo-p-Dioxin (TCDD)

24

1 Introduction

2 Alcohol abuse and non-alcoholic fatty liver diseases are among the predominant causes of chronic liver diseases
3 (CLD). Schematically, these conditions generate injuries of the hepatic tissue thereby leading to inflammation
4 and fibrogenesis. Progression of fibrosis disrupts the liver architecture ultimately leading to cirrhosis, portal
5 hypertension and hepatocellular carcinoma. It is generally accepted that liver fibrosis is the consequence of a
6 sustained wound healing response to chronic injury. It is characterized by an abnormal accumulation of
7 extracellular matrix (ECM) of altered composition that results from an increased production of collagen fibers
8 (Brenner *et al.*, 2000; Lotersztajn *et al.*, 2005). Fibrosis is the consequence of an intense proliferation and
9 accumulation of hepatic myofibroblasts. These cells originate from different populations, among which are
10 activated hepatic stellate cells (HSC), portal fibroblasts and bone marrow-derived cells. Recent *in vitro* and *in*
11 *vivo* investigations using cultured primary hepatocytes or lineage-tracing experiments, have indicated that
12 hepatocytes display significant reversion of their epithelial phenotype and acquire characteristics of
13 mesenchymal cells, which was suggested to be one of several mechanisms leading to liver fibrosis (Choi and
14 Diehl, 2009; Ikegami *et al.*, 2007; Rowe *et al.*, 2011; Taura *et al.*, 2010; Zeisberg *et al.*, 2007).

15 Although several epidemiological studies suggest that the progression of CLD to fibrosis is enhanced by
16 environmental factors (Altamirano and Bataller, 2010; Mallat and Lotersztajn, 2009; Pessione *et al.*, 2001;
17 Zein, 2010), the underlying mechanisms of action are poorly understood. In particular, it is unclear whether
18 increased risk for fibrosis results from the liver toxicity of chemicals, from indirect systemic effects, such as
19 insulin resistance, or from a more targeted effect of some of these chemicals on the fibrogenic pathways. In
20 addition, these observations raise a more general question as to whether environmental pollutants contribute to
21 CLD. One way to address this issue is to explore the effects of xenobiotic signalling pathways on the biological
22 targets associated with the development of fibrosis. One of the most important receptors for xenobiotics is the
23 Aryl hydrocarbon Receptor (AhR) (Barouki *et al.*, 2007), a cytosolic, ligand-activated transcription factor. The
24 AhR has a very large repertory of ligands, including dioxins and polychlorinated biphenyls (PCBs) as well as
25 several hydrocarbons present in cigarette smoke and air particles. Upon activation, the AhR forms a
26 heterodimeric complex with the AhR Nuclear Translocator (ARNT) which binds to specific Xenobiotic
27 Responsive Elements (XRE) and induces the transcription of target genes including xenobiotic metabolizing
28 enzymes (XME) (Barouki *et al.*, 2007). In addition to detoxification functions, recent *in vitro* studies have
29 shown that the activation of the AhR elicits diverse responses such as the alteration of cellular morphology, cell
30 migration and plasticity (Bui *et al.*, 2009; Carvajal-Gonzalez *et al.*, 2009; Diry *et al.*, 2006; Tomkiewicz *et al.*,

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3 12012). These alterations could mediate part of the long-term toxicity of the AhR ligands in humans and animals
4
5 2(Connor and Aylward, 2006; Denison *et al.*, 2011). One of the most potent AhR ligands is 2,3,7,8-
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7 3TetraChloroDibenzo-p-Dioxin (TCDD), a highly persistent AhR activator. Both in humans and in animals,
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9 4exposure to TCDD leads to several toxic effects such as carcinogenesis and alteration of liver functions
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11 5including lipid (steroid, phospholipid and fatty acids) and carbohydrate metabolism (Boverhof *et al.*, 2006; He
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13 6*et al.*, 2013; Mandal, 2005; Marinkovic *et al.*, 2010; Nault *et al.*, 2013; Okey, 2007). Interestingly, one of
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15 7those studies shows that over-activation of the AhR in the liver, leads to an increased expression of several
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17 8fibrogenic genes (He *et al.*, 2013). Therefore, we hypothesized that activation of the AhR could trigger
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19 9biological pathways associated with liver fibrosis.

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21 10In the present work, we show that the AhR ligand, TCDD, regulates the expression of several fibrosis,
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23 11inflammatory, epithelial and mesenchymal markers in murine liver and that exposure to TCDD leads to liver
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25 12fibrosis.

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1Materials and Methods

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4 **2Animals, treatments and tissue extraction.** The animal treatment protocol was approved by a bioethics
5 committee (n° CEEA34.MA.003.12). Male mice (7 weeks old) were injected i.p. once with 200µL of either
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7 3TCDD (25 µg per kg of body weight, which leads to a blood concentration of TCDD after 48h, ranging from
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9 4100 to 1000 ppt according to the Emond's physiologically based pharmacokinetic model (Emond *et al.*, 2005))
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11 5or the vehicle (corn oil). TCDD (99% in Nonane) was purchased from LCG Promochem (Molsheim, France).
12
13 6Subchronic treatment consisted of injections of TCDD (25 µg/kg body weight) or corn oil on days 0 and day 7
14
15 7and mice were sacrificed at day 14. A longer treatment period consisted of injections of TCDD (25 µg/kg b.w.)
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17 8or corn oil on days 0, 7, 14, 21, 28, and day 35 (six total injections) and the mice were sacrificed at day 42. The
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19 9livers were removed immediately and frozen in liquid nitrogen. AhR knockout mice were provided by Dr Pedro
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21 10Fernandez-Salguero (University of Extramadura, Spain). The establishment, characterization and behavioral
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23 11analysis of these transgenic mice have been described previously (Fernandez-Salguero *et al.*, 1995).
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14 **14Histology and immunohistochemistry.** Livers were fixed with paraformaldehyde and embedded in paraffin.
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16 15Tissue sections (4 µm) were stained with hematoxylin-eosin or picro-sirius red for visualization of hepatic
17
18 16collagen. Immunostaining of smooth muscle α -actin was performed using the Vector M.O.M immunodetection
19
20 17kit (Vector Laboratories) and a monoclonal antibody directed against α -SMA (Sigma, 1:1000 dilution) followed
21
22 18by detection with the UltraVision LP detection kit (Thermo Scientific) using metal-enhanced diaminobenzidine
23
24 19(DAB) substrate (Thermo Fisher). Slides were counterstained with aqueous hematoxylin.
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21 **21RNA and miRNA extractions from liver tissue.** Thirty mg of liver tissue were used for the extraction. One
22
23 22mL of trizol (Invitrogen) and 2 stainless steel beads (Qiagen) were used to crush the sample with a Tissuelyser
24
25 23system (Retsch MM300, Germany). After 5 minutes of incubation at room temperature, 200 µL of chloroform
26
27 24were added. The samples were vortexed and then centrifuged at 12,000 rpm for 15 minutes at 4°C. For the RNA
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29 25samples, one volume of ethanol 70% was added to the upper phase. Finally, total mRNA was extracted using the
30
31 26RNeasy mini kit (Qiagen). For the miRNA samples, one volume of isopropanol was added to the upper phase,
32
33 27samples were vortexed and then centrifuged at 12,000 rpm for 30 minutes at 4°C. Pellets were washed with one
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35 28volume of ethanol 70% and centrifuged 15 minutes at 12,000 rpm for 30 minutes at 4°C. Pellets were air-dried
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37 29at room temperature. DNase I (RNase-Free DNase set, Qiagen) treatment at 37°C for 15 minutes was
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39 30performed. Finally, total RNA were dissolved in sterile water.

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3 11:10000). Immunopositive bands were visualized on X-ray films using an alkaline phosphatase-linked
4 secondary antibody (Applied Biosystems, T2191 or T2192, 1:10000) and an enhanced chemiluminescence
5 system CDP-Star® (Applied Biosystems). Quantification was performed with ImageJ
6
7 (<http://rsb.info.nih.gov/ij/>).
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11 **6Gelatin zymography.** Proteins were extracted from the tissues (see immunoblotting), except that protease
12 inhibitors were omitted. Samples (10 µg) were electrophoresed on a 1 mg/ml Gelatine-acrylamide-SDS gel (Bui
13 *et al.*, 2009).. The gel was then incubated at 37°C overnight in an incubation buffer (50mM Tris-HCl pH7.4,
14 95mM CaCl₂, 0.02% NaN₃, 1µM ZnCl₂, 0.015% Brij35, 2.5% Triton X100). After the incubation, the gel was
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16 stained with Coomassie blue for 1h and destained with 10% acetic acid, 40% methanol for 1h at room
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18 temperature and then with 10% acetic acid, 5% methanol for 1h. A clear zone of gelatin digestion indicated
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20 matrix metalloproteinase activity. Finally, the gel was scanned, using a Canoscan Lide 500F.
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26 **14ELISA cytokine assay.** Proteins were extracted from the livers (Procarta cytokine assays, Panomics,
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28 Affymetrix). Equal amounts of total proteins (10 µg) were mixed with antibody-conjugated beads for 30
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30 minutes. A detection antibody was added for 30 minutes, then Streptavidin-Phycoerythrine (PE) for 30 minutes.
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32 Finally, using a Luminex instrument (Luminex 200 Total System, Centre des Cordeliers), we measured the
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34 quantity of cytokines.
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37 **20Chromatin immunoprecipitation assay.** HepG2 cells were seeded into 150-mm-diameter dishes
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39 (approximately 20x10⁶ cells per dish) 48h before being treated or not with 25 nM TCDD for 15, 45 or 75 min.
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41 The cells were then processed for the ChIP assay as previously described (Bui *et al.*, 2009). The XRE site is
42
43 located between -248 and -243 bp but it was impossible to design primers, which encompass the XRE;
44
45 therefore, we designed two different constructs close to the XRE region to perform the ChIP experiments.
46
47 Indeed, ChIP DNA was analyzed by quantitative PCR using specific primers covering the sequences between –
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49 **26239 bp and +4 bp** of the human Snai2 promoter region, located downstream the XRE (forward primer: 5’-
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51 TCACCGAGCGAGGTTAC-3’ and reverse primer 5’-AACTGAGCCCGTTTTGG-3’) and the region between
52
53 **28–195 bp and –48 bp** of the human Snai2 promoter region just after the XRE (forward primer: 5’-
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55 AAACAGCCCATTTTGAACC-3’ and reverse primer 5’- GGCTAAGGATTCAGCAGCAA -3’).
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3 **1Statistical analysis.** The results are expressed as the means \pm standard deviations for at least three different
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5 2experiments. Differences between groups were analyzed by the Mann-Whitney test (non-parametric comparison
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7 3of 2 independent series) or the Kruskal-Wallis test (non-parametric comparison of k independent series). A p-
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9 4value < 0.05 was considered to be statistically significant.
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1 Results

2 TCDD stimulates liver fibrosis.

3 Since several epidemiological studies suggest that environmental factors might enhance fibrosis, we treated
4 mice for a short time (2 weeks of treatment) with TCDD, an environmental pollutant and a ligand of the Aryl
5 hydrocarbon Receptor (AhR) and then measured the expression of two markers for fibrosis in the liver (collagen
6 $\alpha 1$, α -smooth muscle actin) (Akiri *et al.*, 2003; Lotersztajn, Julien, Teixeira-Clerc, Grenard and Mallat,
7 2005). Quantitative RT-PCR showed that the expression of both markers increased significantly after treatment
8 with TCDD (Figure 1C). This is not observed in AhR KO mice (Figure 1C). After 2 weeks of TCDD treatment,
9 we also examined the histology of the livers of the mice using Hematoxylin and eosin staining. There was a
10 cellular infiltration at the periphery of the portal veins upon TCDD treatment (Figure 1A) but without significant
11 signs of fibrosis despite the increase in the expression of markers of fibrosis. Therefore, we investigated whether
12 the increased expression of those markers could lead to fibrosis after longer periods of exposure. The levels of
13 both fibrosis markers after 6 weeks of treatment was greater than that observed 14 days after treatment with two
14 doses of TCDD (Figure 1B). Furthermore, treatment with 25 $\mu\text{g}/\text{kg}/\text{week}$ TCDD for 6 weeks led to changes in
15 the morphology of the liver surface (loss of color, granular surface) (data not shown) and histological analysis
16 reveals liver fibrosis (Figures 1B).

17

18 TCDD stimulates liver inflammation.

19 Since we observed a cellular infiltration at the periphery of the veins upon 2 weeks of treatment with TCDD
20 (Figure 1B), we also measured the hepatic expression of the genes of several inflammatory cytokines which
21 stimulate fibrosis (Coussens and Werb, 2002). After only two weeks of treatment with TCDD, the levels *IL1 β* ,
22 *MCP1* and *TNF α* were increased (10-, 11- and 12-fold, respectively, Figure 2A). In addition, the amounts of the
23 *IL1 β* and *MCP1* proteins were increased (3-fold and 9-fold, respectively Figure 2B). We also quantified the
24 level of Fibroblast Specific Protein 1 (FSP1) (Helfman *et al.*, 2005), which is a marker of activated fibroblasts
25 (Sugimoto *et al.*, 2006). Liver *FSP1* mRNA levels were increased (4-fold) 14 days after treatment with TCDD
26 (Figure 2C). Further, we found that, following TCDD treatment, there was an increase in the expression of
27 *TGF β* (6.3 fold), an inducer of fibrosis, as well as in one of its target genes, *ZEB2* (4.8 fold) (Figure 2D).
28 Recently, it has been shown that *TGF β* inhibits the expression of several members of the microRNA 200 family
29 which are inhibitory regulators of *ZEB2* gene expression. Thus, we measured the level of expression of the five

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3 members of the miR200 family in the livers of treated and untreated mice. The miRNA 200a level was
4 significantly and specifically reduced (51%) in TCDD-treated mice (Figure 2E).

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7 Finally, several fibrosis and inflammatory markers were quantified by RT-PCR after 6 weeks of exposure to
8 TCDD and all were shown to be induced by this treatment (Table 1). In several cases, the level of induction was
9 greater than that following 2-weeks of treatment.

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12 These results strongly suggest that exposure to TCDD induces both liver inflammation and fibrosis molecular
13 markers.

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19 **Epithelial and mesenchymal markers are regulated by TCDD *in vivo*.**

20 We investigated the expression of epithelial and mesenchymal markers in the livers of 2-weeks TCDD-treated
21 mice. Snail family members are transcriptional repressors of epithelial markers. The levels of mRNA of two
22 members of the Snail family, *Snai1* and *Snai2*, were significantly increased by TCDD treatment (6.5 and 2.0
23 fold respectively, Figure 3A) whereas the levels of the mRNA of their targets, *E-cadherin* and *Claudin1*, were
24 significantly decreased (50% and 80% respectively, Figure 3C). In parallel, the amount of Snai1 protein was
25 increased approximately 1.6 fold after TCDD treatment as compared to untreated mice (Figure 3B) whereas a 2-
26 fold decrease in the E-cadherin protein level was observed (Figure 3D).

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29 We next studied the effect of TCDD on mesenchymal markers. The matrix metalloproteinases 2 and 9 (MMP-2
30 and MMP-9) are two gelatinases which are involved in the degradation and remodeling of the extracellular
31 matrix (Chen and Parks, 2009). The levels of both *MMP-2* and *MMP-9* mRNA increased 5- and 8-fold,
32 respectively, following treatment with TCDD (Figure 3E). Measurement of the activity of these enzymes by
33 gelatin zymography showed that the activities of both enzymes were enhanced (2- and 4-fold, respectively)
34 following treatment with TCDD (Figure 3F). We also observed a 6-fold increase in the level of vimentin mRNA
35 (Figure 3G) and a 2-fold increase in the protein (Figure 3H) in the livers of the TCDD-treated animals as
36 compared to the untreated ones (Kokkinos *et al.*, 2007).

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39 Taken together, these data clearly demonstrate that dioxin up-regulates, *in vivo*, the expression of mesenchymal
40 markers and down-regulates epithelial markers.

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52 **TCDD stimulates *Snai2* expression through activation of the AhR**

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54 In order to gain mechanistic insight into the effect of dioxin on the expression of fibrosis related genes, we
55 carried out *in vitro* studies. Since we showed previously that treatment of the human hepatocarcinoma cell line,
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3 1HepG2 with TCDD leads to a decrease of E-Cadherin expression, we investigated whether members of the Snail
4 2family were also regulated in this cell line. We found that the expression of *Snai2* is, indeed, up-regulated by
5 3TCDD, in agreement with the observations of Ikuta *et al* in the human keratinocyte cell line HaCAT (Ikuta and
6 4Kawajiri, 2006). The dose-response analysis showed that, following 48h of treatment, the levels of both *Snai2*
7 5mRNA and protein increased as a function of TCDD concentration and that this increase was maximal at 100
8 6nM (Figure 4A and 4B). A significant hepatic induction of *Snai2* was observed after 48h and 120h of treatment
9 7in mice (supplementary figure 1A). Interestingly, the 48h induction was not observed in AhR KO mice
10 8(supplementary figure 1B) indicating an AhR-mediated effect. Following treatment of HepG2 cells with 25 nM
11 9TCDD, the levels of both *Snai2* mRNA and protein increased as a function of time (Figure 4C and 4D). The
12 10induction of *Snai2* is specific as it is not observed with *Snai1* (supplementary figure 2). We carried out ChIP
13 11experiments in the *Snai2* promoter region, which contains putative XREs, in order to prove that *Snai2* was a
14 12direct transcriptional target of the AhR. AhR binding significantly increased, as compared to controls, 15 and 45
15 13minutes after treatment with TCDD (Figure 4E and 4F). This suggests that the AhR is directly involved in the
16 14regulation of *hSnai2* in the HepG2 *in vitro* model.
17 15Taken together, these results demonstrate that, following activation by its ligand (TCDD), the AhR up-regulates
18 16the expression of *Snai2* both in the HepG2 *in vitro* model and in a mouse *in vivo* model.
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1 Discussion

2 Environmental pollutants have been implicated in liver carcinogenesis, steatosis and in the alteration of liver
3 functions including metabolism (Lee *et al.*, 2010). Little is known about their effects on liver fibrosis despite the
4 public health relevance of this disease. Despite this lack of knowledge, it is assumed that some chemicals
5 (notably CCl₄) could directly activate the biological pathways that elicit a fibrotic state in liver. Here we show
6 that, in the mouse liver, dioxin up-regulates the expression of the genes encoding the fibrosis markers Colla1
7 and α -SMA, and the inflammatory markers IL-1 β , TNF α and MCP1 and elicits histological features of fibrosis
8 following 6 weeks of treatment (one injection of TCDD per week). These data clearly suggest that sustained
9 exposure to TCDD leads to the development of hepatic fibrosis in mice, which is probably mediated by the AhR
10 since this receptor is the only known target of dioxin and since many of these effects are not observed in AhR
11 KO mice.

12 Other studies have focused on liver steatosis and inflammation. Large-scale studies in mice and rats have
13 recently shown that exposure to TCDD in rodents can lead to the disruption of molecular pathways involved in
14 hepatic lipid (steroid, phospholipid and fatty acid) and carbohydrate metabolism (gluconeogenesis), which is
15 consistent with TCDD-elicited hepatic steatosis (Nault *et al.*, 2013; Boverhof *et al.*, 2006). Those observations are
16 important as nonalcoholic steatohepatitis (NASH) is an increasingly prevalent disease characterized by steatosis
17 as well as inflammation and ultimately fibrosis. Its pathogenesis remains incompletely understood. A mouse
18 model based on the methionine- and choline-deficient (MCD) diet, is commonly used to study NASH. A recent
19 investigation by He *et al.*, showed that transgenic or pharmacological AhR activation sensitized mice to MCD
20 diet-induced NASH. Similarly to our study, the authors observed increased expression of fibrogenic (collagen
21 α 1, α 2, α -SMA), inflammatory (IL1 β , IL6), or mesenchymal (TGF- β) markers with a single dose of TCDD
22 (10 μ g/kg) coupled with an MCD diet for 4 to 6 weeks. This is associated with histological features of fibrosis.
23 A two-hit model is commonly proposed to explain the mechanism of NASH. The authors show that AhR
24 activation leads to increased steatosis and oxidative stress due to a decreased activity of superoxide dismutase 2
25 (SOD2). Interestingly, they linked this decreased activity to increased acetylation of this enzyme due to
26 activation of TipARP (TCDD)-inducible poly(ADP-ribose) polymerase, depletion of NAD⁺ and subsequent
27 inactivation of sirtuin deacetylase 3 (Sirt3) (He *et al.*, 2013).

28 The protocol used in the present study (according to the Emond's physiologically based pharmacokinetic model
29 (Emond *et al.*, 2005), it leads to a blood concentration of TCDD after 48h, ranging from 100 to 1000 ppt.) is
30 different from that of He *et al.* since it consists in weekly injections of TCDD instead of a single injection

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3 1coupled to a specific deficient diet. This could explain some of the differences in histological observations.
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5 2However, we cannot exclude that constant exposure to TCDD could alter the metabolism of methionine or
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7 3choline, or lead to repeated production of reactive oxygen species, which setting the stage for liver dysfunction
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9 4and ultimately fibrosis. Current evidence is not sufficient to differentiate between a direct effect of TCDD on
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11 5fibrosis or an indirect effect coupling metabolic disruption, inflammation and disruption of the redox status.
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13 6Several studies have demonstrated the predominant role of stellate cells in fibrosis progression (Bataller *et al.*,
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15 72003; Friedman, 2000; Knittel *et al.*, 1999). However, more recent investigations have suggested that adult
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17 8hepatocytes might also be involved in the increased mesenchymal cells in the liver (Ikegami *et al.*, 2007).
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19 9Indeed, Zeisberg *et al.*, using lineage-tracing experiments, suggested that, upon treatment with CCl₄, which is
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21 10routinely used to induce fibrosis in rodents, a significant number of fibroblasts might be derived from
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23 11hepatocytes in the advanced stages of fibrosis. However, these data have been challenged recently (Taura *et al.*,
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25 122010). Our study did not attempt to address these controversial issues, but rather focused on establishing a
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27 13correlation between the liver fibrosis phenotype elicited by TCDD and the induction by this pollutant of a
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29 14number of genes that are relevant for this condition, including fibrosis and inflammatory markers as well as
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31 15regulatory genes such as those of the Snail family. A very recent report demonstrated that *Snail* expression was
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33 16specifically increased in hepatocytes in response to acute liver challenging and, interestingly, that specific
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35 17hepatocyte inhibition of *Snail* disrupted fibrotic progression (Rowe *et al.*, 2011). In addition, the expression of
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37 18*Snail* triggered growth factor expression, inflammatory processes and biosynthesis of the extracellular matrix
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39 19related to liver fibrosis (Rowe *et al.*, 2011). Recent work from several laboratories, including our own, also have
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41 20shown that TCDD regulates several genes involved in cell motility and plasticity (Bui *et al.*, 2009; Carvajal-
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43 21Gonzalez *et al.*, 2009; Diry *et al.*, 2006; Rowe *et al.*, 2011). We have shown, in particular, that the expression of
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45 22E-cadherin is repressed. Although JNK activation and NEDD9 induction were shown to be involved, the actual
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47 23molecular mechanism of E-cadherin repression by TCDD has not been elucidated completely. Since the Snail
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49 24family of transcription factors is known to be involved in E-cadherin repression and since it is also involved in
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51 25the development of liver fibrosis (Rowe *et al.*, 2011), we have focused, in the present study, on the regulation of
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53 26this family of proteins.
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55 27In order to further delineate the mechanism of TCDD induction of the Snail genes, ie, whether the TCDD effect
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57 28is direct or indirect, we carried *in vitro* experiments using the HepG2 cell line. Using this cell line, we show that
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59 29*Snai2* is induced by TCDD (in agreement with observations made in keratinocytes (Ikuta and Kawajiri, 2006))
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30and that the binding of the AhR to the promoter of the *Snai2* gene increases following treatment with TCDD.

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3 We also show that dioxin up-regulates *Snai1* and *Snai2* (two members of the Snail family), several
4 mesenchymal markers (including MMPs 2 and 9, Fibronectin and Vimentin) and down-regulates two epithelial
5 markers (Claudin1 & E-cadherin). Importantly, *Snai2* is induced by TCDD *in vivo* as early as 48 h of treatment,
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8 in line with the *in vitro* data. This suggests that *Snai2* is a direct target of TCDD and its induction is an early
9 event.

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11 Our data suggest a possible role of *Snai2* induction for the effects of TCDD on liver fibrosis. However, we also
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14 have observed inflammation and this could be triggered by a different mechanism. In addition, we cannot
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17 eliminate the possibility that the liver toxicity elicited by TCDD also could contribute to fibrosis. What our
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20 studies indicate is that at least part of the mechanism of TCDD action is mediated through the direct induction of
21 critical fibrosis-promoting genes.

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23 This paper describes the effects of an AhR ligand on fibrosis *in vivo* and points to a role for activated
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26 AhR in this liver pathology. Interestingly, the AhR KO mice display several developmental abnormalities,
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29 including a decrease in the liver size (Fernandez-Salguero *et al.*, 1995) and a TGF β signaling-dependent fibrosis
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32 (Butler *et al.*, 2012; Zaher *et al.*, 1998). Although these observations may seem paradoxical at first sight, they
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35 do, in fact, support the hypothesis that TCDD and possibly other related xenobiotic ligands exert their effects, at
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38 least partially, by disrupting the normal physiological functions of the receptor (similarly to a knock-out of the
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41 receptor). Interestingly, the phenotypes of AhR KO models and TCDD-treated cells or tissues display other
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44 similarities (Chang *et al.*, 2007). A recent study by Rico-Leo *et al.*, indicates that AhR KO cells (keratinocytes
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47 and mammary cells) display phenotypic abnormalities reminiscent of the epithelial-mesenchymal transition
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50 (EMT) with decreased and increased expression of epithelial and mesenchymal markers respectively (including
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53 for the last category, *Snai2*) (Rico-Leo *et al.*, 2013). Interestingly, Hanada *et al.* also show that liver fibrosis
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56 down-regulates AhR expression which might exacerbate the pathology (Hanada *et al.*, 2012). One interpretation
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59 of these observations is that endogenous ligands of the AhR may display anti-fibrotic effects. In this regard, it is
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noteworthy that other studies have shown that this receptor can bind ligands which antagonize the action of
TCDD and which trigger their own specific effects (Gouedard *et al.*, 2004a; Gouedard *et al.*, 2004b). In line
with this hypothesis, Lehmann *et al.* show that 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester
(ITE), an endogenous AhR ligand, blocks myofibroblast differentiation, a critical step for fibrosis (Lehmann *et al.*, 2011). However, it is also possible that the mechanisms of liver dysfunction and fibrosis in the absence of
the AhR and in the presence of persistent AhR ligands may be different. Indeed, we show that fibrotic genes do

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3 1not respond similarly in TCDD-treated mice and AhR-KO mice (Figure 1). This possibility needs further
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5 2exploration.
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3 **1Author contributions:** Stéphane Pierre & Aline Chevallier were involved in the study concept and design and
4 statistical analysis; Stéphane Pierre was involved in drafting of the manuscript; Stéphane Pierre, Aline
5 Chevallier, Fatima Teixeira-Clerc, Ariane Ambolet-Camoit, Linh-Chi Bui, Anne-Sophie Bats, Jean-Christophe
6 Fournet were involved in the acquisition of data, analysis and interpretation of data; Fatima Teixeira-Clerc,
7
8 Martine Aggerbeck, Sophie Lotersztajn, Pedro Fernandez-Salguero, Robert Barouki were involved in critical
9
10 revision of the manuscript for important intellectual content ; Fatima Teixeira-Clerc, Martine Aggerbeck, Sophie
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12 Lotersztajn, Robert Barouki, Xavier Coumoul were involved in obtained funding ; Martine Aggerbeck, Robert
13
14 Barouki and Xavier Coumoul were involved in study supervision.
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27

28 14

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47 **25Number of figures and tables: 4 figures and 1 table**
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3 **1 Figure legends**

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7 **3 Figure 1: TCDD increases the expression of the hepatic fibrosis markers Coll1A1 and α -SMA and leads to**
8 **4 hepatic fibrosis. A** Wild-type mice were treated on days 0 and 7 with either TCDD (25 μ g/kg) or corn oil
9 5 (control) and sacrificed at day 14. Hematoxylin/eosin staining of liver tissues is shown. Four mice in each group
10 6 were analyzed. Wild-type mice were treated on days 0 – 7 – 14 – 21 – 28 – 35 (6 weeks) with either TCDD
11 7 (25 μ g/kg) or corn oil (control) and sacrificed at day 42. **B.** Picrosirius red staining (upper panel, staining
12 8 collagen) and α -smooth muscle actin (lower panel) staining of liver tissues are shown. Scale bar = 150 μ m. **Four**
13 9 **mice in each group were analyzed. C.** Wild-type or AhR knock-out mice were treated on days 0 and 7 (2
14 10 weeks) or on days 0, 7, 14, 21, 28, and day 35 (6 weeks for the wild-type mice) with either TCDD (25 μ g/kg) or
15 11 corn oil (control) and sacrificed at day 14 or day 42, respectively. Collagen1 α 1 and α -smooth muscle actin
16 12 mRNA levels were measured by qRT-PCR. *, p < 0.05 or **, p < 0.01 as compared to controls. For each qRT-
17 13 PCR experiment, 4 control mice and 4 TCDD-treated mice were used.

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3 **Figure 2: TCDD increases pro-inflammatory cytokines, the expression of FSP1 an hepatic marker of**
4 **2activated fibroblasts and regulates the expression of the hepatic fibrosis inducer, the TGFB gene, and of**
5 **3its gene targets.** Wild-type mice were treated on days 0 and 7 (2 weeks) with either TCDD (25µg/kg) or corn
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7 4oil (control) and sacrificed at day 14. **A.** IL1□, MCP1 and TNF□ mRNA levels were measured by qRT-PCR.
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9 **5B.** The amounts of IL-1□ and MCP1 protein were estimated using an ELISA assay. Proteins are expressed as pg
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11 6of cytokine per mg of total protein. Three mice in each group were used. **C.** FSP1 mRNA levels were measured
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13 7by qRT-PCR. **D.** TGFB and ZEB2 mRNA levels were measured by qRT-PCR. **E.** Treatment with TCDD
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15 8decreased miRNA 200a levels compared to controls as measured by specific qRT-PCR. The levels of mRNA or
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17 9miRNA were normalized to control condition. ***, p < 0.001; **, p < 0.01 and *, p < 0.05 compared to controls.
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19 10Four mice in each group were analyzed for the qRT-PCR experiments.

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3 **1Figure 3: TCDD regulates the expression of epithelial and mesenchymal markers 1 in mouse liver.** Male
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5 2wild-type mice were treated on days 0 and 7 (2 weeks) with either TCDD (25µg/kg) or corn oil (control) and
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7 3sacrificed at day 14. **A.** The *Snai1* and *Snai2* mRNA levels were found to be increased as measured by qRT-
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9 4PCR **B.** The mSnai1 protein was found to be increased following quantification by western blotting **C.** the *mE-*
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11 5*cadherin* and *claudin1* mRNA levels were found to be decreased **D.** and the E-cadherin protein was found to be
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13 6decreased following quantification by western blotting. **E.** The levels of MMP2, MMP9 and **G.** vimentin mRNA
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15 7were found to be increased as compared to controls when measured by qRT-PCR. **F.** The proteolytic activity of
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17 8pro-MMP, MMP2 and MMP9 was found to be increased as compared to controls by gelatin electrophoresis,
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19 9with actin used as a loading control. **H.** Vimentin protein levels were determined to be increased by western
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21 10blotting The levels of mRNA were normalized to control condition. Intensities were normalized by comparison
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23 11to the actin signal using Image J software. ***, $p < 0.001$ **, $p < 0.01$ and *, $p < 0.05$ compared to controls
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25 12($n > 3$). For each experiment, 4 control mice and 4 TCDD-treated mice were used.

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3 **Figure 4: TCDD increases *mSnai2* expression in the mouse liver and in the human HepG2 cell line**
4 **through the AhR pathway. A.** HepG2 cells were treated or not with increasing concentrations of TCDD (0-100
5 3nM) for 48h. The amounts of *Snai2* mRNA as measured by qRT-PCR and **B.** protein as measured by western-
6 4blotting were found to be increased, as compared to controls. **C.** HepG2 cells were treated with 25 nM TCDD or
7 5not for times between 16 and 72h. The amounts of mRNA and **D.** protein were found to be increased as
8 6measured. **E., F.** Chromatin immunoprecipitation (ChIP) was performed on the *Snai2* promoter region. HepG2
9 7cells were treated, or not, with 25 nM TCDD for 15, 45 or 75 min. Gene specific qPCR analysis with two
10 8different couples of primers spanning the AhR binding region of the *hSnai2* gene promoter was performed using
11 9genomic DNA immunoprecipitated with either an AhR or a mock IgG antibody and an equal amount of input
12 10DNA as described in the methods. The results are normalized to the control condition (not treated with TCDD).
13 11*, $p < 0.05$ or **, $p < 0.01$ as compared to controls. Each experiment was repeated 3 times.
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1Table

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3Table 1

mRNA	Fold induction (2w)	Fold induction (6w)
AhR	-	1,6
CYP1a1	-	55372,14*
Collagen 1A1	11,7*	39,31*
α -SMA	3,6*	22,34**
IL-1 β	9,8***	14,07**
MCP1	12,1***	61,82**
TNF- α	13,4***	20,13**
CCR5	-	3,96*
FSP1	4,2***	5,32*
TGF β 1	6,1***	6,49*
Zeb2	4,3**	4,34*
RasGPR1	-	3,98*

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6**Table 1: relative mRNA expression of fibrosis and inflammatory markers in pre-fibrotic and fibrotic**
7**livers of mice subchronically treated with TCDD.** Wild-type mice were treated two times at day 0 and 7 with
8either TCDD (25 μ g/kg) or corn oil (NT) and sacrificed at day 14 (second column) or five times at day 0 – 7 – 14
9– 21 – 28 with either TCDD (25 μ g/kg) or corn oil (NT) and sacrificed at day 35 (third column). Ahr battery
10genes, fibrosis and inflammatory markers (first column) expression were assessed by qRT-PCR. ***, p < 0.001;
11**, p < 0.01 and *, p < 0.05 compared to controls. For each qRT-PCR experiment, 4 control mice and 4 TCDD-
12treated mice were used.

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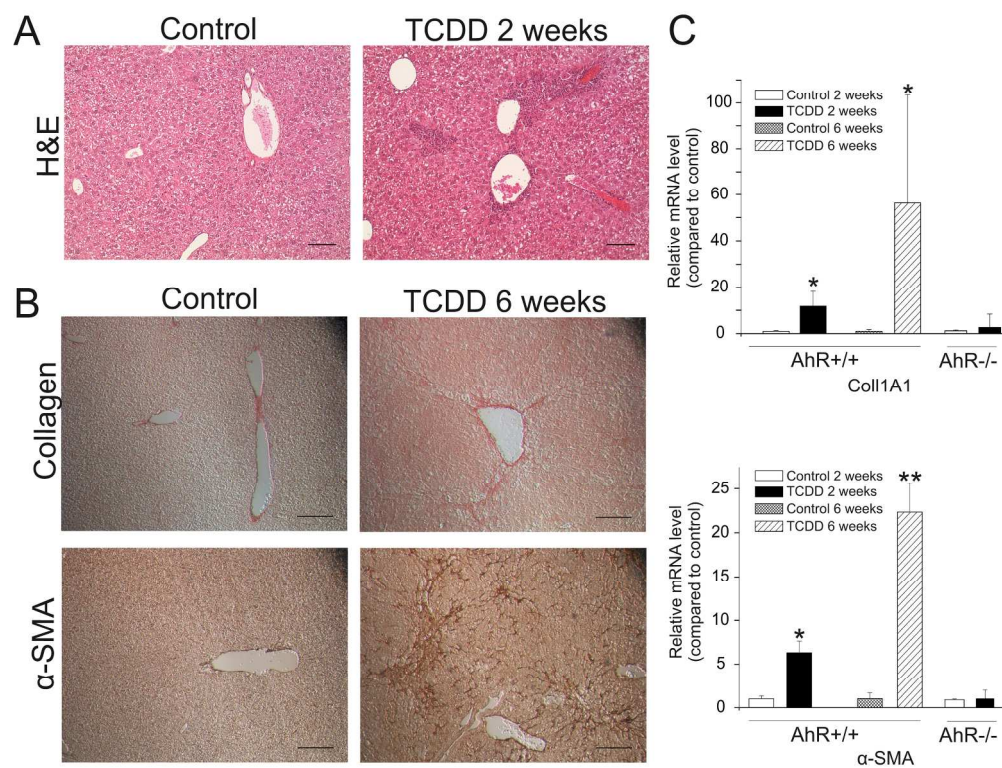
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TCDD increases the expression of the hepatic fibrosis markers Coll1A1 and α -SMA and leads to hepatic fibrosis. A Wild-type mice were treated on days 0 and 7 with either TCDD (25 μ g/kg) or corn oil (control) and sacrificed at day 14. Hematoxylin/eosin staining of liver tissues is shown. Four mice in each group were analyzed. Wild-type mice were treated on days 0 - 7 - 14 - 21 - 28 - 35 (6 weeks) with either TCDD (25 μ g/kg) or corn oil (control) and sacrificed at day 42. B. Picrosirius red staining (upper panel, staining collagen) and α -smooth muscle actin (lower panel) staining of liver tissues are shown. Scale bar = 150 μ m. Four mice in each group were analyzed. C. Wild-type or AhR knock-out mice were treated on days 0 and 7 (2 weeks) or on days 0, 7, 14, 21, 28, and day 35 (6 weeks for the wild-type mice) with either TCDD (25 μ g/kg) or corn oil (control) and sacrificed at day 14 or day 42, respectively. Collagen1a1 and α -smooth muscle actin mRNA levels were measured by qRT-PCR. *, $p < 0.05$ or **, $p < 0.01$ as compared to controls. For each qRT-PCR experiment, 4 control mice and 4 TCDD-treated mice were used.

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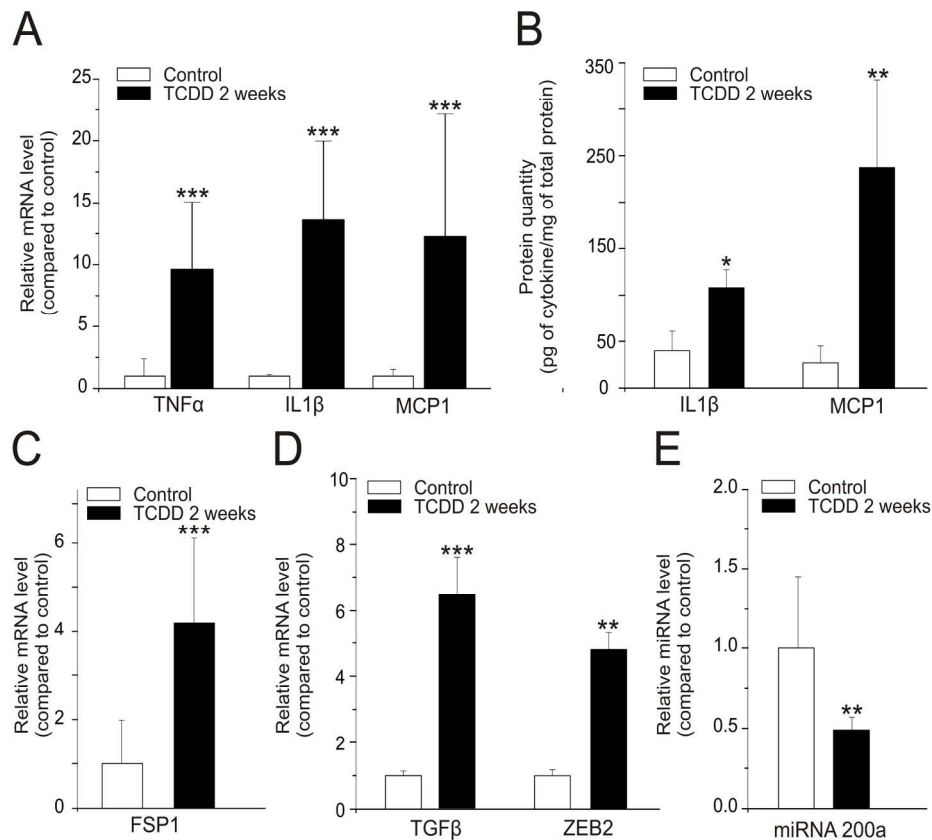


Figure 2: TCDD increases pro-inflammatory cytokines, the expression of FSP1 an hepatic marker of activated fibroblasts and regulates the expression of the hepatic fibrosis inducer, the TGF β gene, and of its gene targets. Wild-type mice were treated on days 0 and 7 (2 weeks) with either TCDD (25 μ g/kg) or corn oil (control) and sacrificed at day 14. A. IL1 β , MCP1 and TNF α mRNA levels were measured by qRT-PCR. B. The amounts of IL-1 β and MCP1 protein were estimated using an ELISA assay. Proteins are expressed as pg of cytokine per mg of total protein. Three mice in each group were used. C. FSP1 mRNA levels were measured by qRT-PCR. D. TGF β and ZEB2 mRNA levels were measured by qRT-PCR. E. Treatment with TCDD decreased miRNA 200a levels compared to controls as measured by specific qRT-PCR. The levels of mRNA or miRNA were normalized to control condition. ***, $p < 0.001$; **, $p < 0.01$ and *, $p < 0.05$ compared to controls. Four mice in each group were analyzed for the qRT-PCR experiments.

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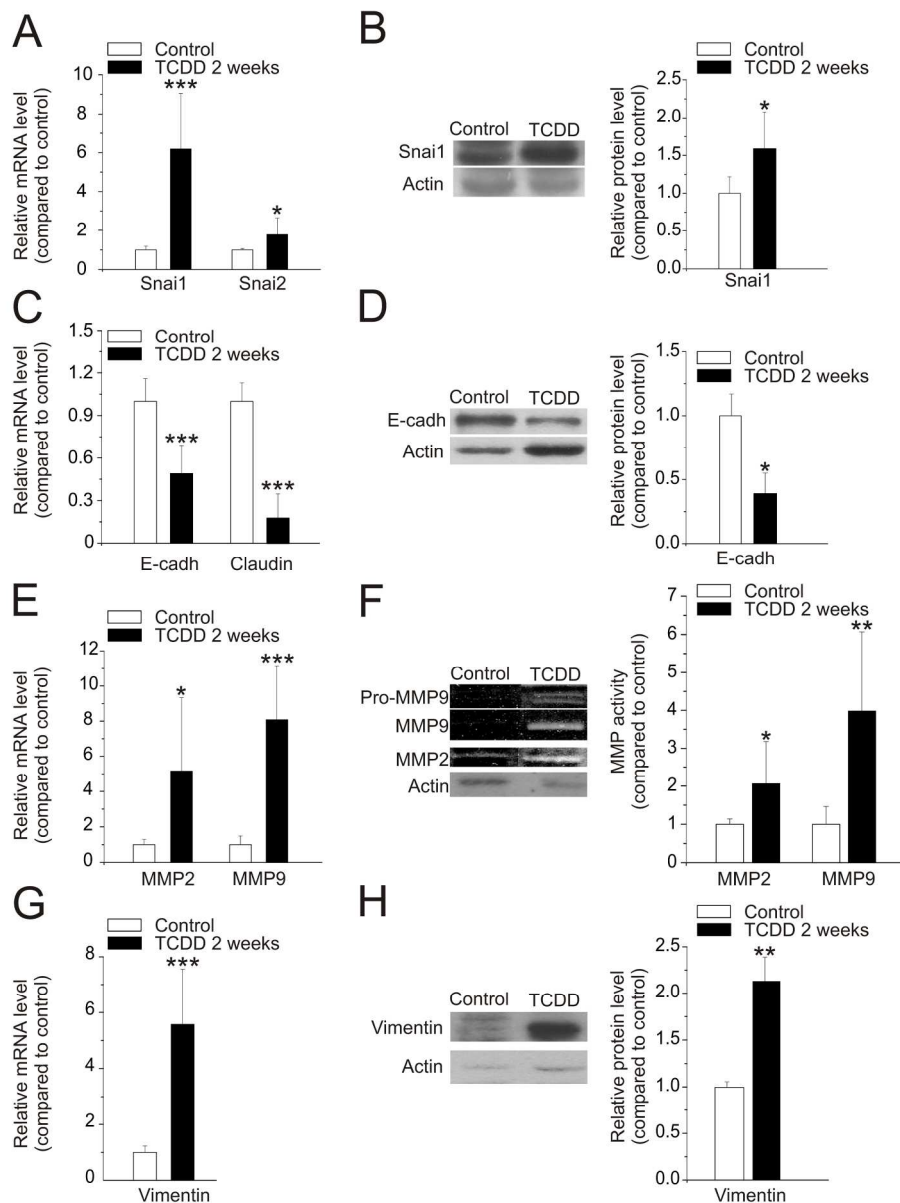


Figure 3: TCDD regulates the expression of epithelial and mesenchymal markers in mouse liver. Male wild-type mice were treated on days 0 and 7 (2 weeks) with either TCDD (25 μ g/kg) or corn oil (control) and sacrificed at day 14. A. The Snai1 and Snai2 mRNA levels were found to be increased as measured by qRT-PCR. B. The mSnai1 protein was found to be increased following quantification by western blotting. C. The mE-cadherin and claudin1 mRNA levels were found to be decreased. D. and the E-cadherin protein was found to be decreased following quantification by western blotting. E. The levels of MMP2, MMP9 and G. vimentin mRNA were found to be increased as compared to controls when measured by qRT-PCR. F. The proteolytic activity of pro-MMP, MMP2 and MMP9 was found to be increased as compared to controls by gelatin electrophoresis, with actin used as a loading control. H. Vimentin protein levels were determined to be increased by western blotting. The levels of mRNA were normalized to control condition. Intensities were normalized by comparison to the actin signal using Image J software. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ compared to controls ($n > 3$). For each experiment, 4 control mice and 4 TCDD-treated mice were used.

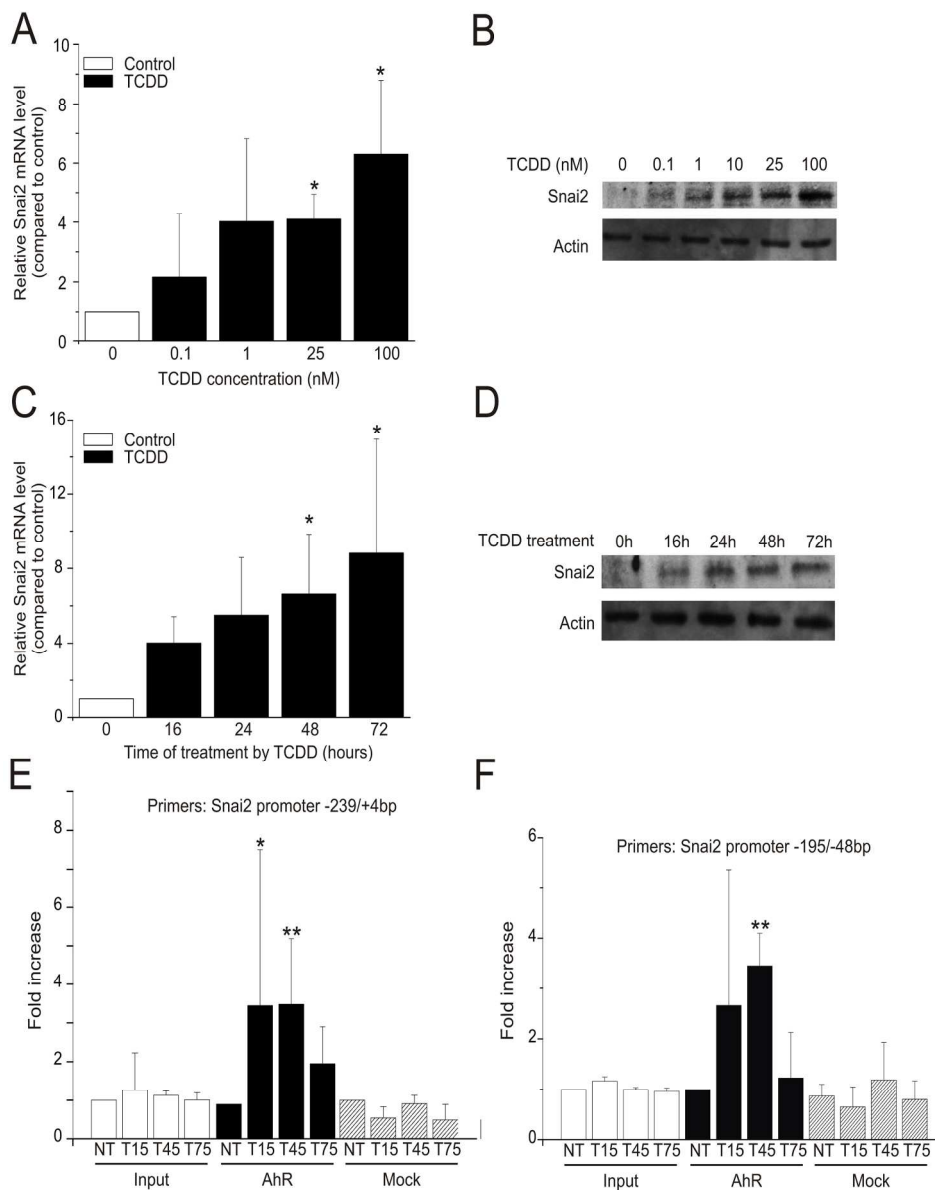


Figure 4: TCDD increases mSnai2 expression in the mouse liver and in the human HepG2 cell line through the AhR pathway. A. HepG2 cells were treated or not with increasing concentrations of TCDD (0-100 nM) for 48h. The amounts of Snai2 mRNA as measured by qRT-PCR and B. protein as measured by western-blotting were found to be increased, as compared to controls. C. HepG2 cells were treated with 25 nM TCDD or not for times between 16 and 72h. The amounts of mRNA and D. protein were found to be increased as measured. E., F. Chromatin immunoprecipitation (ChIP) was performed on the Snai2 promoter region. HepG2 cells were treated, or not, with 25 nM TCDD for 15, 45 or 75 min. Gene specific qPCR analysis with two different couples of primers spanning the AhR binding region of the hSnai2 gene promoter was performed using genomic DNA immunoprecipitated with either an AhR or a mock IgG antibody and an equal amount of input DNA as described in the methods. The results are normalized to the control condition (not treated with TCDD). *, p < 0.05 or **, p < 0.01 as compared to controls. Each experiment was repeated 3 times.

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