Retinoic acid signaling modulates the fibrogenic potential of

activated hepatic stellate cells

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Abstract

Hepatic stellate cells (HSCs), also known as perisinusoidal cells, are pericytes found in the perisinusoidal space of the liver. HSCs are the major cell type involved in liver fibrosis, which is the formation of scar tissue in response to liver damage. When the liver is damaged, stellate cells can shift into an activated state, characterized by proliferation, contractility, and chemotaxis. The activated HSCs secrete collagen scar tissue, which can lead to cirrhosis. Recent studies have also shown that *in vivo* activation of HSCs by fibrogenic agents can eventually lead to senescence of these cells, which may limit fibrosis but may also favor the insurgence of liver cancer. Retinoic acid is a metabolite of vitamin A (retinol) that mediates the functions of vitamin A required for growth and development. HSCs store vitamin A, which decreases progressively in liver injury. Retinoic acid and its receptors (RAR and RXR) are known to act synergistically with peroxisome proliferator-activated receptor gamma (PPAR-gamma) signaling in various cell types, through the activity of transcriptional heterodimers. Here we review the recent advancements in the understanding of how retinoic acid signaling modulates the fibrogenic potential of HSCs.

Introduction

Hepatic stellate cells (HSCs), also known as perisinusoidal cells, Ito cells or lipocytes, are non-parenchymal cells located in the space between the endothelial cells of sinusoids and the hepatocytes, representing approximately the 5-8% of resident liver cells. As suggested by their name, HSCs show a star-like shape with the cell body lying between the parenchymal cells of the liver and a number of cytoplasmic processes embracing sinusoids and making contact with hepatocytes and other HSCs' processes [1, 2]. In the healthy liver, HSCs are found in a quiescent state and exhibit lipid droplets storing large amounts of retinoids, mainly in the form of retinyl esters [3, 4]. Apart from the regulation of retinol homeostasis, the functions of these cells in the healthy adult liver are yet not fully understood. In recent years, new roles for HSCs in liver development during embryogenesis [1, 5] and in immunity, as antigen-presenting cells [3, 6, 7] have been proposed. Well known instead is the pivotal role HSCs play in response to liver injury, upon which they transdifferentiate into activated cells. Activated HSCs acquire a myofibroblast-like morphology, lose their retinol stores, become proliferative and contractile, migrate to the site of injury and begin to produce extracellular matrix to protect against further damage, and cytokines and growth factors which favor the regeneration and replacement of damaged hepatocytes [5]. Chronic activation of HSCs following persistent liver damage (either due to viral hepatitis viruses, alcohol, toxins, or autoimmune disorders) results in promotion of fibrosis, because of the deposition of excessive amounts of scar tissue, which alters the structure and functionality of the liver [5]. Liver fibrosis often represents the starting setting for the development of cirrhosis, which finally may evolve into hepatocellular carcinoma (HCC).

Hepatic stellate cells and senescence

The activation of the HSCs occurring upon liver injury and leading to fibrosis is somehow a self-limiting process. Indeed, besides proliferating, HSCs performing extracellular matrix deposition, also apoptotic and senescent HSCs can be found in fibrotic liver, and this would account for fibrosis reversal [8-10].

Cellular senescence (also called replicative senescence) is a phenomenon in which mitotic cells permanently stop dividing yet remaining metabolically active. Several events have been described as promoters of senescence, among which are telomere shortening after a number of replicative cycles, DNA damage, alteration of chromatin structure, oncogene activation or tumor-suppressor gene loss, oxidative stress and other kinds of stress [11, 12]. Typical features that mark senescent cells are irreversible cell cycle arrest sustained by p53 and p16/Rb pathways, morphological changes (above all increased cell size and flattened morphology), expression of senescence-associated β-galactosidase(SA-βgal), expression of a senescence-associated secretory phenotype (SASP) consisting in the production of cytokines, chemokines, growth factors, proteases and other bioactive molecules acting in a autocrine and paracrine fashion [11, 13].

Interleukin-22 (IL-22) has been recognized as a stimulus triggering senescence in HSCs [14].IL-22 is a cytokine produced by T lymphocytes and Natural Killer (NK) cells and reported to target epithelial cells of the skin, kidney, digestive and respiratory systems and hepatocytes [15]. It acts by binding a heterodimeric receptor composed by IL-10R2 and IL-22R1, then activating the JAK/STAT and MAP kinases pathways and resulting in mediation of immune responses, tissue protection from injury and regeneration [15].

Interestingly, cultured murine and human HSCs were recently found to express the IL-10R2 and IL-22R1 receptors, and when treated with IL-22, underwent senescence [14]. Consistently, in transgenic mice overexpressing IL-22 the degree of liver fibrosis was reduced and the resolution of fibrosis was faster than in wild- type mice. This pro-senescence phenotype triggered by IL-22 occurs via the activation of the STAT3 and the SOCS3 pathways, as already shown in hepatocytes [16], by increasing the phosphorylation levels of these effectors either in primary mouse and human HSCs and in the human LX2 HSC cell line [14]. Moreover, HSCs lacking STAT3 or SOCS3 fail to induce p53 and p21, which are likely responsible for the cell cycle block occurring during senescence [14].

Another inducer of senescence in HSCs is represented by CCN1 (also known as CYR61 or cystein-rich 61) [17], a non-structural protein of the extracellular matrix whose expression is induced upon injury [17, 18]. Mice lacking hepatic CCN1 treated with the hepatotoxic compound carbon tetrachloride (CCl_4), exhibit a more pronounced liver fibrosis and a decreased number of senescent cells, as judged on the basis of the expression of the SA-β-Gal marker, when compared to control mice [17]. Moreover, purified CCN1 administered to isolated activated HSCs pushes them into senescence by binding to $\alpha_1\beta_1$ integrin, inducing the Rac1/Nox1 pathway, and resulting in accumulation of reactive oxygen species (ROS) [17], which are known mediators of senescence [19].

On the other hand, the commitment of HSCs towards senescence is counteracted by adenosine, a molecule rapidly produced in response to cell injury [8]. Binding of adenosine to its A_{2A} receptor on HSCs has a pro-fibrotic effect since it stimulates collagen production [20]. Ahsan *et al.* demonstrated that by binding to the A_{2A} receptor on both LX2 cell line and primary rat HSCs, adenosine activates the

PKA/Rac1/p38MAPK pathway and this results in suppression of p53 and Rb, two proteins required for senescence initiation [8].

Based on the mechanisms described above, it is clear that once activated the fate of HSCs (proliferation, apoptosis or senescence) depends on an ill-known balance between pro- and anti-fibrotic endogenous molecules acting locally.

This feature is taking a centre stage since the secretory pattern of senescent cells can create an inflammatory environment favoring HCC. Although long considered a mechanism to prevent proliferation of mutated cells, it is now clear that senescence may also play the opposite role, triggering tumor development and progression [13, 21, 22]. Indeed, among the factors secreted by senescent cells there are growth factors which can induce cell proliferation, chemokines and metalloproteases which can promote cell migration and invasion [21].

A strong link between HSCs senescence and HCC development has been described in mouse models of obesity [22]. Obesity is a well-known risk factor for cancer, including HCC, but the underlying mechanisms are poorly understood. Yoshimoto *et al.* showed that changes in the intestinal microbiota play a key role in HCC development in both genetically obese and high-fat diet fed mice. In these models, an increase in Gram-positive gut bacteria, especially those belonging to the *Clostridium* clusters XI and XIVa, resulted in increased production of the metabolite deoxycholic acid (DCA). On reaching the liver through the enterohepatic circulation, DCA induces SASP in HSCs, which in turn promotes HCC development [22-24].

Hepatic stellate cells activation and retinoic acid

A hallmark of HSCs in their quiescent state in healthy liver is the storage of large amounts of vitamin A (or retinol) in the form of lipid droplets of retinyl esters, mostly retinyl palmitate [3, 4]. Vitamin A and its metabolites, collectively known as retinoids, are essential for normal cell growth and differentiation, for vision, reproduction and for the proper functioning of the immune system [3, 4, 25].The source of retinoids is represented by animal and plant foods, the latter mainly containing carotenoids to be converted into retinoids. Once absorbed by the intestine, retinoids bound to chylomicrons enter the circulation and are targeted to liver and other organs and tissues, where retinol is converted into retinaldehyde and this, in turn, is oxidized to retinoic acid (RA) [26]. RA is the main biologically active form carrying out the majority of the aforementioned functions [25, 26], especially in its all-*trans* and 9-*cis* isomeric forms [3]. Canonically, RA acts by binding to nuclear retinoic acid receptor -α, -β, or -γ (RARs) working as heterodimer with retinoid X receptor -α, -β, or -γ (RXRs) and resulting in regulating the expression of hundreds of target genes containing a retinoic acid responsive element (RARE) [25, 26]. In addition to this signaling pathway, however, RA is known to also bind another kind of nuclear receptor, namely peroxisome proliferator-activated receptor beta (PPARβ) and even to exert its effects in a non-transcriptional way, by activating kinase cascades rather than gene expression [25, 26].

The liver is the main organ involved in retinoid storage and metabolism. In particular, hepatocytes take up retinoids from the bloodstream, hydrolyze retinyl esters, conjugate the hydrophobic retinol with a retinol binding protein (RBP) and transfer the complex retinol –RBP to HSCs, in which it is again esterified and stored in lipid droplets. HSCs account for the storage of up to 80% of the total body retinol content

[3], and are responsible for its release into the bloodstream when the dietary intake does not meet the body requirement [4, 27]. Beside storing retinol, HSCs also metabolize it and respond to RA since they have been reported to express enzymes for retinol to retinaldehyde and retinaldehyde to RA conversion and both RARs and RXRs for regulating target genes expression [4].

Following liver injury, HSCs undergo a process of activation during which they become myofibroblast-shaped, proliferative and fibrogenic. The activation of HSCs is accompanied by a marked depletion of retinoid stocks [4, 27], even though the meaning of this event is still not fully understood. Whether retinol loss is a cause or a secondary effect of HSCs activation or whether administration of retinol/RA could have a therapeutic effect in liver disease is not clear [27, 28]. A number of reports would suggest a potential use of retinol/RA as anti-fibrotic drug. Studies on cultured cells showed retinoids to inhibit HSCs proliferation and activation [29-32]. *In vivo* studies demonstrated that the administration of retinol or its derivatives decreases liver fibrosis in different experimental models [33-37]. Beta-carotene, a precursor of retinol, was shown to cause a milder pathological phenotype in a rat model of CCl_4– induced hepatic fibrosis when compared to untreated controls [36]. Consistently, retinol administration prevented liver fibrosis induced by CCl_4 by suppressing HSCs activation [34, 37], and retinyl palmitate treatment in rats injured with dimethylnitrosamine or with pig serum decreased the number of activated HSCs thereby preventing collagen deposition and fibrosis in liver [33]. The *all-trans* RA isomer was found to alleviate ethanol-induced liver damage [35]. On the other hand, some conflicting papers exist reporting a hepato-toxic and pro-fibrotic effect of retinoids [38-40].

Another controversy concerns the responsiveness of HSCs to retinoids in their activated state [41]. As already stated, quiescent HSCs express the nuclear receptors

RARs and RXRs through which they modulate gene expression upon RA stimulus [4]. The expression of these receptors, on the contrary, was found to be down-regulated at the mRNA level and undetectable at the protein level in activated HSCs and not to increase upon exogenous RA administration [42, 43], thus suggesting HSCs unresponsiveness to RA during activation. Opposing evidence is provided by Mezaki *et al.*, who showed the protein expression of the receptors RAR-α and RAR-β to be increased in activated rat HSCs, despite lower mRNA levels. Moreover, HSCs were found to become responsive to *all-trans* RA only during activation due to post-transcriptional up-regulation of RAR-α [41].

Other lines of evidence support the importance of HSCs and retinol metabolism in liver health. RA plays a role in the negative modulation of liver fibrosis through the activation of the immune system cells, and namely NK cells. Early activated HSCs express high levels of retinoic acid early inducible gene 1 (RAE-1) which binds the NKG2D receptor on NK cells resulting in their activation [16]. Activated NK cells, in turn, become cytotoxic towards activated HSCs and HSC killing ameliorates fibrosis [4, 16, 44].

Finally, the patatin-like phospholipase domain- containing 3 (PNPLA3) has been identified as a lipase catalyzing the hydrolysis of retinyl esters in HSCs [45], that would account for the retinol stores depletion occurring during cell activation. Interestingly, the I148M polimorphism in PNPLA3 gene in HSCs, causing loss of retinyl esterase activity, is associated with chronic liver disease and hepatocellular carcinoma [45, 46].

Retinoic acid and senescence

As stated above, HSC senescence could represent a mechanism of fibrosis reversal [8-10].

Retinoids have their clinical application in the therapy of some kinds of hematological and solid cancers and in the prevention of pre-neoplastic to neoplastic disease progression [47]. The rationale for this use relies on at least three mechanisms of action: promotion of differentiation of abnormal cells back to normal, induction of apoptosis, block of cell cycle at the G_1 stage [48]. As for the cell cycle arrest, *all trans* – RA was found to repress positive G_1 regulators and to up-regulate the expression of cycle inhibitors, such as p16, p21 and p27 [48]. Since p16 and p21 are involved in the process of senescence, Park *et al*. [48] investigated on whether *all trans* – RA could promote senescence as well. They found it to cause irreversible cell cycle arrest at G_1 phase in the human hepatic cell lines HepG2 and Hep3B, in the human embryonic kidney cell line HEK293 and in the human breast cancer cell line MCF-7.This arrest was in every case dependent on the up-regulation of p16 and/or p21 [48]. Similarly, retinoids were found to induce senescence in T-cell leukemia cells [49].

An interesting evidence linking RA with senescence in stellate cells, was obtained by Froeling *et al.* in pancreatic stellate cells (PSCs) [50], a kind of cells located in the periacinar, perivascular and periductal regions of the exocrine pancreas, and sharing many morphological and functional features with HSCs [51]. As their liver counterpart, indeed, PSCs in their quiescent state store retinol-rich lipid droplets, which are depleted upon cell activation [52]. Once activated, PSCs acquire a myofibroblast-like shape, become motile and proliferative and produce huge amounts of extracellular matrix components driving the fibrogenic process [52]. Interestingly, *all trans*- RA administration was shown to restore a quiescent state in PSCs, both *in vitro* and *in vivo,* thus slowing pancreatic cancer progression [50].

Although until now no reports are available about a putative ability of retinoids to induce senescence in HSCs, one might speculate that retinoid treatment could

potentially activate the senescence program in these cells too, and this would contribute to reverse HSCs activation and thus to reverse liver fibrosis.

Retinoic acid and PPAR-gamma signaling

As discussed above, the canonical signaling of RA is through its receptors RARs and RXRs, the latter being prone to form heterodimers with other nuclear receptors, among which PPARs [53].

PPARs are a family of nuclear ligand– activated transcription factors regulating metabolism, above all the lipid one, in inflammation, cell growth and differentiation [54-56]. This family includes three members, namely PPARα, PPARβ/δ and PPARγ, differing from each other for tissue distribution, ligand selectivity and biochemical properties [54]. The PPARs work as heterodimers with RXRs and they are activated upon binding of either natural or synthetic lipidic compounds, which induce structural changes making the PPAR-RXR complex able to bind to specific responsive sequences on DNA and to allow target gene expression [55, 56]. PPARγ is the most studied among PPARs. It exists in two splicing-generated isoforms, PPARγ1 and PPARγ2, the former being ubiquitously expressed while the latter nearly restricted to adipose tissue [54]. PPARγ is mainly known for its central role in driving adipogenesis and lipid metabolism, since it promotes the expression of genes involved in lipid uptake and storage [55]. Moreover, PPARγ regulate adipose secretions such as adiponectin, leptin, resistin, TNF-α and it takes part in glucose homeostasis and response to insulin [54], as it upregulates the glucose transporter 4 (GLUT4) [57]. The latter aspect has a considerable importance in the clinical field, where a class of drugs known as

thiazolidinediones (TZD), used as insulin sensitizers in the treatment of type 2 diabetes, have revealed to be PPARγ activators [58-60].

Unlike PPARα and PPARβ/δ, PPARγ is poorly expressed in the liver [61], nevertheless it is essential for lipid metabolism in this organ. Studies performed in liver-specific knock-out mouse models demonstrated that liver ablation of PPARγ impairs triglyceride clearance, increases body fat mass [62], decreases the expression of genes involved in lipogenesis, lipid transport and β-oxidation and protects from the development of hepatic steatosis [62, 63]. Moreover, PPARγ has a protective role against liver fibrosis, since *in vivo* fibrogenesis upon injury turned out to be decreased in PPARγ- depleted and enhanced in PPARγ- overexpressing rat livers [64, 65]. This modulation of liver fibrosis by PPARγ is likely due to its effect on HSCs, since PPARγ agonists or ectopic expression of PPARγ in activated HSCs were shown to inhibit proliferation, induce apoptosis and cell cycle arrest, and promote reversal from an activated to a quiescent state [66-69].

Whether PPARγ effects on HSCs are somehow related to retinol metabolism in these cells is still uncertain, although they share the same trend: as retinol, also PPARγ is abundant in quiescent HSCs and dramatically decreases upon activation [69, 70]. Furthermore, several analogies subsist between HSCs and adipocytes: both cell types store lipid droplets, both undergo a process of differentiation/transdifferentiaton in which PPARγ is a master regulator. Both preadipocytes and activated HSCs have a fibroblast-like shape, and express the same types of interstitial collagens. Because of all these similarities, it is currently believed that these cell types are strongly interrelated and maybe regulated by the same master transcription factor, namely PPARγ [71, 72]. Some reports support the existence of a combined and synergistic action of RA and PPARγ in HSC fate and, thus, in the course of liver fibrosis [73, 74].

Combined treatment with the PPARγ ligand 15-Δ12,13-prostaglandin L(2) and the RXR ligands *all trans*-RA and *9cis*-RA on rat primary HSCs led to cell cycle arrest, inhibition of lipid droplets release and down-regulation of fibrotic markers such as collagen Iα1 and αSMA to an extent which is greater than the effect produced by each ligand alone [74].

The reciprocal interconnection between PPARγ and retinoids, however, is quite controversial. The *all trans*- RA was found to protect mice from diet-induced hepatic steatosis through a cascade culminating in the suppression of the lipogenic PPARγ2: *all trans*- RA binding to RAR activates the repressor hairy and enhancer of split 6 (Hes6) which, in turn, represses PPARγ2 expression [75]. Moreover, RA and the synthetic retinoid fenretinide block the C/EBPα-PPARγ pathway in the adipogenic process [76-78] and, accordingly, the RARα antagonist Ro 41-5253 promotes pre-adipocytes differentiation into mature adipocytes by working as activator of PPARγ [79].

Discordantly, in myeloid cells retinoids were reported to enhance PPARγ expression and activity, which is required for the differentiation of this lineage [80]. This would suggest that the cross-talk between retinoids and PPARγ is cell type specific.

Much less information is available for the inverse relationship between retinoids and PPARγ, i.e. whether PPARγ can modulate retinoid metabolism. In human dendritic cells PPARγ activation induces the expression of genes responsible for retinol metabolism and conversion to *all trans*- RA, which is at last able to modulate immune cell functions [81].

In light of all these observations, and based on its expression in the HSCs quiescent state and on its repression in activated HSCs, PPARγ has been proposed to potentially represent a new molecular target in the reversal of liver fibrosis [70, 71].

Conclusion

Liver fibrosis is a scarring process taking place as a consequence of chronic damage and inflammation of this organ and represents a major clinical challenge since it degenerates into cirrhosis with consequent liver failure and poor prognosis. The main responsible cells for fibrogenesis are HSCs which, upon liver injury, undergo an activation process and begin to produce and deposit extracellular matrix forming scar tissue.

Fibrosis has long been considered an irreversible mechanism but recently it has been uncovered that HSCs may undergo a phenotype reversion accounting for fibrosis regression [9].

The hallmark of HSCs is the storage of large amounts of retinol in the form of retinyl esters in lipid droplets which are depleted upon cell activation, generating metabolically active forms of RA [4, 27]. Concomitantly, PPARγ expression, which is substantial in quiescent HSCs, significantly decreases following activation [64, 65]. Since RA acts through retinoid receptors which synergize with PPARγ, and since both retinoids and PPARγ have proven to be able to reverse HSCs activation and liver fibrosis [73, 74], it is tempting to propose that a combined treatment with retinoid receptor agonists and PPARγ agonists may potentially represent a new and more beneficial strategy in the clinical management of liver fibrosis (Figure 1).

Compliance with ethical standards
Conflict of interest The authors declare that they have no conflict of interest.

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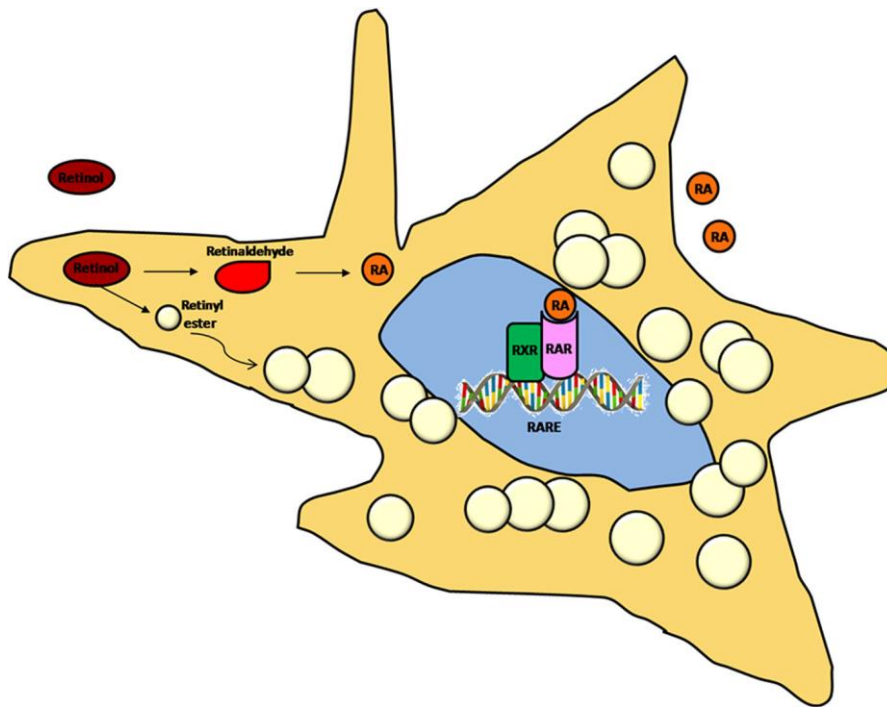
Figure 1

HSCs are able to metabolize retinol to give both retinyl esters (then stored in lipid droplets) and retinoic acid. Moreover, HSCs express the nuclear receptors for retinoic acid RARs, which, together with RXRs, bind to DNA on Retinoic Acid Responsive Elements (RARE) and modulate target genes expression.

Table 1:

Characteristics of the studies showing the anti- or the pro-fibrotic effects of retinoids on liver

| Type of retinoid	Organism/cells	Effect	References
Anti-fibrotic effect of retinoids			
13-cis-retinoic acid	Cultured rat HSCs	Inhibition of proliferation and transformation	Chi et al. [67]
13-cis-retinoic acid	Rat and cultured rat HSCs	Inhibition of activation	Davis et al. [68]
Vitamin A	Cultured rat HSCs	Inhibition of proliferation	Davis et al. [69]
All-trans retinoic acid	Rat HSC line	Inhibition of proliferation and collagen production	Ye and Dan[71]
Retynyl palmitate	Rat	Suppression of hepatic fibrosis induced by dimethylnitrosamine/pig Serum	Mizobuchi et al [72]
Vitamin A	Rat	Suppression of CCl4-induced HSCs activation and fibrosis	Noyan et al. [73]
All-trans retinoic acid	Rat	Amelioration of ethanol-induced liver injury	Pan et al. [74]
Beta carotene	Rat	Alleviation of CCl4-induced hepatic inflammation and fibrosis	Seifert et al. [75]
Vitamin A	Rat	Suppression of hepatic fibrosis induced by CCL4/pig serum	Senoo and Wake [76]
All-trans retinoic acid	Mouse	Amelioration of CCl4-induced liver fibrosis	Hisamori et al. [77]
Pro-fibrotic effect of retinoids			
9-cis-retinoic acid	Rat	Exacerbation of pig serum-induced liver fibrosis	Okuno et al. [79]
Vitamin A (high levels)	Rat	Exacerbation of CCl4-induced liver fibrosis	Vollmar et al. [80]
Vitamin A	Humans	Dose-dependent chronic liver damage	Geubel et al. [78]
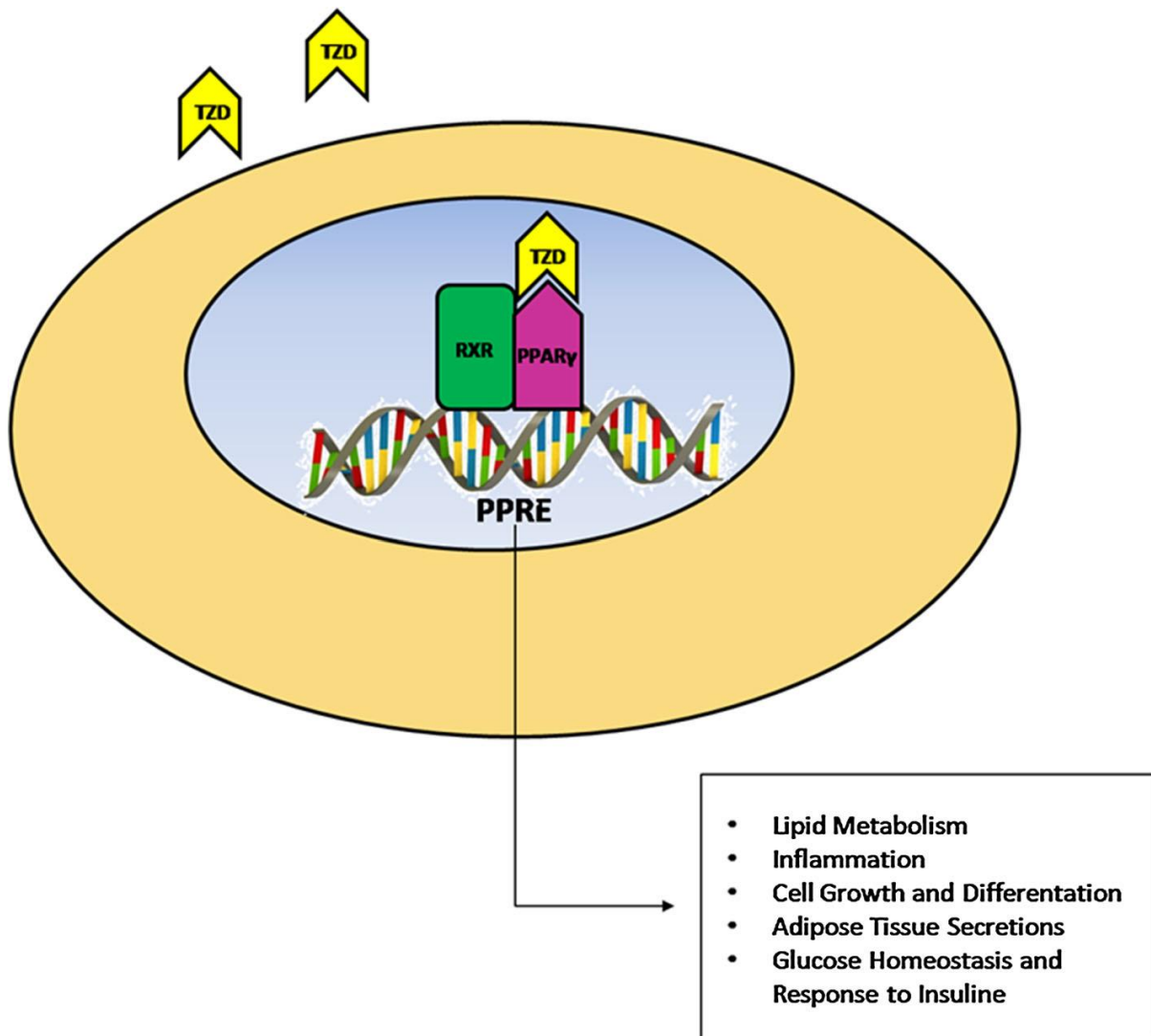
Figure 2
Natural or synthetic lipid compounds, such as thiazolidinediones (TZD) are agonists for the transcription factor PPARc, which heterodimerizes with RXR, binds to DNA on PPAR responsive elements (PPRE) and drives transcription of a number of target genes involved in lipid and glucose metabolism, inflammation, cell growth.
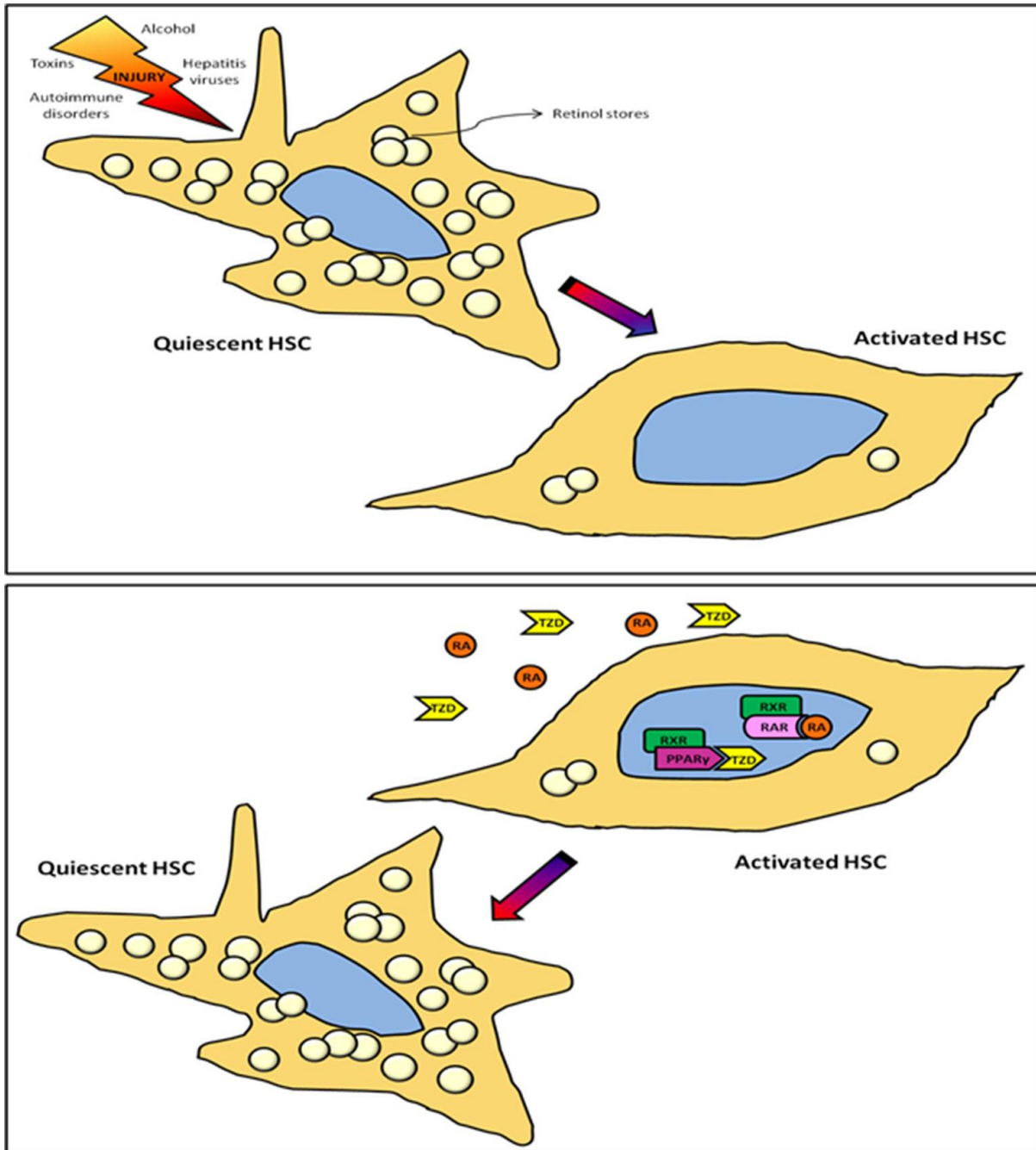
Figure 3
Alcohol, toxins, hepatic viruses, autoimmune disorders and other kinds of injuries cause the transdifferentiation of hepatic stellate cells (HSCs) from a quiescent to an activated, pro-fibrotic state. The combined action of retinoid receptor agonists (such as RA) and PPARc agonists (such as TZDs) may synergistically bring activated HSCs back to a quiescent phenotype, accounting for fibrosis reversal.