Master's Thesis of Pharmacy

Kynurenine Negatively Regulates *TGFB1* Transcription in Hepatic Stellate Cells through AhR

간 성상세포에서 Kynurenine에 의한 AhR 매개 억제적 *TGFB1* 전사 조절

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Abstract

Hepatocyte injury is coupled to the activation of hepatic stellate cells (HSCs), contributing to the pathogenesis of liver fibrosis. In hepatocytes, tryptophan 2,3-dioxygenase (TDO2) is the primary enzyme responsible for the conversion of tryptophan to kynurenine, an endogenous ligand of aryl hydrocarbon receptor (AhR), which may control the activation of HSCs. Transforming growth factor beta (TGF-β) is a key mediator of HSC activation. There are conflicting reports on the effect of AhR on TGF-β regulation depending on cell types and conditions. This study investigated TDO2 expression in hepatocytes, the effect of kynurenine on HSCs, and the role of AhR in TGFB1 transcription in HSCs. When animals were challenged as a single or multiples doses by liver toxicant, TDO2 protein and mRNA levels were both significantly diminished. In GEO database analyses, TDO2 expression was inhibited in patients with alcoholic hepatitis or cirrhosis, suggestive of the inverse correlation between TDO2 level and TGFB1 expression. Kynurenine treatment prevented TGFB1-dependent increase in TGFB1 mRNA in LX-2 cells, a human HSC cell-line, which was reversed by chemical inhibition or knockdown of AhR. Similar results were obtained using TGFB1 promoter reporter analysis. Chromatin immunoprecipitation (ChIP) and TGFB1 promoter reporter assays using putative XRE mutants showed that AhR directly represses TGFB1 transcription by binding to the DNA binding site located in the intronic region. The ability of kynurenine to inhibit HSC activation was corroborated by changes other HSC markers.

Conclusions: Kynurenine, a tryptophan metabolite produced primarily by TDO2, inhibits HSC activation, which may result from AhR-mediated negative regulation of TGFB1 gene in HSCs, supporting the concept that kynurenine produced in healthy hepatocytes serves as a paracrine mediator that maintains HSC quiescence.

Keywords: AhR, Kynurenine, TDO2, TGF-β, smads, transcriptional control

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List of Abbreviations

AhR	aryl hydrocarbon receptor
TDO2	tryptophan-2,3-dioxygenase
IDO	indoleamine 2,3-dioxygenase
Tgfβ	transforming growth factor beta
α-SMA	alpha smooth muscle
ACTA2	gene encoding α- SMA
HFD	high-fat diet
CCl_4	carbon tetrachloride
CM	conditioned Media
Kyn	kynurenine
KMO	kynurenine 3-monooxygenase
FICZ	6-formylindolo[3,2-b]carbazole
Tm	tunicamycin

I. Introduction

TGF-β1 is a major effector of wound healing and fibrogenesis in the liver [1]. Activated hepatic stellate cells (HSCs) are the major source of TGF-β1 within the liver during fibrogenesis, and are fully stimulated via autocrine TGF-β1 signalling [2]. TGF-β1 audoinduces the transcription of its own encoding gene, *TGFB1*. Excessive TGF-β1 signaling results in cell death, making TGF-β1 transcriptional control particularly important. However, the mechanisms involved in controlling *TGFB1* transcription, particularly those transcription in HSCs. remain unknown.

Accumulating evidence suggest the importance of hepatocyte-HSC crosstalk in both healthy and diseased liver. Healthy hepatocytes confer beneficial effects to HSCs in preventing HSC activation in vitro, while damaged hepatocytes exacerbate HSC activation[3]. In the opposite manner, activated HSCs directly influence hepatocytes. Small signaling molecules, cell surface receptors and transcription factors in HSCs are involved in fibrogenesis, which leads to increased cytokines such as TGF-β1 and platelet-derived growth factor (PDGF) [3-6]. However, the mechanisms involved in the control of this crosstalk, including compensatory mechanisms, are poorly understood.

Recent studies have confirmed that kynurenine (kyn) is an endogenous agonist of AhR, has functions as a small signaling molecule in cancer cell survival and immune tolerance [7]. The liver is main organ responsible for the production of kyn, primarily through tryptophan-2,3-dioxygenase (TDO2) [8]. While TDO2 and kyn are both typically abundant in healthy liver[9], their roles in liver homesostasis and disease are not well-studied, and have not been studied in fibrosis.

AhR is a well-studied transcription factor, and its functions in the metabolism and processing of toxins are well-described [10]. While AhR mediates the negative toxic

effects of aryl hydrocarbons such as dioxin, its knockout led to liver fibrosis and increased levels of TGF-β1 [11, 12]. Previous studies have investigated the relationship between AhR and TGF-β1 signaling [13-15]. However, reports have been contradictory depending on the cell-type investigated, and the mechanisms of interaction between the two, remain unclear. Further, there are no current studies indicating direct AhR modulation of TGF-β1 transcription. Kyn has been recently verified as a natural ligand of AhR with endogenous functions [8, 16]. This study investigated whether kynurenine, a tryptophan derivative produced in hepatocytes by TDO2, regulates HSC transcription of TGF-β1. Our findings show a novel transcriptional role of AhR in the expression of the *TGFB1* gene, and may assist in understanding liver pathobiology and designing a strategy for the treatment of liver fibrosis.

II. Materials and Methods

1. Reagents and antibodies

TDO2 and IDO antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while TGF-β antibody was obtained from Cell Signaling Technology (MA, USA). AhR (ab2770) and α-SMA (ab5694) antibodies were obtained from Abcam (Cambridge, UK). β-actin antibody (Cat No. A5441), L-Kynurenine (K8625), the AhR agonist FICZ (SML1489), and AhR antagonist CH-22319 (Cat No. C8124) were purchased from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were purchased from Zymed Laboratories (San Francisco, Ca). Human recombinant TGFβ1 was purchased from R&D Systems (Minneapolis, MN, USA).

2. Cell Culture

The LX-2 cell line (a human HSC line) was kindly supplied by Dr. S.L. Friedmann (Mount Sinai School of Medicine, NY, USA). Primary HSCs and hepatocytes were isolated from mouse liver according to a previously published method [17]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone, UT, USA), 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Mouse primary hepatocytes and hepatic stellate cells were isolated according to previously reported protocols [18].

3. Immunoblot analysis

Cell lysates were prepared as previously described [19] and proteins were resolved

by SDS-polyacrylamide gel electrophoresis, and immobilized proteins were immunoblotted with the antibodies of interest. Bands were visualized using the ECL chemiluminescence system (GE Healthcare, Buckinghamshire, UK). Equal loading of protein was verified by immunoblotting for β-actin. Relative protein levels were determined by scanning densitometry. Cell lysates were prepared as previously described [19].

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4. Transient transfection

Cells were plated in six-well plates overnight, serum-starved for 3 h, and transiently transfected with plasmid in the presence of FuGENE HD Reagent (Roche, Mannheim, Germany). Transfected cells were then incubated in Eagle's minimum essential medium containing 1% fetal bovine serum for 18 h. For reporter gene assays, the cells were transfected with pGL3-TGFB1 encoding TGFB1 (or TGFB1 with mutations) for 4 h in the presence of FuGENE HD reagent, and the activity of luciferase was measured by adding luciferase assay reagent (Promega, Madison, WI).

5. Reporter Assays

For reporter gene assays, the cells were transfected with pGL3-TGFB1 encoding *TGFB*1 (or *TGFB1* with mutations) for 4 h in the presence of FuGENE HD reagent, and the relative activity of luciferase was determined by adding luciferase assay reagent (Promega, Madison, WI).

6. siRNA Knockdown

To knock down target molecules, cells were transfected with an siRNA directed against human AhR (Darmachon) or a non-targeting control siRNA (100 pmol/ml)

using FugeneHD according to the manufacturer's instructions. After transfection over 48 h the cells were treated with vehicle or kyn in the presence or absence of TGF-β1 for 18 h.

7. Chromatin Immunoprecipitation (ChIP) assay

All ChIP assays were carried out usin EZ ChIP kits according to the manufacturer's instructions. Briefly, cell lysates were fixed with formaldehyde; DNA was sheared by sonication, and incubated with the antibody of interest (AhR, H3k27ac), negative control IgG antibody, and Pol II (positive control) antibody overnight with rotation at 4°C. Following incubation with protein G-agarose, the antigen–antibody complex was immunoprecipitated according to instructions. Purified DNA was obtained by elution following the kit instructions, and PCR amplification (55 cycles) was followed by gel electrophoresis and viewing of PCR product by chemiluminescence. Following the same method, histone acetylation was checked using an anti-H3K27ac antibody, followed by PCR amplication with 60 cycles using human TGFB1 primer for the XRE3 region (Figure 5D). Quantitative chIP PCR was carried out using the same DNA samples to verify quantitatively histone acetylation in the XRE 3 promoter region.

8. Primers

Primers used had the following oligonucleotide sequences.

Human GAPDH,

5'-GAAGATGGTGATGGGATTTC-3' (sense)

5'-GAAGGTGAAGGTCGGAGTC-3' (antisense);

Human β-actin,

5’-ACCCACACTGTGCCCATCTAC-3’ (sense)

5’-TCGGTGAGGATCTTCATGAGGTA-3’ (antisense);

Human α-SMA,

5’-CGTGGCTATTCCTTCGTTAC-3’ (sense)

5’-TGCCAGCAGACTCCATCC-3’ (antisense);

Human TGFB1,

5’- GGCAGTGGTTGAGCCGTGGA-3’ (sense)

5’-TGTTGGACAGCTGCTCCACCT-3’ (antisense);

Human β-actin,

5’- GATGAGATTGGCATGGCTTT-3’ (sense)

5’-GTCACCTTCACCGTTCCAGT-3’ (antisense);

Human TDO2,

5’- TGGGAACTA CCTGCATTT GGA-3’ (sense)

5’-TCG GTG CAT CCG AGA AAC AA-3’ (antisense);

Mouse β–actin,

5’-CTGAGAGGGAAATCGTGCGT-3’ (sense)

5’-TGTTGGCATAGAGGTCTTTACGG-3’ (antisense);

Mouse Tdo2,

5’- CTGGGGGATCCTCAGGCTAT-3’ (sense)

5’- TGT CAC TGT ACT CGG CTG TG-3’ (antisense);

Mouse Ido1,

5’-TGCTTACTCTCTTTTCCCTTCC-3’ (sense)

5’-CAT CAG ACC TGG TGC TTCA-3’ (antisense);

Mouse α-SMA,

5’- GGCTCTGGGCTCTGTAAGG-3’ (sense)

CTCTTGCTCTGGGCTTCATC-3’ (antisense);

Mouse TGF-β1,

5’-GCCCTGGATACCAACTATTGC-3’ (sense)

5’-GCAGGAGCGCACAATCATGTT-3’ (antisense);

Mouse Tdo2,

5’-CTGGGGGATCCTCAGGCTAT-3’ (sense)

5’- TGT CAC TGT ACT CGG CTG TG-3’ (antisense).

9. Bioinformatic and statistical analysis

Gene expression data were supplied from the Gene Expression Ombinus (GSE28619 and GSE25097) available in the public domain (http://www.ncbi.nlm.nih.gov/gds). Expression of genes (TDO2, IDO1, TGFB1) in disease and normal subjects was analysed using One-way analysis of variance (ANOVA) or Student t tests to assess the significance of differences among disease or healthy groups, or between treatment groups for cell based assays. The data were expressed as means ±S.E.M. The criterion for statistical significance was set at $*P<0.05$ or $**P<0.01$.

III. Results

1. Kynurenine represses HSC activation markers.

LX-2 are a well-established TGF-β1 responsive human HSC cell line, with treatment resulting in increased expression of TGFB1 transcription [20]. We treated LX-2 human stellate cells (HSCs) with kyn (100μM) with or without recombinant human TGF-β1 (5ng/ml). As expected, the TGF-β1 treated group showed a marked increase in TGF-β1 transcription by autoinduction as previously described. However, this was significantly reversed by co-treatment of kyn (Figure 1A)

To verify the significance of this finding, and if kyn may affect HSC activation, we carried out further investigations into the effect of AhR activation on the fibrosis marker αSMA. In addition, in LX-2 cells, ACTA2 expression was increased as expected by TGF-β treatment, and this was reversed with co-treatment of kyn (Figure 1B). Furthermore, the cytosolic protein levels of αSMA were also increased by TGF-β which was also reduced by treatment with kyn (Figure 1C). We found that tunicamycin (TM), an ER-stress inducer, increased expression of ACTA2, the gene encoding αSMA, which was in line with previous reports. However, co-treatment with Kynurenine 100μM repressed ACTA2 expression (Figure 1D).

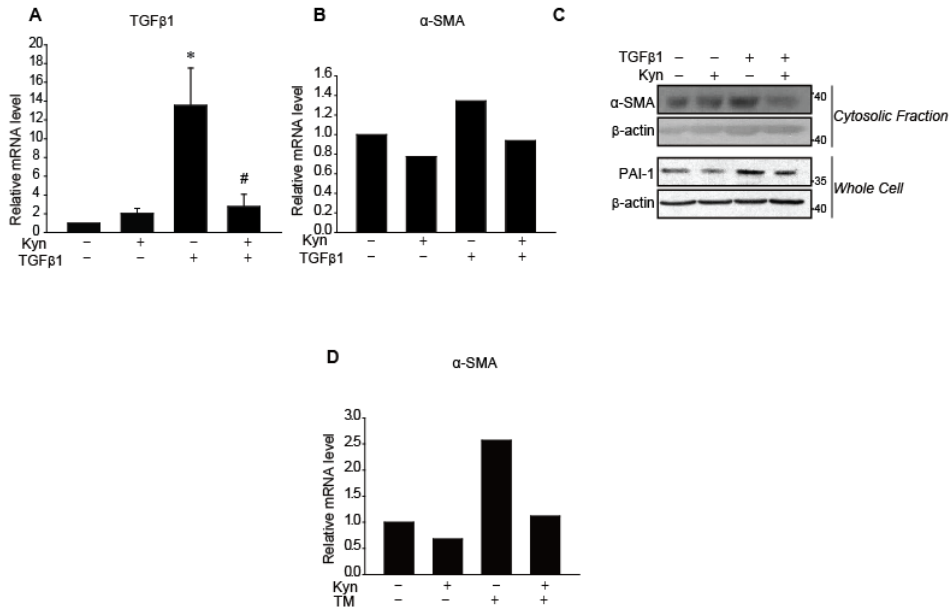

Figure 1. Kynurenine represses HSC activation markers.

(A) Relative TGFB1 mRNA expression in LX-2 cells following 18 h treatment of Kynurenine (100μM) and/or recombinant human TGF-β (5ng/ml), versus control (no treatment). Data represent the means ± SEM from at least 3 separate experiments. Significantly different as compared to control: *P < .05, **P<.01, and compared to TGF-β1 treatment #P < .05, ##P<.01

(B) Relative ACTA2 mRNA expression in LX-2 cells following 18 h treatment of Kynurenine (100μM) and/or tunicamycin (5ng/ml), versus control (no treatment), in an ER-stress model. n=1 for each group in this experiment.

(C) αSMA cytosolic fraction protein levels of LX-2 cells analysized by western following 18h treatment of Kynurenine (100μM) and/or recombinant human TGF-β (5ng/ml), versus control (no treatment).

(D) Relative ACTA2 mRNA expression in LX-2 cells following 18 h treatment of Kynurenine (100μM) and/or recombinant human TGF-β (5ng/ml), versus control

(no treatment). n=1 for each group in this experiment.

2. Activation of AhR represses *TGFB1* transcription.

We hypothesized that kyn's effects in LX-2 cells may be mediated through AhR, a well-known receptor for kyn. We treated LX-2 cells with the AhR agonist FICZ (100μM) in the same manner as in Figure 1, over 18 hours, and found that FICZ also significantly reduced TGF-β1 transcription (Figure 2A). To confirm that kyn reduces TGF-β1 transcription through AhR activation, we used the AhR inhibitor CH-22319. We found that kyn's repressive effects on TGF-β1 transcription were eliminated by co-treatment of CH-23319 10μM (Figure 2B). This effect was repeated with the use of siRNA against AhR, which similarly eliminated kyn's repression of TGF-β1 transcription when compared to a control siRNA group (Figure 2C). Furthermore, kyn treatment in the presence of exogenous TGF-β (5ng/ml) inhibited the luciferase activity in a TGFB1 WT reporter assay (Figure 2D). We therefore concluded that kyn inhibits TGFB1 transcription through the activation of AhR.

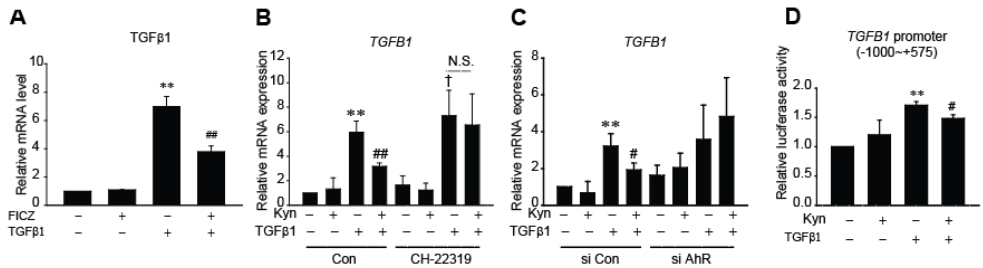

Figure 2. Activation of AhR represses *TGFB1* transcription.

(A) Relative TGFB1 mRNA expression in LX-2 cells following 18 h treatment of FICZ (100μM) and/or recombinant human TGF-β (5ng/ml), versus control (no treatment).

(B) Relative TGFB1 mRNA expression in LX-2 cells following 18 h treatment of Kynurenine (100μM) and/or recombinant human TGF- β (5ng/ml), with or without co-treatment of AhR inhibitor CH-22319 (10μM), versus control (no treatment).

(C) Relative TGFB1 mRNA expression in LX-2 cells following 18 h treatment of Kynurenine (100μM) and/or recombinant human TGF-β (5ng/ml), in siControl and siAhR groups.

(D) Relative TGFB1 luciferase assay of LX-2 cells following 18 h treatment of Kynurenine (100μM) and/or recombinant human TGF-β (5ng/ml), versus control (no treatment).

3. AhR negatively controls TGFB1 transcription by direct binding to the TGFB1 promoter region

Next, we set out to determine the precise mechanism through which AhR negatively regulates *TGFB1* transcription in stellate cells. We hypothesized that the AhR might bind to the *TGFB1* promoter and alter its transcription activity. As outlined in previous studies, the XRE sequences consisting of conserved core sequences 5'-(A/T)NGCGTG-3' are found in the regulatory regions of a variety of genes. [16] In *in silico* promoter analysis, we identified three putative sites at which AhR may bind to the *TGFB1* promoter region. Of the putative DNA-binding sites (XRE1, XRE2 and XRE3), AhR was found to interact with the sites XRE1 and XRE3, as indicated by ChIP assays (Figure 3A). To confirm the function of these XREs in the auto-induction of TGF-β1 gene expression, reporters with mutated XRE1 and/or XRE3 (MT1, MT3, MT1/3) were constructed (Figure 3B). Kyn treatment inhibited the luciferase activity in the *TGFB1* WT reporter, but lost its inhibitory effects in MT1, MT3 and MT1/3 (Figure 3C). Collectively, these data indicate that the XRE1 and XRE3 sites were both responsible for the AhR function. To further confirm that AhR activation and binding to the TGFB1 promoter repressed TGFB1 transcription, we carried out a histone acetylation assay. LX-2 cells were treated with or without kyn and/or TGF-β1 (5ng/ml) in the same manner as previously mentioned (over 18 h). The H3K27ac level at the *TGFB1* gene body was repressed by kyn treatment, indicating that transcriptional activity of *TGFB1* is inhibited by kyn. This is consistent with the results of qRT-PCR and reporter assays.

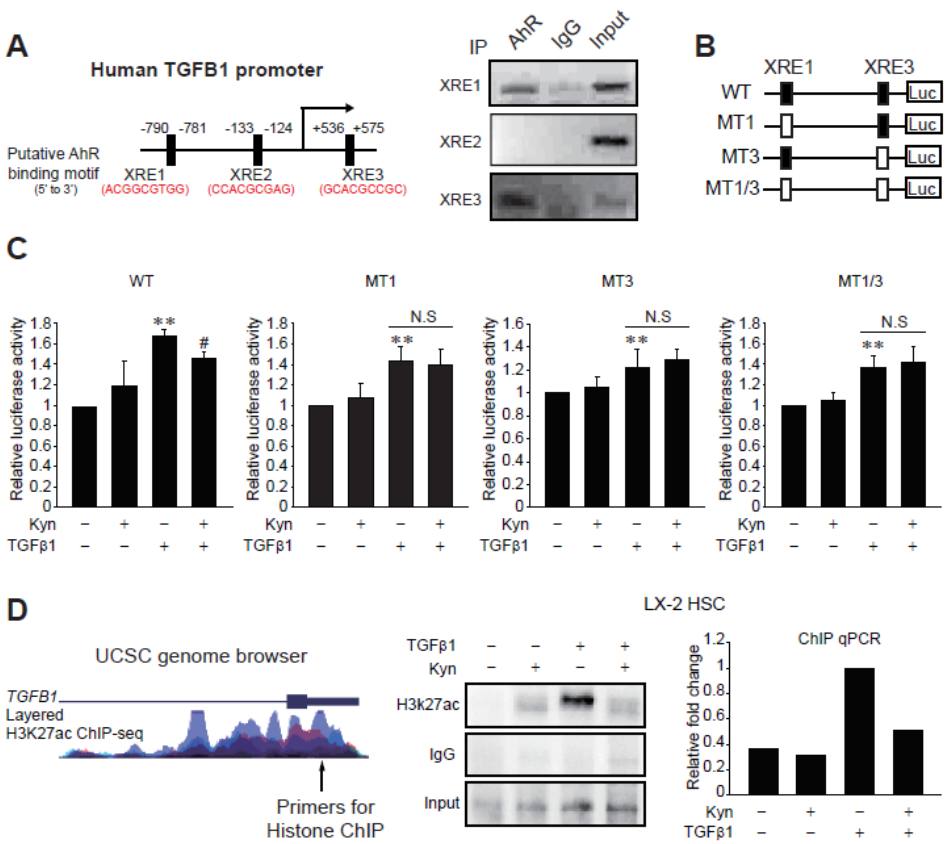

Figure 3. AhR negatively controls TGFB1 transcription by direct binding to the TGFB1 promoter region.

(A) Putative AhR binding sites at the *TGFB1* promoter, termed XRE1, XRE2, and XRE3, based on analysis of UCSC genome browser data (*left*). *Right*, ChIP assay from LX-2 non-treated cells using three primers designed based on the XRE1, XRE2, and XRE3 putative sites. Results following immunoprecipitation using Ahr, IgG (negative control), or Pol II (positive control) antibodies, and input.

(B) Design of four *TGFB1* luciferase systems with mutated sites (white indicates mutated site). WT, wild-type (no mutation); MT1, mutation at putative XRE1 site;

MT3, mutation at putative XRE3 site; MT1/3, mutation at both XRE1 and XRE3 sites.

(C) Luciferase activity of LX-2 cells transfected with TGFB1 WT, MT1, MT3, or MT1/3 luciferase genes, and pre-treated for 4h with kyn (where indicated), and then recombinant human TGF-β1 (5ng/ml) for 6 h, versus control group (no treatment). Data represent the means ± SEM from at least 3 separate experiments. Significantly different as compared to control: *P < .05, **P<.01, and compared to TGF-β1 treatment #P < .05, ##P<.01

(D) UCSC genome browser analysis of transcriptional activity at the *TGFB1* promoter by Layered H3K27ac ChIP-sequencing, and the region upon which ChIP primers for *TGFB1* were designed (*left*); *centre*, ChIP assay of LX-2 cells treated for 18 h with or without kyn (100μM) and/or recombinant human TGF-β1 (5ng/ml), and immunoprecipitation using antibodies for H3k27ac or IgG (negative control), input, followed by PCR amplification using Primer 3 TGFB1 primer. *Right*, Chip qPCR fold enrichment of samples immunoprecipitated with H3k27ac antibody, relative to inputs, following amplification using primer 4 TGFB1 primer. n=1 for each group in this experiment.

4. TDO2 is the primary enzyme responsible for the production of Kynurenine in the hepatocyte and exerts an influence on TGFB1 in hepatic stellate cells.

As TGF-β1 is primarily synthesized and secreted by activated HSCs [20], and kyn is produced in the liver primarily by the enzyme TDO2 (Figure 4A), we set out to investigate whether TDO2 affects HSC transcription of TGF-β1. As TDO2 is highly expressed in the liver and IDO is not, we analysed the UCSC browser for TDO2's cell expression profile, and found that it is primarily expressed in hepatocytes and minimally expressed in HSCs (Figure 4B). This pattern was confirmed by our own qRT-PCR assays of mouse primary liver cells (Figure 4C). As TDO2 has been shown to affect the modulation of cytokines [7], we hypothesized that a connection may exist between the activity of TDO2 in hepatocytes and *TGFB1* transcription in HSCs, as described in Figure 4D. We first checked TDO2 expression in 2 human hepatocyte cell-lines, HepG2 and Huh7. We found that while expressed in both lines, Huh7 cells better expressed TDO2 (data not shown), and so chose this line to carry out further experiments. Hepatocyte-HSC interaction was assessed by coculture experiments of Huh7 (hepatocytes) and LX-2 HSC cells separated by a transwell insert. TGF-β1 transcription in LX-2 cells was decreased in the presence of hepatocytes, in accordance with previous reports (Figure 4E). However, inhibition of TDO2 led to a significant increase in TGF-β1 when compared to the control (non-inhibitor) group. We collected conditioned media following 24 h-incubation with primary mouse hepatocytes with or without the TDO2 inhibitor 680C91 (10μM), and added the media to LX-2 cells for a period of 18 h. We found that, as expected, conditioned media from hepatocytes led to a reduction in the transcription of *TGFB1* following treatment with recombinant human TGF-β1 protein (5ng/ml) compared to the non-

CM group (Figure 4F). However, with conditioned media gathered from hepatocytes in the presence of 680C91, the reduction of *TGFB1* transcription was ablated. We concluded that TDO2 production by hepatocytes has a role in inhibiting the transcription of *TGFB1* in HSCs, which may be through kyn and AhR.

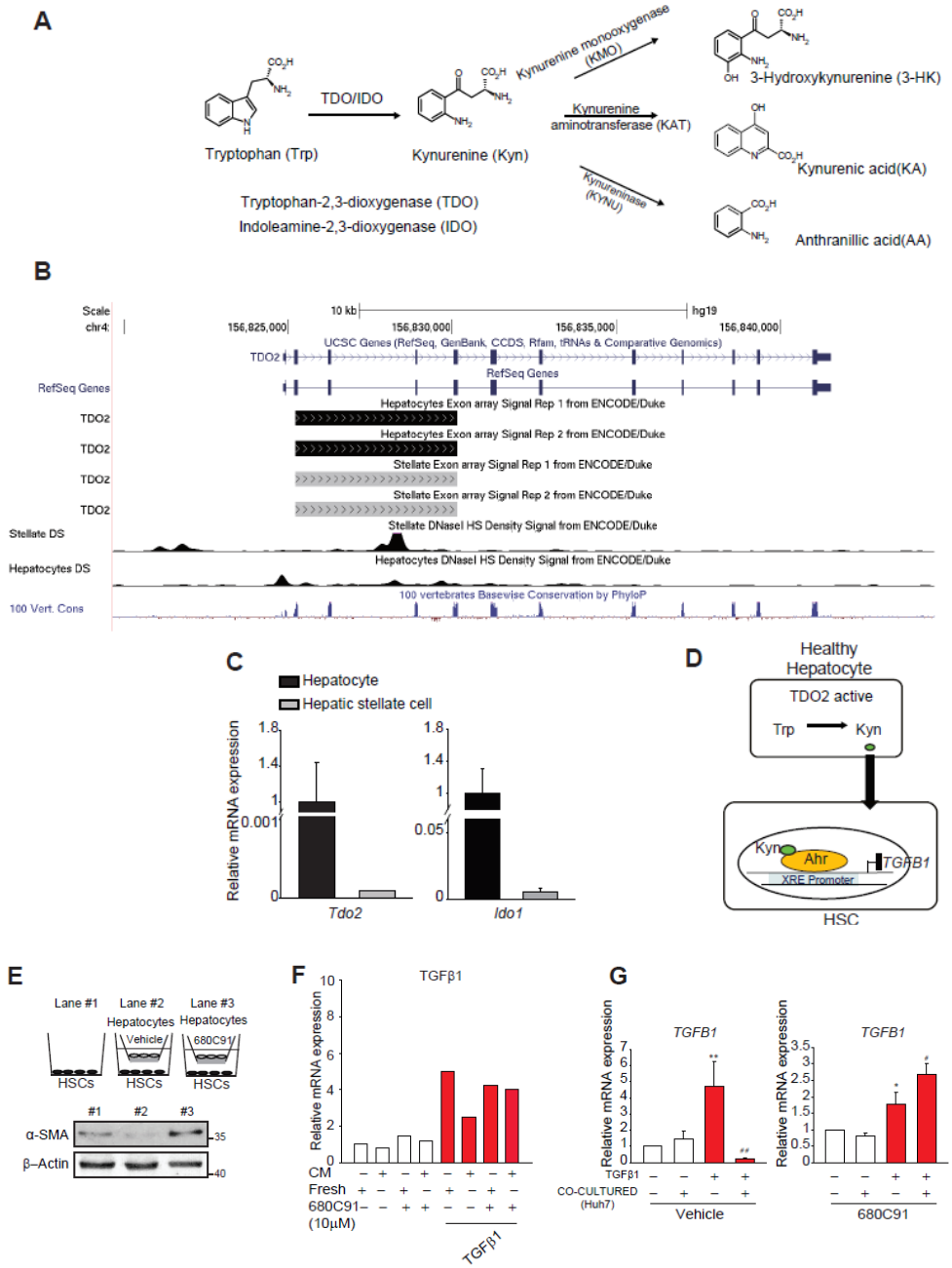

Figure 4. TDO2 is the primary enzyme responsible for the production of kyn in the hepatocyte and exerts an influence on TGFB1 in hepatic stellate cells.

(A) Kynurenine synthesis and degradation pathways.

(B) Relative expression levels of TDO2 enzyme in hepatocytes and HSCs in humans

(C) TDO2 mRNA levels in mouse primary hepatocytes and HSCs.

(D) Proposed scheme for Kyn's role in repression of HSC activation based on data from figures 1-4. Kyn is produced in healthy hepatocytes with a normal TDO2 activity, followed by uptake by HSCs, activation of AhR in the HSC, and finally repression of *TGFB1* transcription.

(E) Co-culture of primary HSCs with primary hepatocytes. The benefits exerted by hepatocytes in repressing *TGFB1* transcription in HSCs are reversed by co-treatment of the TDO2 inhibitor 680C91 (10μM).

(F) TGF-β1 mRNA levels in LX2 cells with or without conditioned media (CM). Conditioned Media was collected following 24 h of incubation with primary hepatocytes with or without *TDO2* inhibitor 680C91 (10μM), or fresh (non-conditioned media) then added to LX-2 cells for 18 h with or without treatment of recombinant human TGF-β1 (5ng/ml).

(G) Co-culture of LX-2 HSC and Huh-7 hepatocyte cell-lines experiment. The benefits exerted by hepatocytes in repressing TGFB1 transcription in HSCs are reversed by co-treatment of the TDO2 inhibitor 680C91 (10μM). Data represent the means ± SEM from at least 3 separate experiments. Significantly different as compared to control: *P < .05, **P<.01, and compared to TGF-β1 treatment #P < .05, ##P<.01

5. TDO2 inversely correlates with hepatic inflammation and fibrosis markers in animals

Having confirmed TDO2 repression of *TGFB1* transcription in HSCs *in vitro*, we next investigated TDO2 in a mouse fibrosis model. Mice were treated for 6 weeks with twice weekly intraperitoneal injections of the fibrosis inducing agent carbon tetrachloride (CCL_4) or vehicle. Following sacrificing and whole liver extraction, tissue homogenate was prepared for immunoblotting. Our results showed that TDO2 protein levels in mouse liver was significantly reduced in CCL_4-treated mice compared to that of vehicle-treated mice (Figure 5A). Moreover, TDO2 protein expression inversely correlated strongly with those of the active form of TGF-β1 (mature TGF-β1). IDO protein levels correlated in a positive manner with mature TGF-β1, which may be explained by the increased IDO production in non-parenchymal cells, such as dendritic cells, in response to inflammation. As expected, α-SMA, a marker of fibrosis, was increased significantly in CCl_4-treated mice, and this was inversely correlated to TDO2 protein levels as shown in Fig. 5A. Again, α-SMA was positively correlated with IDO protein levels. As TDO2 levels were decreased in all models of fibrosis, and was inversely correlated to TGF-β1 and *TGFB1* mRNA, we hypothesized that TDO2 is likely regulator of TGF-β1 in fibrosis.

Next, in a mouse sub-chronic fibrosis model, mRNA of *TDO2* also correlated in an inverse manner with that of *TGFB1* (Figure 5A, *bottom right*). However, *IDO1* transcipt levels in the same set did not correlate in any in any identifiable manner with that of *TGFB1* (data not shown). Interestingly, in the same set of mice, kyn levels were increased, while tryptophan (tryp) levels were decreased (Figure 5B). This resulted in an increased kyn/tryp ratio. Meanwhile, the kyn degradation enzyme

kynurenine 3-monooxygenase (KMO) was not significantly changed (Figure 5B, *right*).

Next, we conducted an acute model of liver toxicity by treating mice with a single dose of CCL4, and found that after 24 h the levels of TDO2 in the livers of treated mice were significantly decreased compared to those of the untreated group (Figure 5C). In a different set, treatment of carbon tetrachloride (CCl_4) resulted in a significant decrease in the mRNA levels of the genes encoding both TDO2 and IDO enzymes from 24-72 h following a single intraperitoneal injection (Figure 5D).

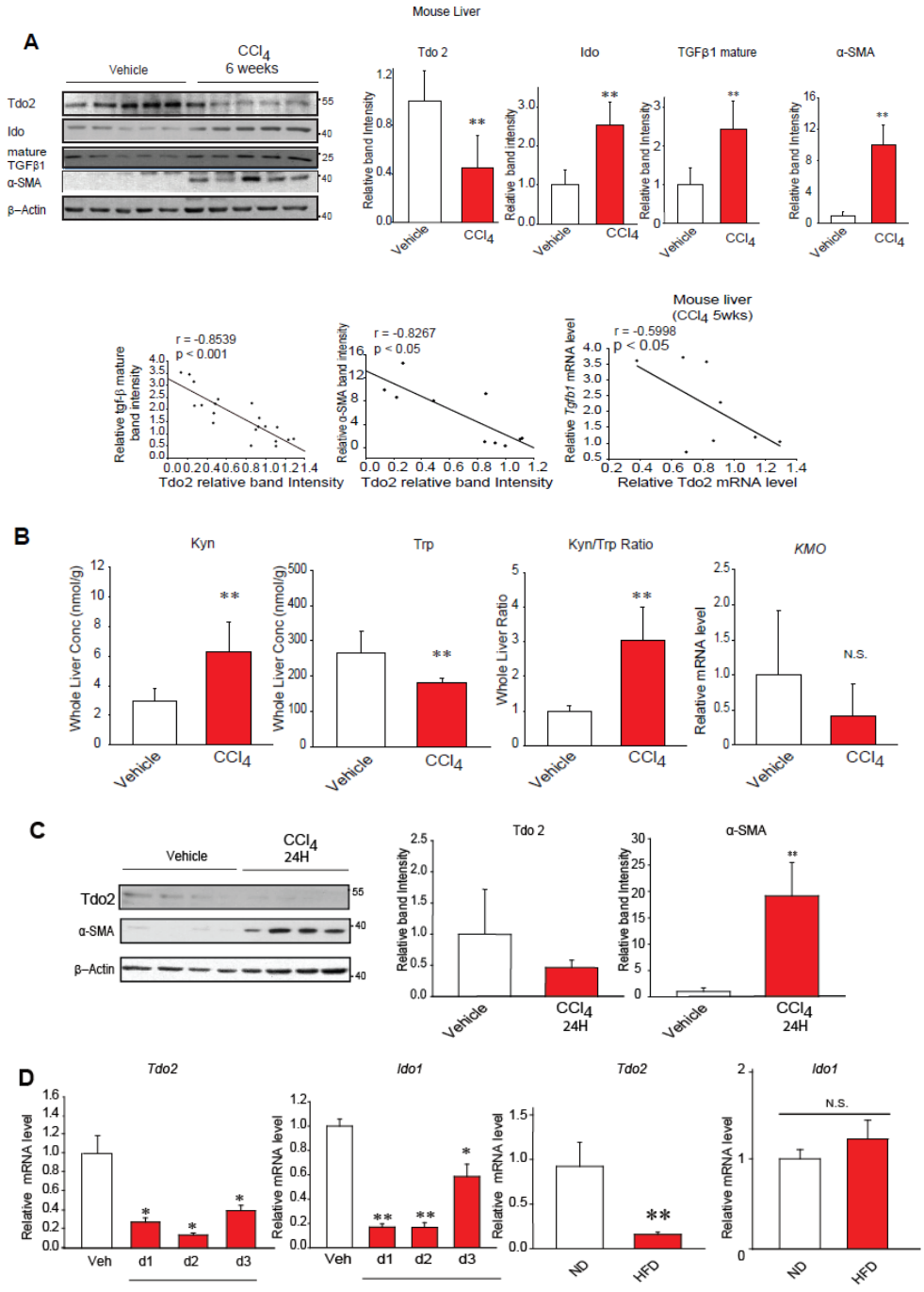
Mouse Liver
A
Vehicle
CCl4 6 weeks
Tdo2
Ido
mature TGFβ1
α-SMA
β–Actin
Tdo 2
Ido
TGFβ1 mature
α-SMA
Relative band Intensity
Vehicle
CCl4
Mouse liver (CCl4 5wks)
r = -0.8539
p < 0.001
Relative tgf-β mature band intensity
Tdo2 relative band Intensity
r = -0.8267
p < 0.05
Relative α-SMA band intensity
Tdo2 relative band Intensity
r = -0.5998
p < 0.05
Relative Tgfb1 mRNA level
Relative Tdo2 mRNA level
B
Kyn
Trp
Kyn/Trp Ratio
KMO
Whole Liver Conc (nmol/g)
Whole Liver Ratio
Relative mRNA level
N.S.
C
Vehicle
CCl4 24H
Tdo2
α-SMA
β–Actin
Tdo 2
α-SMA
Relative band Intensity
D
Tdo2
Ido1
Relative mRNA level
Veh
d1
d2
d3
ND
HFD
N.S.

Figure 5. TDO2 inversely correlates with hepatic inflammation and fibrosis markers in animals.

(A) Whole liver western blotting of tdo2, IDO, mature TGF-β, and α-SMA (top left), and, right, relative band intensities for TDO2, mature TGF-β, and α-SMA in mice (mouse experimental set 1, vehicle n=9, CCl4 n=10). Below, TDO2 band intensities inversely correlate with those of TGF-β and α-SMA in experimental set 1, and right, TDO2 mRNA levels inversely correlate with Tgfb1 mRNA in mouse whole liver experimental model set 2.

(B) Effect of CCl4 treatment (0.75ml/kg) in a chronic treatment model over 6 weeks (experimental set 1) on liver kynurenine, tryptophan, and kynurenine:tryptophan ratio, as measured by HPLC. Right, whole liver mRNA levels of the kyn degradating enzyme KMO from the same set.

(C) Tdo2 and α-SMA protein levels in whole liver 24 h after vehicle or CCl4 treatment (*left*), and relative band intensities of the same proteins (*right*).

(D) *Left*, effect of one-off CCl4 dose of 0.6ml/kg 1-3 days after injection on Tdo2 and Ido1 mRNA levels. *Right*, effect of a high fat diet (HFD) compared to that of normal diet (ND) on mouse liver expression of Tdo2 and Ido1 mRNA for mice fed on a ND (N=9) or HFD (N=15) for 9 weeks. (A and C) Significantly different band intensities as compared to vehicle-treated mice: *P < .05, **P<.01 (D) Significantly different as compared to control: *P < .05, **P<.01

6. TDO2 inversely correlates with liver inflammation and TGFB1 in human patients.

We sought to identify the potential mechanisms by which TDO2 and IDO enzymes may play a role in hepatic inflammation and fibrosis. We found that in the datasets (GSE28619 and GSE25097), mRNA expression of TDO2 inversely correlated with that of *TGFB1* (Figure 6A,B). IDO1 had a positive correlation with *TGFB1* in the cirrhosis set, with no clear correlation identified in the alcohol hepatitis group.

TDO2 is the primary enzyme responsible for the production of kyn in the liver. Previous reports have implicated TDO2 in cancer progression, and it has been shown to influence cytokine production [7]. We identified TDO2 as a potential regulator in liver fibrosis by analysis of human GEO datasets. Analysis of both a human alcoholic hepatitis dataset (GSE28619) and human cirrhosis dataset (GSE25097) showed that the mRNA expression of *TDO2* was significantly downregulated in disease in both sets (Figure 6C,D). *IDO*, the other enzyme that produces kyn, is present mainly outside the liver, and in the same datasets, its mRNA expression showed no conclusive trend.

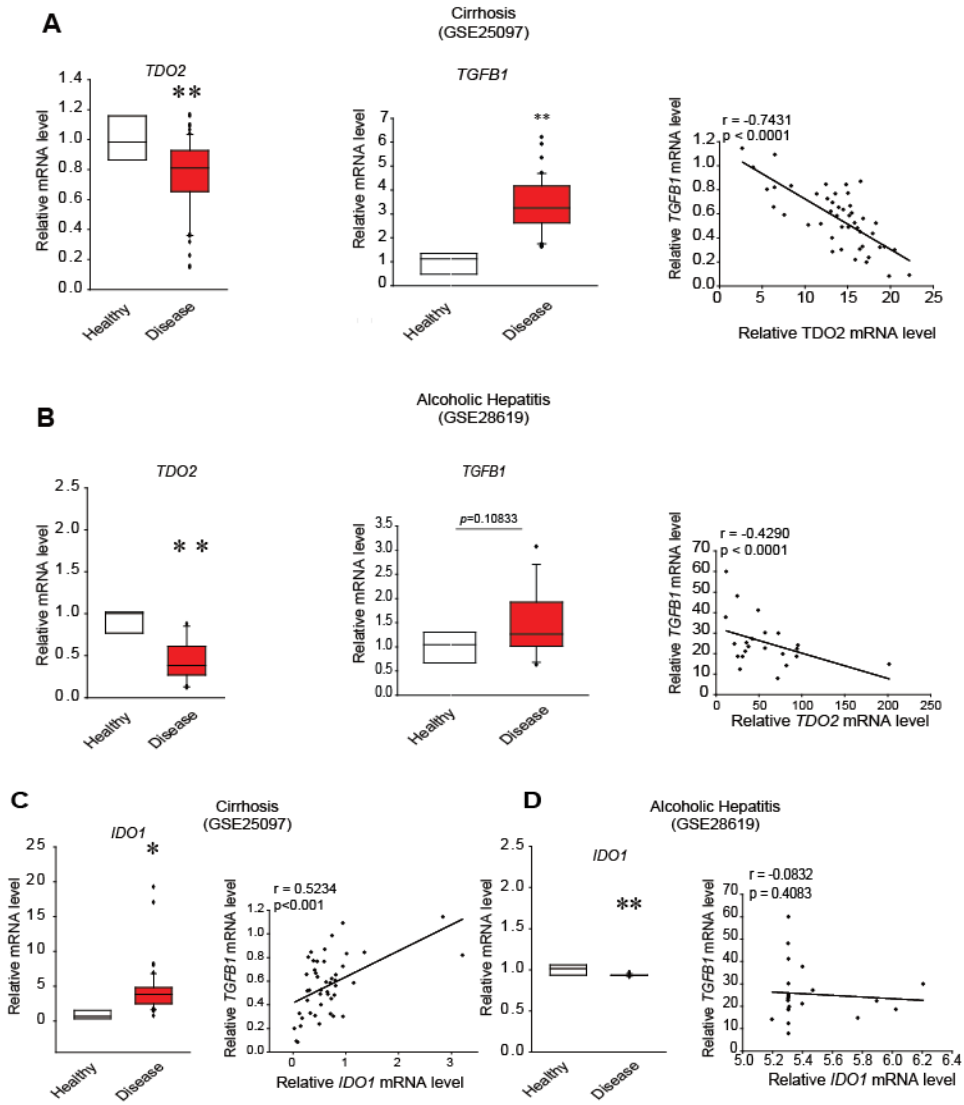

Figure 6. TDO2 inversely correlates with liver inflammation and TGFB1 in human patients.

(A) GEO dataset analysis of TDO2 and IDO1 mRNA expression levels in normal and diseased humans in a human cirrhosis set (GSE25097) (N =46) (left); and right, Pearson correlation analyses of the same dataset.

(B) GEO dataset analysis of relative TDO2 and TGFB1 mRNA expression levels in normal and diseased humans in an alcoholic hepatitis set (GSE28619) (N=22) (left); right, Pearson correlation analyses of the same dataset.

(C) IDO1 relative mRNA expression levels in normal and diseased humans in the cirrhosis dataset (GSE25097) (left) right, Pearson correlation analyses of the same dataset.

(D) IDO1 relative mRNA expression levels in normal and diseased humans from the alcoholic hepatitis (GSE28619) dataset (left); right, Pearson correlation analyses of the same dataset.

IV. Discussion

This study identifies a previously unidentified mechanism of hepatocyte-HSC crosstalk mediated through kynurenine. Most importantly, kynurenine is identified as a repressor of TGFB1 transcription. Of the three TGF-β isoforms, TGF-β1 is is the most implicated in liver disease[21]. TGF-β1 production following liver injury is required to promote wound repair, a process which triggers apoptosis in damaged cells and encourages tissue regeneration[1]. Impaired wound repair occurs when this process is poorly regulated, leading to innapropriate TGFβ-1-driven cell damage and apoptosis[1, 2, 21]. TGF-β1 ligation of its receptor results in autoinduction of *TGFB1* transcription through the smad pathway as well as the AP-1 complex [21, 22].Therefore tight regulatory control is needed to prevent uncontrolled TGF-β1 transcription and signaling in HSCs and the damage this causes. In acute liver injury quiescent HSCs produce TGF-β and exhibit negative feedback regulation of TGF-β signaling through the induction of Smad7, helping to terminate TGF-β/Smad signaling and allow cell repair [23, 24]. In contrast, activated HSCs lack this negative feedback regulation through Smad7 [23]. Other negative feedback regulatory systems have not been described, particularly that for *TGFB1* transcriptional control. Our findings intrigueingly suggest a novel mechanism which may negatively mediate *TGFB1* transcription in HSCs.

Parenchymal and non-parenchymal liver cells actively communicate in a bidirectional manner in both healthy and disease states[3]. HSCs respond to signals from hepatocytes, endothelial cells, as well as Kupffer cells. For example, healthy hepatocytes repress HSC activation through production of Wnt and jagged1 ligands[3]. Stromal cell–derived factor-1 (SDF1) released from endothelial cells attracts HSCs to a stem cell niche known as the *Space of Disse*[3]. However cell

intercellular signaling mechanisms regulating *TGFB1* expression in HSC have not been studied.

Kyn, a tryptophan metabolite produced by TDO2 and/or IDO1, was recently identified as an endogenous AhR ligand [7]. Due to its small size it diffuses through cellular membranes and act as an intercellular signaling molecule. Indeed, certain cancer cells can survive through TDO2 overexpression and kyn production[7]. Further, dendritic cells and macrophages express IDO which confers immune tolerance through inhibition of T cell proliferation [25, 26]. While TDO2 is the primary enzyme functioning in the liver conversion of tryptophan to kyn in a healthy state[9], their roles in liver homesostasis and disease are not well-studied, and have not been studied in fibrosis. We found TDO2 levels in primary hepatocytes to be significantly higher than in stellate cells, which was negligible. Our results show that blocking TDO2 activity in a hepatocyte cell line resulted in significantly higher expression of TGF-β1 mRNA in a HSC line. Consistently, direct kyn treatment completely repressed TGF-β1-induced *TGFB1* transcription through AhR. Taken together, our data suggest that kyn plays a role in HSC *TGFB1* transcriptional control as an intercellular communicator.

Notably, important foundational AhR studies found that AhR whole-body knock out mice are susceptible to spontaneous liver fibrosis and express TGF-β1in the liver in significantly larger quantities than wild-type mice [11, 12]. However, the underlying mechanisms explaining these results have never been fully elucidated. Despite several reports of interaction between AhR and TGF-β signaling [13-15], there are no previous studies showing a direct link between AhR and *TGFB1* at a transcriptional level in HSCs. For the first time this study reveals direct binding of AhR at two sites on the *TGFB1* promoter represses TGF-β1 autoinduction of *TGFB1*

transcription in HSCs. Interestingly, kyn treatment alone somewhat increased *TGFB1* transcription. Our speculation therefore is that AhR activity in HSCs forms a compensatory negative feedback mechanism regulating *TGFB1* transcription.

Our data showed that TDO2 was significantly downregulated on mRNA and protein levels following single injection of the liver toxicant CCL_4. Furthermore, chronic repeated CCL_4 treatment study showed TDO2 downregulation appears to be sustained. TDO2 is a well-known hepatocyte identity gene which, following hepatocyte injury, is believed to be downregulated by dedifferentiation [27]. This idea is supported by our other results showing TDO2 downregulation in a High fat diet mouse model. This corresponds with our analysis of the GEO database of human fibrosis and alcohol hepatitis patients.

While TDO2 expression was reduced in all disease models, the other kyn producing enzyme, IDO, was increased in our sub-chronic model, likely the result of inflammatory processes. While TDO2 activity is believed to be regulated by tryptophan and cortisol only, IDO, in contrast, is highly inducible, responding to cytokines such as interferon-γ (IFNγ), lipopolysaccharide (LPS), and tumor necrosis factor (TNF), as well as TGFβ [28]. Our HPLC data showed kyn levels were increased in parallel with IDO induction. Together with our results indicating kyn as a negative regulator of TGF-β1 transcription in HSCs, we propose that compensatory induction of IDO in liver may repress *TGFB1* expression in HSCs during liver inflammation. It is noteworthy that our delineation of an intercellular kyn signaling pathway to repress *TGFB1* expression in HSCs is also consistent with recent studies involving IDO in liver pathophysiology. In a high-fat model of liver injury, IDO knockout in mice led to increased TGF-β1 mRNA levels and appeared to render mice more vulnerable to HFD-induced hepatic fibrosis[29], while a

separate study indicated that chemical inhibition of IDO in mice resulted in increased fibrosis in a chronic CCL_4 model[30]. Also, liver production of IDO is increased in the liver of hepatitis B virus (HBV) patients[31] and higher IDO levels are a positive prognostic marker[32]. These studies support our hypothesis that production of kyn and Ahr activation in HSCs can fine-tune *TGFB1* transcription.

The present study demonstrates for the first time the role of TDO2/IDO, kynurenine, and AhR activity on HSC transcription of TGF-β1. While further study is required to clarify whether kyn has therapeutic value, our findings provide an insight into the bidirectional crosstalk between hepatocytes and HSCs in the liver inflammatory response (Figure 7).

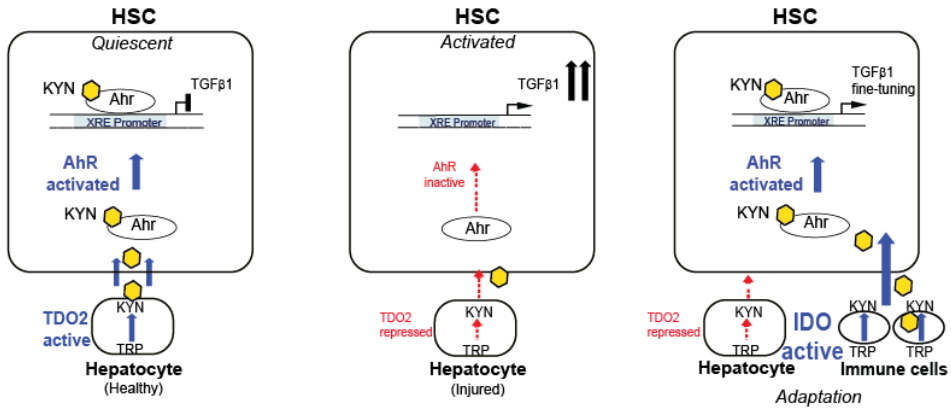

Figure 7. Proposed scheme for role of kyn and AhR in HSCs.

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국문 초록

간 성상세포에서 Kynurenine에 의한 AhR 매개 억제적 *TGFB1* 전사 조절

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간세포 손상은 간성상세포 활성화를 유도하여 간섬유화를 일으킨다. 간세포에서는 tryptophan 2,3-dioxygenase(TDO2)가 트립토판을 AhR(Aryl Hydrocarbon Receptor)의 내인적 리간드인 kynurenine으로 전환시키는 주요한 효소로 작용한다. TGFβ는 성상세포 활성화에 핵심적인 역할을 한다. 현재 AhR의 TGFβ 조절 역할 및 기전이 세포 유형이나 실험조건에 따라 상반된 방향으로 보고되어 있다. 본 연구에서는 간세포의 TDO2 발현과 간성상세포에 미치는 kynurenine의 영향, 간성상세포에서 AhR 활성화에 의한 TGFB1 전사조절 기전을 통합적으로 연구하고자 하였다. 마우스에 간독성자극 물질을 단회 혹은 수회 투여한 모델에서 TDO2 단백질과 mRNA 양이 모두 유의적으로 감소하였다. 또한 GEO 데이터베이스 분석으로부터 TDO2 발현이 알코올성 간염환자나 간경변증 환자의 간에서 억제되어있는 것을 발견하였고 이는 마우스 모델과 더불어 TDO2의 양과 TGFB1의 전사발현 간에 역상관관계가 있음을 시사하였다. LX-2 인간 간성상세포주에서 kynurenine 처치는 TGFβ 유도성 *TGFB1* 전사증가를 억제하였으나 AhR의 억제제를 같이 처치하거나 RNAi를 이용하여 AhR

발현을 억제하였을 경우 이러한 현상이 나타나지 않았다. TGFB1 프로모터 리포터 분석으로부터도 비슷한 결과를 얻었다. ChIP과 TGFB1 추정적 XRE 변이체를 이용한 프로모터 리포터 분석에서 AhR이 직접 TGFB1 DNA의 프로모터 부분에 결합하여 전사를 억제하였다. Kynurenine에 의한 성상세포 활성화 억제 현상은 α-SMA와 PAI-1 단백질의 발현 정도를 통해 검증하였다. 종합하면 본 연구에서는 TDO2에 의해 생산된 트립토판 대사체인 kynurenine은 간성상세포 활성화를 억제하며 그 기전으로 간성상세포에서 활성화된 AhR이 TGFB1 발현을 전사적으로 억제함을 제시하였고, 이러한 신호전달체계를 통해 건강한 간세포에서 생산되는 kynurenine이 간성상세포의 휴지상태를 유지하는 주변분비물질로 작용함을 규명하였다.

주요어: AhR, Kynurenine, TDO2, TGF-β, smads, 전사조절
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