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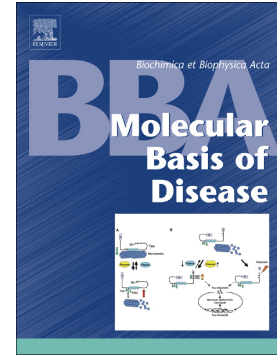
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The role of AdipoR1 and AdipoR2 in liver fibrosis

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Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; ALT, alanine aminotransferase; α SMA, alpha-smooth muscle actin; APN, adiponectin; AdipoR2, adiponectin receptor 2; AdipoR1, adiponectin receptor 1; AST, aspartate aminotransferase; BrdU, bromodeoxyuridine; CCl₄, carbon tetrachloride; Col1- α 1, collagen type 1 α 1; ECM, extracellular matrix; HSCs, hepatic stellate cells; HDL, high-density lipoproteins; IL-10, interleukin 10; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; NASH, non-alcoholic steatohepatitis; ns, non-significant; Scram, Scrambled; siRNA, small interfering ribonucleic acid; TIMP-1, tissue inhibitor of metalloproteinase-1; TGF β 1, transforming growth factor β ; WT, wild-type.

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Abstract

Activation of the adiponectin (APN) signaling axis retards liver fibrosis. However, understanding of the role of AdipoR1 and AdipoR2 in mediating this response is still rudimentary. Here, we sought to elucidate the APN receptor responsible for limiting liver fibrosis by employing AdipoR1 and AdipoR2 knock-out mice in the carbon tetrachloride (CCl₄) model of liver fibrosis. In addition, we knocked down receptor function in primary hepatic stellate cells (HSCs) *in vitro*. Following the development of fibrosis, AdipoR1 and AdipoR2 KO mice had no quantitative difference in fibrosis by Sirius red staining. However, AdipoR2 KO mice had an enhanced fibrotic signature with increased Col1- α 1, TGF β -1, TIMP-1, IL-10, MMP-2 and MMP-9. Knockdown of AdipoR1 or AdipoR2 in HSCs followed by APN treatment demonstrated that AdipoR1 and AdipoR2 did not affect proliferation or TIMP-1 gene expression, while AdipoR2 modulated Col1- α 1 and α -SMA gene expression, HSC migration, and AMPK activity. These findings suggest that AdipoR2 is the major APN receptor on HSCs responsible for mediating its anti-fibrotic effects.

1. Introduction

Hepatic fibrosis results from the excess deposition of extracellular matrix (ECM) proteins such as collagen in response to chronic liver injury from insults such as viral hepatitis, excess alcohol and non-alcoholic steatohepatitis (NASH) [1]. If the underlying injury persists, liver fibrosis can progress to cirrhosis where swirls of ECM around clusters of hepatocytes can promote portal hypertension and liver failure, and ultimately result in the development of liver cancer. The major cell type responsible for elaborating excess collagen is the hepatic stellate cell (HSC) which differentiates into a myofibroblast, proliferates and migrates to regions of hepatocyte injury. Simultaneously, these cells secrete ECM proteins and inhibit ECM degradation by the release of inhibitors such as tissue inhibitor of metalloproteinase-1 (TIMP-1), together promoting the formation of excess scar tissue [2].

Adiponectin (APN) is an adipocytokine produced by adipocytes and shown to have an array of biological functions [3]. APN is secreted as low, medium or high molecular weight forms that have different magnitudes of activity through the binding to three receptors: adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2), and T-cadherin [4]. AdipoR1 is found predominantly in muscle and AdipoR2 principally in liver, while T-cadherin is found in diverse tissues and cell types. Little is known about the signaling events of T-cadherin in the liver, but AdipoR1 and –R2 signaling and downstream events have been extensively characterized. Importantly, the binding of APN to AdipoR1 and –R2 leads to the phosphorylation and activation of adenosine monophosphate-activated protein kinase (AMPK) to modulate cellular energy utilisation.

Numerous groups have reported that APN has strong hepatic anti-fibrotic activity [5]. APN null mice treated with carbon tetrachloride (CCl₄) develop more liver fibrosis than wild type mice and APN overexpression limits fibrosis *in vivo* [6]. Further, the application of recombinant APN to activated HSCs can limit their fibrotic signature as exemplified by reduced α -smooth muscle actin (α SMA), collagen type 1 α 1 (Col1- α 1) and transforming growth factor β (TGF β) expression

(markers of HSC activation), AMPK activation, and reduced HSC proliferation and migration [6-10]. Nevertheless, little is known about the identity of the APN receptor(s) through which anti-fibrotic responses are mediated in liver. Given that APN agonists reduce CCl₄-induced liver fibrosis in mice [9] and APN has diverse activities in different tissues, a greater understanding of receptor activity is important if APN agonists are considered as therapeutic targets. We sought to elucidate the role of APN and AdipoR1 and -R2 interactions in liver fibrosis *in vitro* and *in vivo*. Our results demonstrate that AdipoR2 is the major receptor mediating APN's protective responses during fibrosing liver injury.

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2. Materials and Methods

2.1. Materials: Recombinant full-length APN was sourced from BioVendor (Evropska, Czech Republic). Small interfering ribonucleic acid (siRNA) against Scrambled (Scram), SiGENOME Rat AdipoR1 siRNA-SMARTpool (Cat. No. M-100692-01-0005) and AdipoR2 siRNA-SMARTpool (Cat. No. M-095646-02-0005) were purchased from Thermo Fisher Scientific.

2.2. Animals: All animal experiments were undertaken according to protocols approved by the Western Sydney Area Health Service Animal Ethics Committee and conducted in accordance with the guidelines of the Australian Council on Animal Care. Male wild-type (WT), AdipoR1 and AdipoR2 knock-out mice (6-8 per group) were sourced from Jackson Laboratory and bred on the C57B/6 background for at least 6 generations. Mice were housed under standard conditions with a 12-h light/dark cycle. Liver fibrosis was induced by the intraperitoneal injection of carbon tetrachloride (300 μ l/kg) in corn oil, twice per week for 12 weeks as previously described [6]. The livers harvested 48 hours after the final injection. Controls were WT mice never treated with CCl₄.

2.3. Primary rat HSC isolation and culture: HSCs were isolated from the livers of male adult Sprague-Dawley rats by *in situ* perfusion and purified by single-step density gradient centrifugation as described [11]. HSCs were seeded in Dulbecco's modified eagle medium containing 20% Fetal bovine serum (FCS), penicillin (100 IU/mL) and streptomycin (100 mg/mL).

2.4. Gene expression studies: Total RNA was isolated from the livers and cells using a FavorPrep Tissue Total RNA Extraction Mini Kit, according to the manufacturer's protocol. Complementary deoxyribonucleic acid (cDNA) was synthesized from total RNA with the Superscript III cDNA First-Strand Synthesis system (Invitrogen). Real-time PCR was performed on a Corbett 6000 rotor gene platform (Corbett).

2.5. Western Blot Analysis: Liver tissues and HSC cell lysates were generated, subjected to electrophoresis and western blotting and as previously described [12]. Primary antibodies diluted in

milk TBST buffer against AdipoR1 (1/500; cat.no. sc-46748; Santa Cruz), AdipoR2 (1/500; cat. No sc-46755; Santa Cruz), adenosine monophosphate-activated protein kinase alpha (1/1000; AMPK α ; cat no. 2532), Phospho-AMPK α (1/1000; p-AMPK α ; cat no. 2535; Cell Signaling), and β -actin (1/1000; Sigma; cat no. A2228) were used to quantitate protein levels. Protein expression was quantified using ImageJ software analysis and normalised against β -actin.

2.6. siRNA Knockdown of AdipoR1 and AdipoR2 *In Vitro*

siRNA knockdown was performed with the Lipofectamine RNAiMAX reagent (Life Technologies) and cells treated for 24 hours, serum-starved overnight and followed by subsequent treatment with recombinant full-length APN (5 μ g/ml) for 16 hours. The scram siRNA was used as a negative control. For time course studies, cells were treated with full-length APN over 5, 10, 20, 30 and 60 minutes.

2.7. Immunohistochemistry

Liver tissues were fixed in formalin and embedded in paraffin. 5 μ m sections were cut and stained with haematoxylin and eosin or Sirius red and photographed using the Nuance Multispectral Imaging System. Image quantification was performed using ImageJ software, as previously described [8, 13].

2.8 Migration Assay

Activated HSCs (7 days) were treated with Scram, AdipoR1 or AdipoR2 siRNA, and serum-starved overnight. 2.5×10^4 /ml cells in serum-free medium were plated on the upper chamber of Boyden chambers (8 μ m pores; Becton Dickinson) and the lower chamber was filled with 200 μ l of serum-containing media. APN (5 μ g/ml) was supplied to the upper chamber and the chamber incubated for 24 hours at 37°C. The cells were fixed and stained with hematoxylin and counted over six random fields on a phase contrast microscope.

2.9 Cell Proliferation and Apoptosis Assays

Activated HSCs (7 days) were treated with Scram, AdipoR1 or AdipoR2 siRNA, serum-starved overnight and treated with APN (5 $\mu\text{g/ml}$) for 16 hrs. Cell proliferation or apoptosis was assessed using a bromodeoxyuridine (BrdU) kit (Roche Applied Science) or the FAM-FLICA caspase 3 and 7 staining kit (ImmunoChemistry Technologies), respectively, according to the manufacturer's instructions.

2.10. Data Analysis and Statistics

Quantitative data were analysed using GraphPad Prism software and data are presented as mean \pm standard error of the mean. For multiple group analyses, the one-way analysis of variance and Tukey's multiple group comparison tests were used. The student's t-test was employed when comparing two groups. $p < 0.05$ was statistically considered significant.

3.0. Results

3.1. AdipoR1 and AdipoR2 KO mouse fibrosis characterization

Fibrosis was induced in WT, AdipoR1 and AdipoR2 KO mice for 12 weeks and gross hepatic pathology, serum measures of liver damage and lipogenesis were evaluated. Compared to WT control, AdipoR1 KO mice body weight was 6.3% less ($p < 0.05$). No difference in body weight was noted between the other groups. The liver weights were similar between groups but the liver to body weight ratio was 13% greater in CCl₄ treated AdipoR1 KO mice compared their controls ($p < 0.05$). Liver triglyceride levels were increased in CCl₄ AdipoR1 KO (9%; $p < 0.01$) and CCl₄ AdipoR2 KO (5%; $p < 0.01$) versus CCl₄ WT, and versus placebo elevated in AdipoR1 KO (12%; $p < 0.05$) and AdipoR2 KO (17%; $p < 0.01$) mice. No differences were observed between groups for serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, high-density lipoproteins (HDL) and the cholesterol/HDL ratio. Compared to CCl₄ WT, CCl₄ AdipoR1 KO mice had increased plasma total cholesterol (16%, $p < 0.05$; **Table 1**).

3.2. AdipoR2 mediates APN's anti-fibrotic actions *in vivo*

An examination of H&E and Sirius red staining, suggested no apparent difference in hepatic pathology, inflammation, and fibrosis between WT, AdipoR1 KO and AdipoR2 KO mice receiving CCl₄ (**Figures 1A and B**).

To more thoroughly interrogate a role for AdipoR1 or AdipoR2 in hepatic inflammation and fibrosis, qPCR for the fibrotic and inflammatory markers collagen type 1, $\alpha 1$ (Col1- $\alpha 1$) transforming growth factor beta-1 (TGF $\beta 1$), tissue inhibitor of metalloproteinase-1 (TIMP-1), interleukin 10 (IL-10), macrophages (CD68), matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), were performed. There were no differences in hepatic gene expression between the placebo groups of WT, AdipoR1 KO and AdipoR2 KO.

Comparing CCl₄ WT versus CCl₄ AdipoR1 KO, there were no differences in Col1- $\alpha 1$, TGF $\beta 1$,

TIMP-1, IL-10, MMP-2 and MMP-9, and CD68 was reduced 1.4-fold (ns). In contrast, CCl₄ AdipoR2 KO exhibited increased Col1- α 1 (2.8-fold; p<0.05), TGF β 1 (2.2-fold; p<0.05), IL-10 (2.6-fold; p<0.05), CD68 (2.2-fold, ns), MMP-2 (2.3-fold; p<0.01) and MMP-9 (2.3-fold; p<0.001). Differentiating CCl₄ AdipoR1 KO and CCl₄ AdipoR2 KO, the later had increased Col1- α 1 (2.3-fold, p<0.05), TIMP-1 (2.4-fold, p<0.05), IL-10 (4.5-fold, p<0.01), CD68 (1.5-fold, ns), MMP-2 (3.2-fold, p<0.01) and MMP-9 (7.9-fold, p<0.001).

Compared to placebo AdipoR2 KO, CCl₄ AdipoR2 KO livers had increased Col1- α 1 (4.7-fold, p<0.05), TIMP-1 (3.1-fold, p<0.05), TGF β 1 (3.2-fold, p<0.01), CD68 (1.9-fold, ns), MMP-2 (3.6-fold, p<0.01) and MMP-9 (3.4-fold, p<0.05; **Figure 2A-G**). Only Col1- α 1 was increased in CCl₄ AdipoR1 KO versus control AdipoR1 KO (4.3-fold; p<0.05).

To examine for receptor redundancy between AdipoR1 and AdipoR2, qPCR was performed for each gene. In CCl₄ AdipoR2 KO livers, AdipoR1 gene expression was increased compared to CCl₄ WT and AdipoR2 KO controls by 2.6-fold (p<0.05) and 2.4-fold (p<0.05), respectively (**Figure 2H**). In AdipoR1 KO livers, AdipoR2 was reduced, but not significantly (**Figure 2I**). Given that the markers of fibrosis and inflammation were increased in AdipoR2 KO and not in AdipoR1 mouse livers after CCl₄ treatment, this suggests that AdipoR2 is the major receptor mediating the protective actions of APN during liver fibrosis.

3.3. AdipoR2 mediates the anti-fibrotic action of APN *in vitro*

To further test the role of these receptors, we silenced AdipoR1 and AdipoR2 in rat primary activated HSCs with specific siRNAs, and tested their role in fibrosis *in vitro*. Validation experiments confirmed that the siRNAs reduced the gene expression of AdipoR1 and AdipoR2 by approximately 70% compared to control and Scram siRNA-treated cells, respectively (**Figure 3A and B**). To confirm qPCR knock-down, western blot and densitometry analysis was performed and reduced AdipoR1 (3.5-fold less; p<0.01) and AdipoR2 (7.5-fold; p<0.05) protein was observed compared to scram, after the respective siRNA treatment (**Figure 3C and D**).

In order to understand the temporal effect of HSC activation on AdipoR1 and AdipoR2 gene levels, qPCR for these receptors was performed at 0, 2, 7 and 10 days after plating (**Figure 3E**) in HSCs from WT rats. Compared to day 0, the gene expression of AdipoR1 was significantly reduced after 2 (2.3-fold, $p<0.05$), 7 (5.8-fold, $p<0.05$) and 10 (6-fold, $p<0.05$) days. In contrast, the gene expression of AdipoR2 was unaltered at all days compared to day 0.

Next, we determined the role of APN and AdipoR1 or AdipoR2 interactions on HSC function *in vitro*. Activated HSCs were treated with siRNA and subsequently recombinant APN (5 $\mu\text{g/ml}$) and qPCR performed. Scram siRNA had no effect on Col1- α 1 and α -SMA gene expression compared to control cells. In contrast, APN application reduced Col1- α 1 1.2-fold ($p<0.05$) and α -SMA 2-fold ($p<0.01$). AdipoR1 siRNA had no effect on Col1- α 1 and α -SMA gene expression and in conjunction with APN reduced Col1- α 1 1.5-fold ($p<0.05$) and 2.7-fold ($p<0.001$), respectively. AdipoR2 siRNA had no effect on Col1- α 1 and α -SMA gene expression, however the addition of APN increased Col1- α 1 gene expression 1.5-fold ($p<0.05$) and α -SMA 1.7-fold ($p<0.01$; **Figure 3G and F**).

Significantly, in the comparison of siRNA knock-down after APN treatment there were: (i) for Scram versus AdipoR1 no differences in Col1- α 1 and α -SMA gene expression, (ii) for Scram versus AdipoR2, AdipoR2 knock-down increased gene expression of Col1- α 1 1.8-fold ($p<0.01$) and α -SMA 3.4-fold ($p<0.001$); and (iii) AdipoR1 versus AdipoR2, AdipoR2 knock-down increased gene expression of Col1- α 1 2.4-fold ($p<0.001$) and α -SMA 5.3-fold ($p<0.001$) (**Figure 3F and G**). These data further supports the notion AdipoR2 mediates the anti-fibrotic responses of APN.

We then extended our experiments to TIMP-1 which we have previously shown is upregulated by APN *in vitro* and *in vivo* models of fibrosis [8]. Compared to control cells Scram, AdipoR1 or AdipoR2 siRNA had no effect on TIMP-1 gene expression. The application of APN in conjunction with Scram, AdipoR1 or -R2 siRNA increased TIMP-1 gene expression by similar levels, 1.4-fold, 1.4-fold and 1.3-fold, respectively (all $p<0.05$). There were no differences between groups and

these data therefore suggest that neither receptor alone is responsible for mediating APN's induction of TIMP-1 (**Figure 3H**).

3.4. AdipoR2 mediates HSC migration

APN can regulate HSC survival, proliferation and migration [7, 8, 14]. To understand the involvement of AdipoR1 and AdipoR2 in these events, AdipoR1 or AdipoR2 was selectively knocked-down in activated HSCs and apoptosis, proliferation and migration assessed. In initial experiments, treatment of HSCs with Scram siRNA, or Scram siRNA and APN did not promote HSC apoptosis (**Figure 4A**). The subsequent application of hydrogen peroxide readily promoted HSC apoptosis but Scram, AdipoR1 or AdipoR2 siRNAs alone or with full length APN did not trigger HSC apoptosis (**Figure 4B**).

Treatment with Scram, AdipoR1 or AdipoR2 siRNA had no effect on HSC proliferation compared to control cells. The addition of recombinant APN to Scram, AdipoR1 or AdipoR2 siRNA treated cells reduced HSC proliferation by 1.3 ($p<0.05$), 1.6 ($p<0.001$) and 1.4-fold ($p<0.01$), respectively, and there was no difference between the groups (**Figure 4C**).

Treatment with Scram, AdipoR1 or AdipoR2 siRNA had no effect on HSC migration compared to control cells. However, the addition of full-length APN to Scram or AdipoR1 siRNA reduced HSC migration by 3.6-fold ($p<0.001$) and 4.2-fold ($p<0.001$), respectively. In contrast, AdipoR2 siRNA followed by APN treatment reduced migration by only 1.6-fold ($p<0.01$). Between groups this represented 2.3-fold ($p<0.001$) and 3.1-fold ($p<0.001$) less migration than that observed with respective Scram or AdipoR1 siRNA and APN treatment (**Figure 4D**). These data show that APN regulates HSC migration principally through AdipoR2.

3.5. AdipoR2 regulates AMPK activity

Previous studies have shown that APN can partly mediate anti-fibrotic responses in HSCs by activating AMPK [7, 14]. Given that we saw a limited role for AdipoR1 in *in vivo* and *in vitro* in

fibrotic responses, we evaluated the function of AdipoR2 in AMPK signaling. Cells were treated with either Scram or AdipoR2 siRNA, after which time-dependent treatment with APN (5 $\mu\text{g/ml}$) was performed. Through western blot analyses, less AMPK phosphorylation was detected in AdipoR2 siRNA and APN-treated cells, compared to Scram siRNA and APN, at 10 minutes (1.4-fold, $p < 0.05$), 20 minutes (1.4-fold, $p < 0.05$), 30 minutes (1.4-fold, $p < 0.05$) and 60 minutes (1.4-fold, $p < 0.05$; **Figure 4D**). These data suggest that APN through binding to AdipoR2 can modulate AMPK activity.

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4. Discussion

We have elucidated the roles of AdipoR1 and AdipoR2 in *in vivo* and *in vitro* models of hepatic fibrosis. We show that the absence of AdipoR1 or AdipoR2 is not essential for liver fibrosis *in vivo*, while AdipoR2 loss is associated with an enhanced fibrotic signature. In activated HSCs, AdipoR2 is necessary for mediating APN's anti-fibrotic responses and cell migration. Furthermore, APN was unable to activate AMPK in the absence of AdipoR2 *in vitro*, suggesting that of the G-protein like APN receptors, AdipoR2 is the major receptor transmitting APN intracellular signaling in HSCs.

Since the discovery of the APN receptors by Yamauchi et al., a large body of literature has demonstrated the role of APN mediated activation of diverse intracellular signaling pathways in various cells and tissues [15]. In the liver, APN is a potent modulator of hepatic lipid and glucose metabolism, and is protective against fibrosis [6, 15-17]. However, few publications have addressed the identity and functionality of the receptor mechanisms that mediate APN's anti-fibrotic activity at the genetic and cellular levels. To address this, we subjected AdipoR1 and AdipoR2 knock-out mice to CCl₄ treatment and evaluated liver fibrosis. Only the loss of AdipoR2 resulted in an enhanced fibrotic signature. AdipoR1 gene expression was upregulated in AdipoR2 KO CCl₄ treated mice, suggesting that AdipoR2 may modulate AdipoR1 gene expression, and that genetic redundancy by AdipoR1 could possibly explain the unaltered collagen staining we observed. However, this was insufficient to perturb increases in the gene expression profile of pro-fibrotic genes. Moreover, given that the over-expression of AdipoR2 reduces steatohepatitis and fibrosis in the methionine and choline-deficient model, our *in vivo* observations support a greater role for AdipoR2 in liver diseases [18].

At the cellular level, studies by Ding et al., [14] have shown that AdipoR1 and AdipoR2 are expressed in primary quiescent HSCs and on activation, AdipoR1 mRNA expression was halved and AdipoR2 was maintained. In a separate study, Caligiuri et al., [10] used primary passaged HSCs, and illustrated at the mRNA level that AdipoR1 was more abundant than AdipoR2. In contrast, we observed in primary non-passaged activated HSCs over a 10 day time course that

AdipoR2 gene expression was maintained and AdipoR1 decreased, and at the protein level both receptors were expressed in activated HSCs. Together these data suggest that culturing conditions may influence receptor gene expression, however on the basis of our *in vitro* and *in vivo* data AdipoR2 would appear to be the major APN receptor in primary HSCs. Nevertheless, prior studies have focused on APN's action through AdipoR1. Reduced levels of tyrosine phosphatase 1B (PTPB1) protein were found in APN KO mouse livers, and adenoviral over-expression of APN suppressed focal adhesion kinase activity in CCl₄ treated APN KO mice. In passaged primary rat HSCs, APN binding to AdipoR1 and not AdipoR2 promoted PTP1B activity, and separately Src homology region 2-containing protein tyrosine phosphatase 2 (Shp2) phosphorylation to dephosphorylate focal adhesion kinase (FAK) [19, 20]. Concerning Shp2 and FAK, this suggests that APN through AdipoR1 can disrupt focal adhesion complexes and integrin activity, to limit HSC function. However, these studies did not stringently test the basic properties of fibrotic HSC's, namely proliferation, migration and activation that assist in liver repair and protect damaged regions from further injury insults.

To investigate these facets of HSC function, we referred to Kamada's original work where he illustrated that APN limited primary HSC proliferation, migration and fibrogenic gene expression [6]. In replicating these data in serum free growth conditions and the absence of any mitogenic factor, we confirmed recombinant APN could reduce these parameters in primary HSCs. Given that APN receptor function in the context of active fibrosis *in vitro* had not been tested, we ablated AdipoR1 or AdipoR2 in HSCs and show for the first time that AdipoR2 and not AdipoR1 is responsible for mediating APN's suppression of HSC activity and migration.

In agreement with our recent publication, we find that APN decreases HSC proliferation and increases TIMP-1 gene expression [8]. However, given that neither receptor modulated APN's anti-proliferative activity or the induction of TIMP-1 expression, the downstream pathways of APN activation appear to be complex. A plausible explanation for this is that APN's anti-proliferative action and induction of TIMP-1 require the combined action of both AdipoR1 and AdipoR2.

Previously, the action of both AdipoR1 and AdipoR2 in double knock-out mice has been shown to generate greater insulin resistance than AdipoR1 or AdipoR2 KO loss alone [21]. We attempted to generate AdipoR1 and AdipoR2 double KO mice. However, we were unable to attain double homozygous littermates in our line of C57B/6 mice. Similarly, unfortunately, in primary HSCs the combined knock-down of both AdipoR1 and AdipoR2 with siRNAs affected cell viability, limiting experimental evaluation (data not shown). Thus, it remains open as to whether both receptors are required to mediate some of the anti-fibrotic effects of adiponectin.

Apart from limiting HSC activation, APN can activate hepatic AMPK *in vivo* and in HSCs [7, 8, 10, 22]. Since we demonstrated that AdipoR1 did not mediate APN's anti-fibrotic or migratory activities, we focused on AdipoR2, and found that in its absence APN treatment was associated with reduced AMPK activation. In our hands, the knock-down of AdipoR2 did not affect HSC proliferation, but did mediate AMPK activity, suggesting that the limiting of HSC proliferation by APN is AMPK independent in this model. It is also tenable that both AdipoR1 and AdipoR2 receptors are required for optimal AMPK activity to affect proliferation.

An interesting observation was the increased gene expression levels of the anti-inflammatory cytokine IL-10 in CCl₄ treated AdipoR2 KO mice. Previous reports have shown recombinant APN to increase IL-10 in concanavalin A treated wild-type mice, and in Kupffer cells, leading to the establishment of an APN/IL-10/heme oxygenase-1 signaling axis [23][24]. We speculate that AdipoR2 is involved in an inflammatory switch as CD68 levels are non-significantly increased on CCl₄ treatment. However, further in-depth studies using both wild-type and AdipoR2 depleted hepatic parenchymal and non-parenchymal cells are required to consider the significance of APN and AdipoR2 interactions in inflammation.

APN has now been established as a potential anti-fibrotic as APN agonists can limit fibrosis *in vivo* [9]. AdipoR1 can mediate APN signaling and disrupt focal adhesion assembly, however these events appear to be independent of HSC activity and migration. In contrast, we demonstrate that AdipoR2 primarily mediates APN's anti-fibrotic and -migratory effects. It remains open as to

whether there are alternative receptor mechanisms or interactions between APN receptors that regulate HSC proliferation and TIMP-1 expression. In sum, this work provides a foundation for future studies that aim to elucidate these mechanisms.

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Table 1. The effect of chronic CCl₄ treatment on markers of liver damage in WT, AdipoR1 KO and AdipoR2 KO mice.

	WT		AdipoR1 KO		AdipoR2 KO	
	Placebo	CCl ₄	Placebo	CCl ₄	Placebo	CCl ₄
Body Weight (g)	28.24 ± 0.4	26.77 ± 0.88	26.45 ± 0.06*	27.31 ± 0.77	29.31 ± 0.25	28.34 ± 0.99
Liver Weight (g)	1.21 ± 0.07	1.28 ± 0.05	1.26 ± 0.04	1.48 ± 0.04	1.45 ± 0.11	1.40 ± 0.03
Liver/Body weight Ratio	4.28 ± 0.21	4.79 ± 0.18	4.79 ± 0.17	5.43 ± 0.16 #	4.93 ± 0.32	4.96 ± 0.14
Liver Trig (μmol/g)	349.4 ± 8.8	324.3 ± 11.1	341.5 ± 9.2	381.7 ± 12.7### ^	313.5 ± 49.2	365.8 ± 17.7### ^^
ALT (U/L)	51.25 ± 4.7	55.56 ± 6.04	71.82 ± 13.03	61.64 ± 3.39	63.33 ± 17.8	70.57 ± 12.44
AST (U/L)	17.89 ± 2.65	17.30 ± 2.17	17.58 ± 2.57	14.45 ± 2.02	24.14 ± 7.39	17.86 ± 1.16
Triglycerides (mmol/L)	0.60 ± 0.03	0.60 ± 0.07	0.58 ± 0.04	0.6 ± 0.07	0.71 ± 0.07	0.54 ± 0.07
Cholesterol (mmol/L)	1.73 ± 0.04	1.65 ± 0.08	1.87 ± 0.12	2.09 ± 0.08 ♦	1.86 ± 0.14	1.77 ± 0.17
HDL Chol (mmol/L)	1.65 ± 0.06	1.43 ± 0.17	1.67 ± 0.13	1.83 ± 0.08	2.10 ± 0.2	1.97 ± 0.2
Cholesterol/HDL ratio	1.08 ± 0.03	1.23 ± 0.09	1.17 ± 0.07	1.1 ± 0.04	1.00 ± 0.06	1.13 ± 0.08

Statistical significance was determined by one-way ANOVA: * p < 0.05 versus WT controls; # p < 0.05 versus AdipoR1 control; ### p < 0.01 vs WT control; ^ p < 0.05 vs AdipoR1 control; ^^ p < 0.01 vs AdipoR2 control; ♦ p < 0.05 versus WT on CCl₄; and Tukey's multiple group comparison tests. Trig: triglyceride, Chol: cholesterol. The data are expressed as mean ± SEM.

Table 2. Mouse qPCR primer sequences

Gene	Primers
GAPDH	Forward GTCGTGGATCTGACGTGCC Reverse TGCCTGCTTCACCACCTTC
TIMP-1	Forward GCAAAGAGCTTTCTCAAAGAC Reverse AGGGATAGATAAACAGGGAAACACT
TGF β	Forward GTGGGGACTTCTTGGCACT Reverse GAGTGTCCACGACGGTGAG
Coll- α 1	Forward AGGAGAACCAGGTGACGAAG Reverse CCCAGCTTCTCCTTTCTCT
AdipoR1	Forward TTTGCCACTCCCAAGCAC Reverse ACACCACTCAAGCCAAGTCC
AdipoR2	Forward TCTCAGTGGGACATGTTTGC Reverse AGGCCTAAGCCCACGAAC
IL10	Forward AGGCGCTGTCATCGATTTCT Reverse AGGAAGAACCCTCCCATCA
MMP-2	Forward CCAGACAGGTGACCTTGACC Reverse AAACAAGGCTTCATGGGGGC
MMP-9	Forward AAAGGCAGCGTTAGCCAGAA Reverse ACAACTCGTCGTCGTCGAAA
CD68	Forward TGACCTGCTCTCTCTAAGGCTACA Reverse TCACGGTTGCAAGAGAAACATG

Table 3. Rat qPCR primer sequences

Gene	Primers
β -actin	Forward CTGGCTCCTAGCACCATGA Reverse TAGAGCCACCAATCCACACA
Coll- α 1	Forward CAGATGTCCTATGGCTATGATGAG Reverse CCACGAGGACCAGAAGGAC
α SMA	Forward TTCAATGTCCCTGCCATGTA Reverse CCATCTCCAGAGTCCAGCAC
TIMP1	Reward TTTCCGGTTCGCCTACAC Reverse CGGTTCTGGGACTTGTGG
AdipoR1	Reward AGCACCGGCAGACAAGAG Reverse GGTGGGTACAACACCACTCA
AdipoR2	Forward ATGTTTGCCACCCCTCAGT Reverse GATTCCACTCAGACCCAAGC

Figure Legends

Figure 1. AdipoR1 or AdipoR2 loss does not affect pathology or fibrosis. (A), Representative images of H & E CCl₄ and Sirius red staining from corn oil and CCl₄ treated WT, AdipoR1 KO, and AdipoR2 KO mice. Scale bars: 500 μ m. (B), Graph illustrating the percentage of collagen deposition as represented by Sirius red staining in liver sections after CCl₄ treatment. In AdipoR1 KO and AdipoR2 KO mice receiving CCl₄, the extent of liver fibrosis was not significantly different to the WT. Statistical significance was determined by one-way ANOVA. The data are expressed as mean \pm SEM (n=6).

Figure 2. Loss of AdipoR2 increases fibrotic and inflammatory markers. qPCR for fibrotic and inflammatory genes from WT, AdipoR1 KO, and AdipoR2 KO control and CCl₄ treated mice showed, (A), Compared to controls Col1- α 1 increased in AdipoR2 KO (6.4-fold; p<0.05), AdipoR1 KO (2.8-fold; p<0.05) and WT (3-fold; p<0.05). CCl₄ AdipoR2 KO Col1- α 1 gene expression was increased versus CCl₄ AdipoR1 KO (2.3-fold; p<0.05) and WT (2.8-fold; p<0.05). (B), TGF β gene expression in CCl₄ AdipoR2 KO was increased versus WT CCl₄ (2.2-fold; p<0.01), AdipoR2 control (3.2-fold; p<0.01), and CCl₄ AdipoR1 (1.8-fold; not significant). (C), TIMP-1 gene expression in CCl₄ AdipoR2 KO was increased versus CCl₄ AdipoR1 (2.4-fold; p<0.05) and control AdipoR2 (3.1-fold; p<0.05). (D), IL-10 gene expression in CCl₄ AdipoR2 KO was increased versus CCl₄ WT (2.6-fold; p<0.05) and CCl₄ AdipoR1 (4.5-fold; p<0.01). (E), CD68 gene expression was unchanged between groups (F), MMP-2 gene expression in CCl₄ AdipoR2 KO was increased versus CCl₄ WT (2.3-fold; p<0.01), CCl₄ AdipoR1 (3.2-fold; p<0.01), and control AdipoR2 (3.6-fold; p<0.01). (G), MMP-9 gene expression in CCl₄ AdipoR2 KO was increased versus CCl₄ WT (2.3-fold; p<0.001), CCl₄ AdipoR1 (7.9-fold; p<0.001), and control AdipoR2 (3.4-fold; p<0.05). (H), In CCl₄ AdipoR2 KO mice AdipoR1 gene expression was increased versus CCl₄ WT (2.6-fold; p<0.05) and control AdipoR2 (2.4-fold; p<0.05). (I), In CCl₄ AdipoR1 KO mice AdipoR2 gene expression was unchanged. Differences between groups (n=6) were analysed using a one-way ANOVA with Tukey test (* p<0.05, ** p<0.01, *** p<0.001).

Figure 3. APN Mediates Fibrotic Responses through AdipoR2. (A and B), specific siRNA reduced the gene expression of AdipoR1: compared to untreated (3.2-fold, $p < 0.001$) and Scram siRNA-treated (3.0-fold, $p < 0.01$), and AdipoR2: compared to untreated (3.5-fold, $p < 0.001$) and Scram siRNA-treated (3.5-fold, $p < 0.01$). (C and D), Western blot and densitometry confirmed that specific siRNA treatment reduced the protein levels of AdipoR1: compared to untreated (4.0-fold, $p < 0.01$) and Scram siRNA-treated (3.0-fold, $p < 0.01$), and AdipoR2: compared to untreated (35-fold, $p < 0.001$) and Scram siRNA-treated (30-fold, $p < 0.01$). (E), Time course analyses of AdipoR1 and AdipoR2 gene expression in HSCs, shows AdipoR1 decreased at days 2 (2.3-fold; $p < 0.05$), 7 (5.8-fold; $p < 0.05$) and 10 (6-fold; $p < 0.05$). AdipoR2 expression was unchanged. In activated HSCs: (F), APN reduced Col1- α 1 mRNA in Scram (1.2-fold; $p < 0.05$) and AdipoR1 siRNA (1.5-fold; $p < 0.05$) treated cells. Treatment with APN and AdipoR2 siRNA increased Col1- α 1 gene expression (1.5-fold; $p < 0.05$); (G), APN reduced α -SMA mRNA in Scram (2.0-fold; $p < 0.01$) and AdipoR1 siRNA (2.7-fold; $p < 0.001$) treated cells. Treatment with APN and AdipoR2 siRNA increased α -SMA gene expression (1.7-fold; $p < 0.01$). (H), TIMP-1 gene expression increased after co-treatment with Scram, AdipoR1 and AdipoR2 siRNA, and APN, 1.4-, 1.4- and 1.3-fold, respectively (all $p < 0.05$). Differences between groups ($n=6$) were analysed using a one-way ANOVA with Tukey test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 4. APN through AdipoR2 mediates HSC migration and AMPK activity. In activated HSCs: (A), APN did not induce apoptosis in Scram siRNA treated cells; (B), 0.1% H₂O₂ induced apoptosis compared to Scram siRNA and the combination of APN with AdipoR1 or AdipoR2 treatment had no effect on apoptosis. (C), In proliferations assays: Scram, AdipoR1 or AdipoR2 siRNA had no effect on HSC proliferation compared to control cells. The application of APN to Scram, AdipoR1 or AdipoR2 siRNA treated HSCs reduced proliferation by 1.3 ($p < 0.05$), 1.6 ($p < 0.001$) and 1.4-fold ($p < 0.01$), respectively, and there was no difference between these groups. (D), In migration assays: Scram or AdipoR1 siRNA alone had no effect. Co-treatment with APN

reduced migration in Scram (3.6-fold; $p < 0.001$), AdipoR1 (4.3-fold; $p < 0.001$), and AdipoR2 siRNA (1.6-fold; $p < 0.01$). (E), Western blot analysis of a time course of AdipoR2, AMPK and p-AMPK in Scram siRNA and AdipoR2 siRNA groups with APN treatment at 0, 5, 10, 20, 30 and 60 minutes. APN application reduced AMPK phosphorylation in AdipoR2 siRNA cells at 10 (1.4-fold; $p < 0.05$), 20 (1.3-fold; $p < 0.05$), 30 (1.4-fold; $p < 0.05$) and 60 (1.4-fold; $p < 0.05$) minutes. Differences between groups ($n=6$ for qPCR and $n=3$ for western) were analysed using a one-way ANOVA with Tukey test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Data are expressed as mean \pm SEM ($n=3$).

ACCEPTED MANUSCRIPT

Figure 1

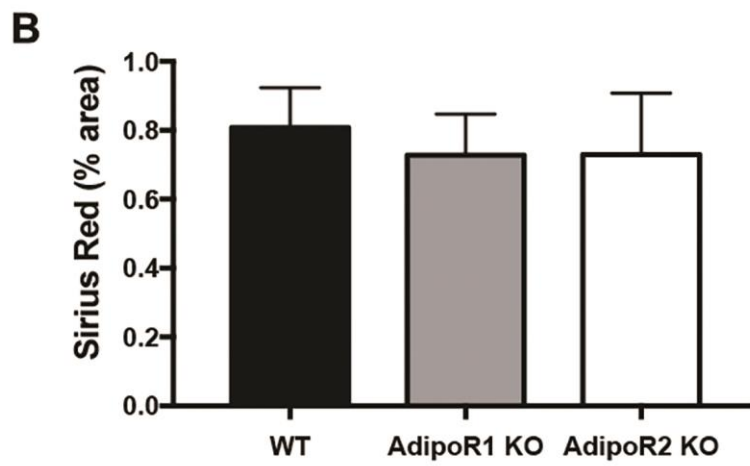
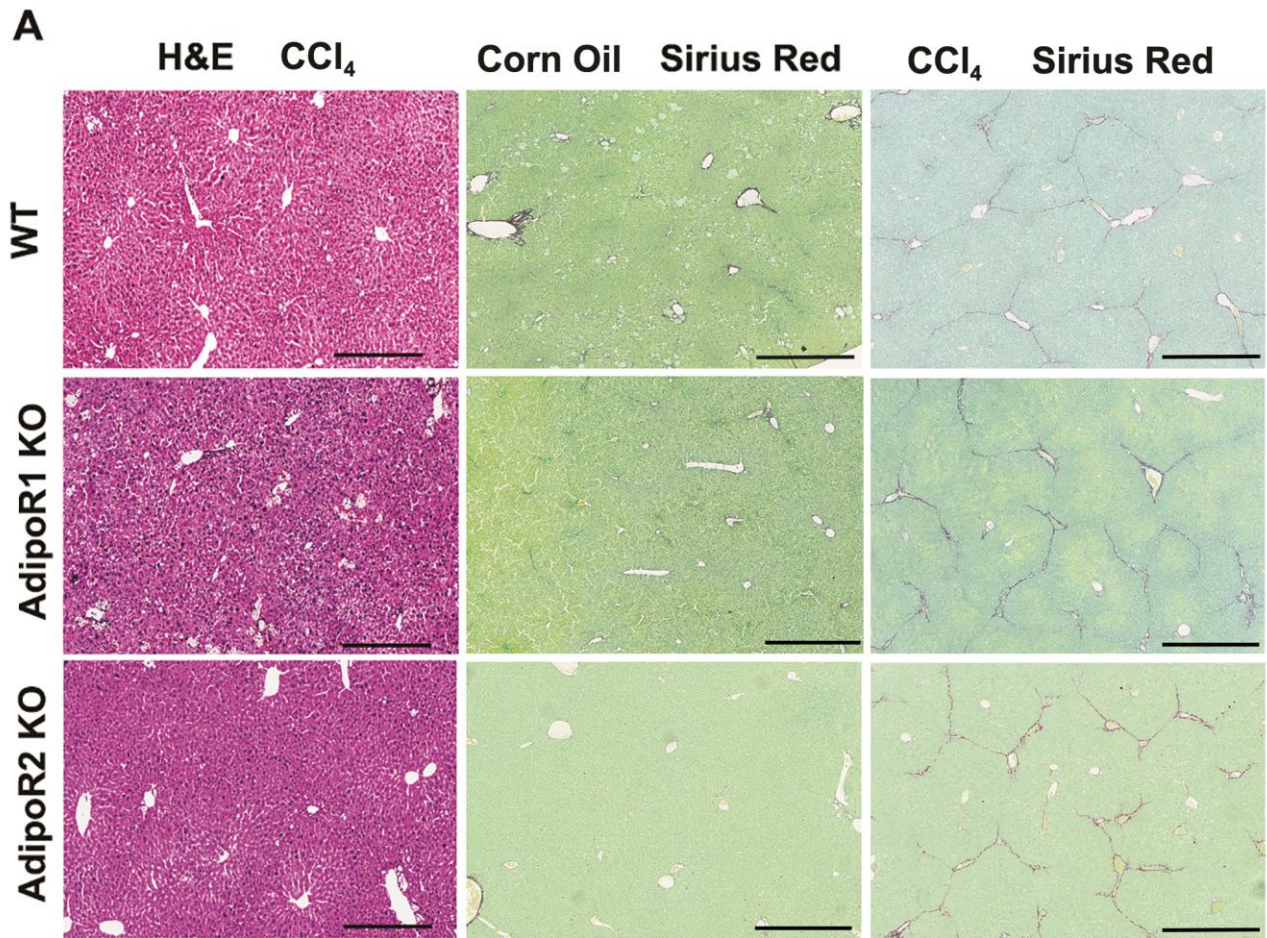


Figure 2

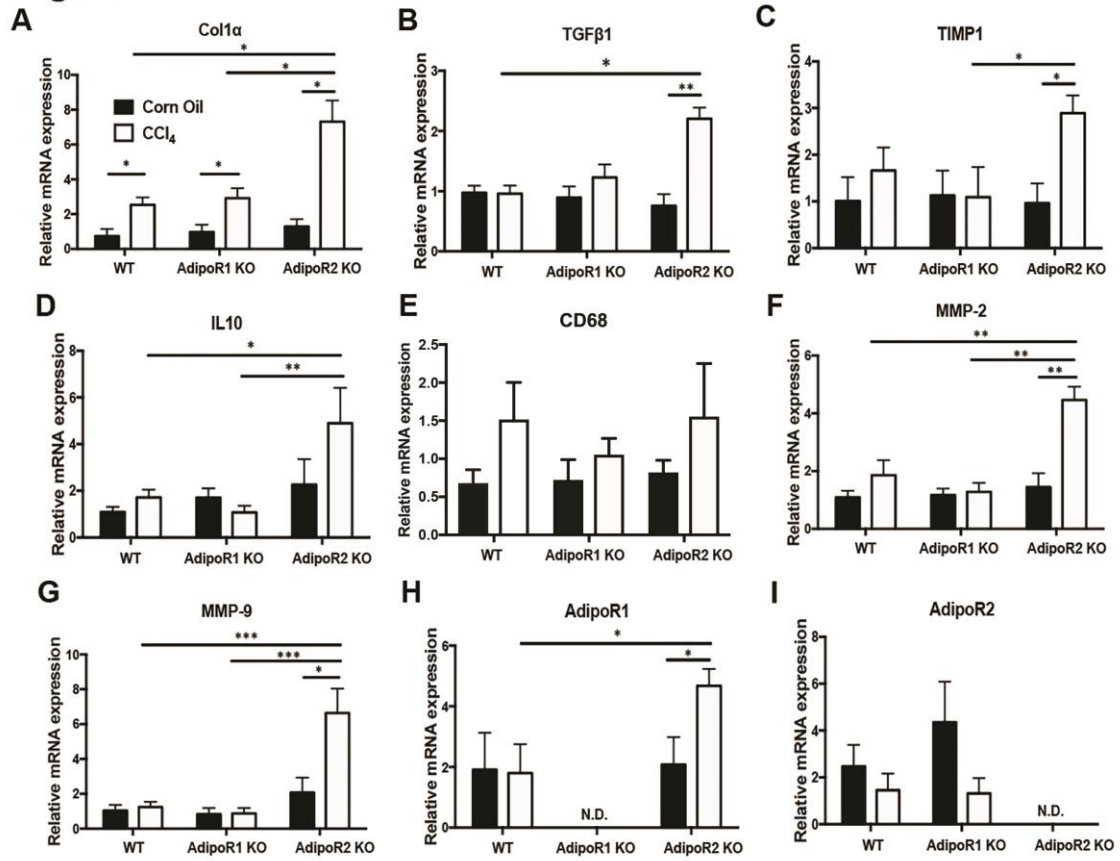


Figure 3

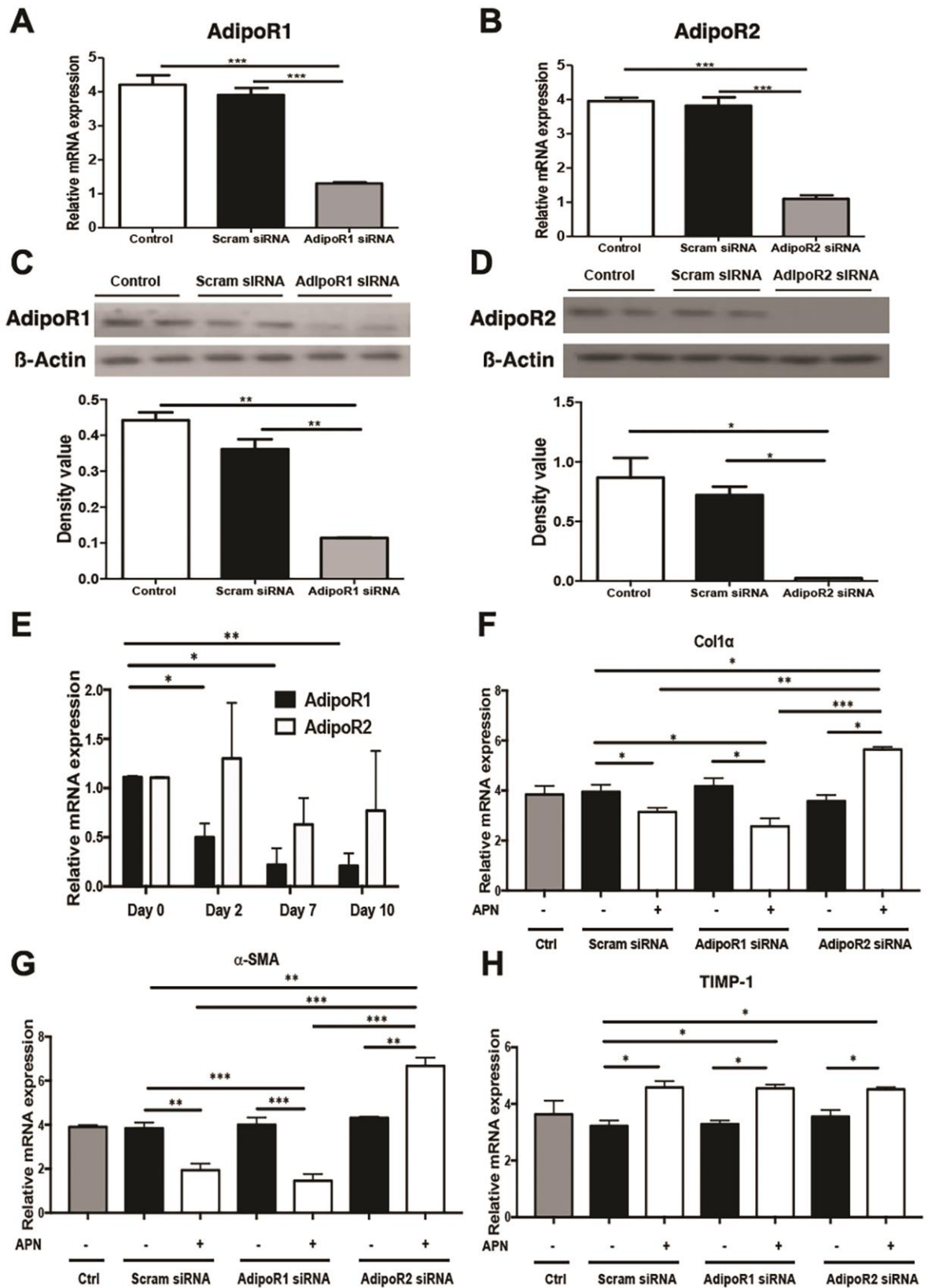
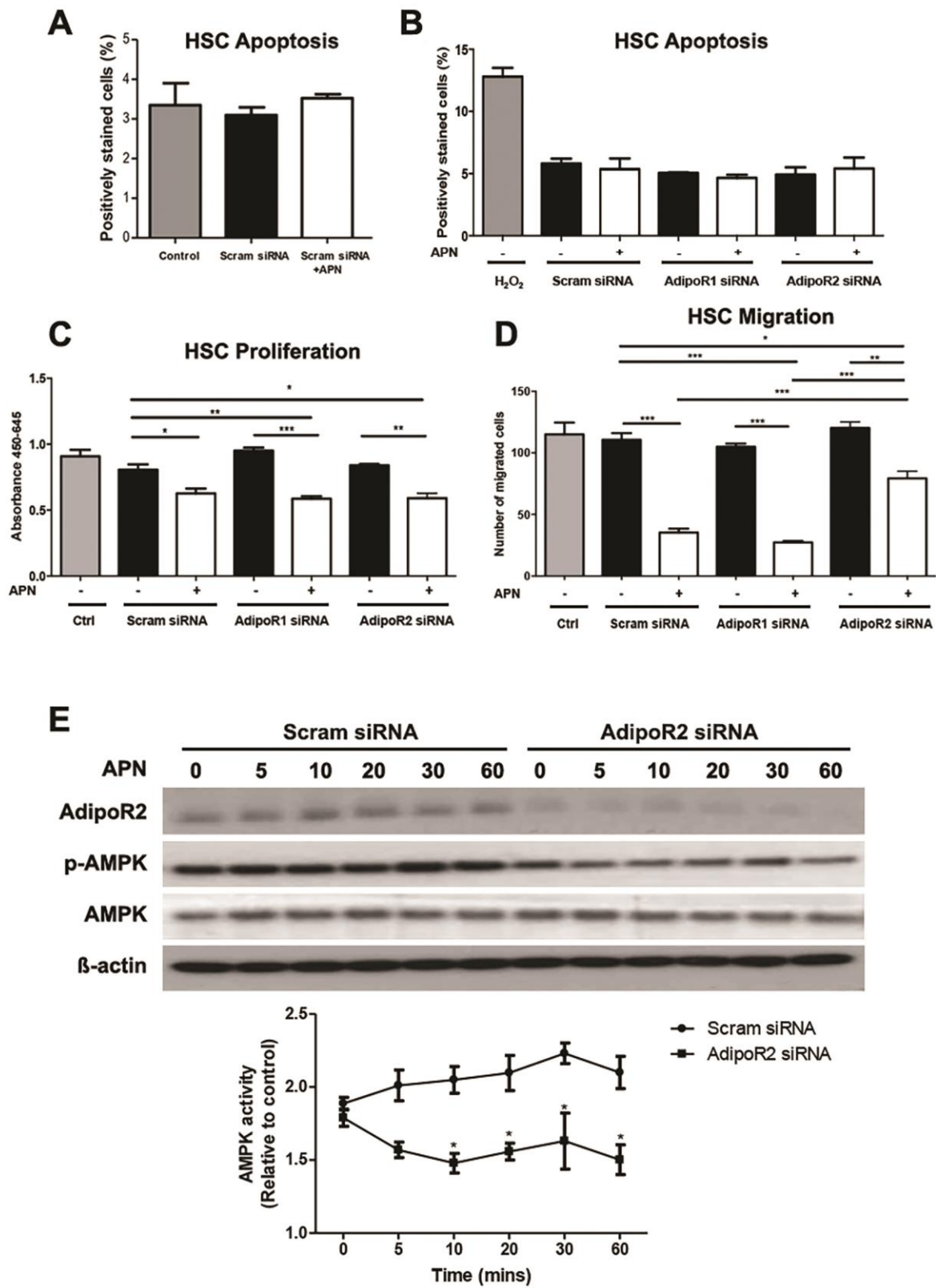


Figure 4



Highlights

- The absence of AdipoR1 or AdipoR2 is not essential for liver fibrosis *in vivo*.
- AdipoR2 loss is associated with an enhanced fibrotic signature.
- In activated HSCs, AdipoR2 is necessary for mediating APN's anti-fibrotic responses and cell migration.
- APN is unable to activate AMPK in the absence of AdipoR2 *in vitro*.

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