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Melatonin suppresses activation of hepatic stellate cells through RORα-mediated inhibition of 5-lipoxygenase

Abstract: Liver fibrosis is scar tissue resulting from an uncontrolled wound-healing process in response to chronic liver injury. Liver damage generates an inflammatory reaction that activates hepatic stellate cells (HSC) that transdifferentiate from quiescent cells that control retinol metabolism to proliferative and migratory myofibroblasts that produce excessive amounts of extracellular matrix proteins, in particular collagen 1a1 (COL1A1). Although liver fibrosis is reversible, no effective drug therapy is available to prevent or reverse HSC activation. Melatonin has potent hepatoprotective properties in a variety of acute and chronic liver injury models and suppresses liver fibrosis. However, it remains unclear whether melatonin acts indirectly or directly on HSC to prevent liver fibrosis. Here, we studied the effect of melatonin on culture-activated rat HSC. Melatonin dose-dependently suppressed the expression of HSC activation markers *Col1a1* and alpha-smooth muscle actin (αSMA, *Acta2*), as well as HSC proliferation and loss of lipid droplets. The nuclear melatonin sensor retinoic acid receptor-related orphan receptor-alpha (RORα/*Nr1f1*) was expressed in quiescent and activated HSC, while the membranous melatonin receptors (*Mtrn1a* and *Mtrn1b*) were not. The synthetic RORα agonist SR1078 more potently suppressed *Col1a1* and *αSma* expression, HSC proliferation, and lipid droplet loss, while the RORα antagonist SR1001 blocked the antifibrotic features of melatonin. Melatonin and SR1078 inhibited the expression of *Alox5*, encoding 5-lipoxygenase (5-LO). The pharmacological 5-LO inhibitor AA861 reduced *Acta2* and *Col1a1* expression in activated HSC. We conclude that melatonin directly suppresses HSC activation via RORα-mediated inhibition of *Alox5* expression, which provides novel drug targets to treat liver fibrosis.

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Introduction

Liver diseases are typically characterized by the loss of functional liver tissue, while at the same time, a wound-healing process is initiated to regenerate the organ. Liver injury can have a wide range of etiologies, including viral infections, alcohol or drug abuse, fatty diets, and autoimmune-related causes. Many liver diseases follow a chronic time course and lead to an uncontrolled wound-healing response that causes liver fibrosis. Liver fibrosis is characterized by the accumulation of extracellular matrix (ECM) proteins in the liver, mainly collagens and fibronectins. The ECM is produced by hepatic myofibroblasts that may arise from multiple cell types, but the hepatic stellate cells (HSC) are considered to be the main source. In the healthy liver, HSC reside in the space of Disse, in between the endothelial cells and the hepatocytes, and control whole-body retinol metabolism [1–3]. Up to 80% of all vitamin A is stored as retinyl esters in HSC in large cytosolic lipid droplets containing also cholesterol esters and triglycerides [4]. Upon liver injury, HSC transdifferentiate to highly proliferative and mobile myofibroblasts that secrete abnormal amounts of ECM proteins, while losing their retinyl ester-containing lipid droplets. Liver fibrosis may progress to cirrhosis, where the complex liver architecture is irreversibly disturbed and predisposes for liver cancer. At the precirrhotic stages, liver fibrosis is considered reversible and may fully resolve if the liver-damaging conditions are removed. Unfortunately, no effective therapeutic drugs are available yet to support the resolution of liver fibrosis [1].

Melatonin (N-acetyl-5-methoxytryptamine), a tryptophan-derived biomolecule, is a main product of the pineal gland, but is also locally synthesized in the digestive system and other extrapineal tissue [5]. Many studies have shown strong hepatoprotective effects of melatonin in a variety of liver injury models, including viral hepatitis, obstructive cholestasis, hepatectomy, septic shock, ischemia reperfusion, drug-induced liver injury, and radiation [6–15]. The therapeutic effects are generally reflected in a reduction in serum markers of liver damage (AST, ALT, γGT, ALP, LDH), bilirubin, hepatic lipid peroxidation, inflammatory markers, and/or increased survival rates. In chronic models of liver injury, melatonin therapy reduces liver fibrosis. Melatonin has potent antioxidant properties [16]. Recent research, however, shows that many of its therapeutic actions act through membrane (MTNR1a and

MTNR1b), cytosolic (MT3/NQO2), or nuclear (RORα) receptors. Both the membrane and the nuclear receptors have been shown to mediate the cytostatic features of melatonin and suppress cancer cell proliferation and tumor growth [17].

Despite the well-established hepatoprotective properties of melatonin, the molecular mechanisms that are involved in its therapeutic action are not fully understood, let alone that we know the main target cells of melatonin in the liver. The antifibrotic action of melatonin may be an indirect effect if it protects hepatocytes and thereby suppresses downstream inflammatory and/or oxidative stress-mediated signaling that promotes HSC activation. Alternatively, melatonin may act directly on the hepatic myofibroblasts in suppressing their activation and/or proliferation. The latter option may allow the development of melatonin as an antifibrotic drug irrespective of the etiology of the liver disease.

Thus, we set out to study the direct effect of melatonin on the activation and proliferation of primary rat HSC in vitro. We found that melatonin suppresses the expression of typical HSC activation markers collagen 1a1 (*Col1α1*) and alpha-smooth muscle actin (αSMA/*Acta2*) and suppresses HSC proliferation and depletion of lipid droplets during the activation process. Rat HSC lack expression of *Mtnr1*a and *Mtnr1b*, but do stably express *Nr1f1* in various stages of the activation process. RORα antagonists blunt the action of melatonin, while RORα agonists appear even more potent in suppressing HSC activation. Both melatonin and SR1078 suppressed the expression of the RORα target gene *Alox5,* encoding 5-lipoxygenase (5-LO), which is a pro-inflammatory enzyme involved in hepatic inflammation and fibrosis [18–20]. In line, pharmacological inhibition of 5-LO using AA861 also suppressed *Col1a1* and *Acta2* expression in activated HSC. These data show for the first time the direct antifibrotic action of melatonin and the molecular mechanisms involved.

Materials and methods

Animals

Specified pathogen-free male Wistar rats (350–400 g; Charles River Laboratories Inc., Wilmington, MA, USA) were kept under standard laboratory conditions with free access to standard laboratory chow and water. All experiments were performed according to the Dutch law on the welfare of laboratory animals and guidelines of the ethics committee of university of Groningen for care and use of laboratory animals.

Primary rat hepatocyte and hepatic stellate cell isolation and culture

Primary rat hepatic stellate cells were isolated by pronase (Merck, Amsterdam, the Netherlands) and collagenase-P (Roche, Almere, the Netherlands) perfusion of the liver. Afterward, cells were purified by Nycodenz (Axis-Shield POC, Oslo, Norway) gradient centrifugation as described before [21]. HSC were cultured in Iscove's Modified Dulbecco's Medium with Glutamax (Invitrogen, Brenda, the Netherlands) supplemented with 20% heat-inactivated fetal calf serum (Invitrogen), 1 mmol/L sodium pyruvate (Invitrogen), 1 × MEM nonessential amino acids (Invitrogen), 50 μg/mL gentamicin (Invitrogen), 100 U/mL penicillin (Lonza, Vervier, Belgium), 10 μg/mL streptomycin (Lonza), and 250 ng/mL Fungizone (Lonza) in a humidified incubator at 37°C with 5% CO_2. Primary rat hepatocytes were isolated by collagenase (Sigma-Aldrich, Zwijndrecht, the Netherlands) perfusion and cultured in William's E medium as described before [22]. Rat brain was taken out, snap-frozen in liquid nitrogen, and stored at −80°C. The frozen tissue was crushed and dissolved in TRI reagent (Sigma-Aldrich) for RNA isolation and qPCR analyses.

Treatments

Melatonin (Sigma-Aldrich), SR1078 (Merck Millipore, Billerica, MA, USA), SR1001 (Sigma-Aldrich), and AA861 (Sigma-Aldrich) were dissolved in DMSO (Merck) and diluted with medium for final concentrations. Treatment concentrations were 10 μM melatonin, 10 μM SR1078, 10 μM SR1001, and 10 μM AA861, unless stated otherwise. Isolated HSC were treated 4 hr after plating. Every day medium was changed and supplied with fresh melatonin, SR1078, SR1001, AA861, or DMSO as control group. Activated HSC were trypsinized and recultured for 3 days. Treatment was started 4 hr after plating.

RNA isolation and quantitative polymerase chain reaction

RNA was isolated using TRI reagent according to the manufacturer's instructions. Reverse transcription was performed on 2.5 μg of total RNA using random nanomers (Sigma-Aldrich) in a final volume of 50 μL. Real-time quantitative PCR (qPCR) was performed on the Gel Doc™ XR+ System (Bio-Rad Laboratories, Hercules, CA, USA) using the TaqMan protocol. mRNA levels were normalized to the housekeeping gene 18S and further normalized to the mean expression level of the control group. The primers and probes are shown in Table 1. mRNA expression of *Mtnr1a* and *Mtnr1b* is presented as the $2^{\Delta CT}$ values.

Immunofluorescence microscopy

HSC were cultured on coverslips and fixed with 4% paraformaldehyde (Merck Millipore). Coverslips were incubated with primary antibodies and labeled with secondary antibodies (Table 2). At the end, slides were mounted in fluorescence mounting medium S3023 (DAKO, Heverlee, Belgium). Images were captured using a Leica DMI6000 and analyzed by ImageJ (ImageJ; National Institutes of Health, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/) and Adobe Photoshop CS6.

BrdU incorporation ELISA

Quiescent HSC were seeded in a 96-well plate for 5 days. Proliferation was assessed at day 5 using a BrdU incorpo-

Table 1. Sequences of rat primers and probes used for real-time quantitative PCR analysis

Gene	Sense 5′–3′	Antisense 5′–3′	Probe 5′–3′
18S	CGGCTACCACATCCAAGGA	CCAATTACAGGGCCTCGAAA	CGCGCAAATTACCCACTCCCGA
Col1α1	CGGCTCCTGCTCCTCTTAGG	CTGACTTCAGGGATGTCTTCTTGG	CCACTGCCCTCCTGACGCATGG
Acta2	GCCAGTCGCCATCAGGAAC	CACACCAGAGCTGTGCTGTCT T	CTTCACACATAGCTGGAGCAGCTTCTCGA
RORα	TCAGGAATCCATTATGGTGTCATTAC	GAAAAGTCAAATATGGAGCTGATGAG	TCGCTGCAGCCCAAGGCTCG
ADFP	GTACGTGACTCGATGTGCTCAA	GTACGTGACTCGATGTGCTCA A	CTACGACGACACCGATGAGTCCCAC
Alox5	CTGTATAAGAACCTAGCCAACAAGATTTG	CTTGAACGCACCCAGATTTTG	CCATCGCCATCCAGCTCAACCAA
MTNR1b	TaqMan® Gene Expression Assay: Rn01447987_m1		
MTNR1a	TaqMan® Gene Expression Assay: Rn01488022_m1		

ration ELISA kit (Roche) in accordance with the manufacturer's instructions. The plate was read using spectrophotometer. Absorbance wavelength was 450 nm (BioTek Instruments, Inc., Bad Friedrichshall, Germany).

Real-time monitoring of cell proliferation

Proliferation of HSC was monitored from the beginning of the activation process using the xCELLigence system (RTCA DP; ACEA Biosciences, Inc., San Diego, CA, USA). Quiescent HSC were plated in E plates having interdigitated gold microelectrodes to constantly record cell proliferation, according to manufacturer's instructions [23]. Treatment was started 4 hr after attachment. Results were recorded and analyzed by RTCA software.

Oil red O staining

HSC were cultured on coverslips and fixed with 4% paraformaldehyde (Merck Millipore). Coverslips were rinsed with 60% isopropanol and incubated with Oil Red O solution (Sigma-Aldrich) for 10 min. Afterward, cells were rinsed with 60% isopropanol and incubated with hematoxylin (Sigma-Aldrich) and tap water for 1 and 5 min, respectively. Slides were mounted with Kaiser's glycerol gelatine (Merck Millipore), scanned with Aperio Scanscope CS slide scanner (Leica Biosystems), and analyzed by Imagescope (Leica Biosystems, http://www.leicabiosystems.com/pathology-imaging/aperio-epathology/integrate/imagescope/).

Statistical analysis

Results are presented as the mean of at least three independent experiments ± S.D. (n = 3). Control groups with sample size 20 (n = 20) were normally distributed. For statistical analyses, it is assumed that the treatment groups are similarly normally distributed. One-way ANOVA followed by Tukey's multiple comparison test or two-tailed Mann–Whitney test was used. Results were considered significant at *P*-value less than 0.05 ($P < 0.05$). All data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

First, we analyzed the effect of melatonin on the HSC activation process. Freshly isolated (quiescent) HSC were cultured in the absence or presence of melatonin (10 or 100 μM) for 7 days until control HSC were fully transdifferentiated, followed by quantitative PCR to determine the expression of HSC activation markers, αSMA (*Acta2*) and *Col1a1* (Fig. 1A). Melatonin dose-dependently

Table 2. Antibody dilutions for immunofluorescence microscopy

Antibody	Dilutions	Company
Mouse αSMA	1:400	Sigma-Aldrich, St. Louis, MO, USA
Goat Collagen type 1	1:400	Southern Biotech, Birmingham, AL, USA

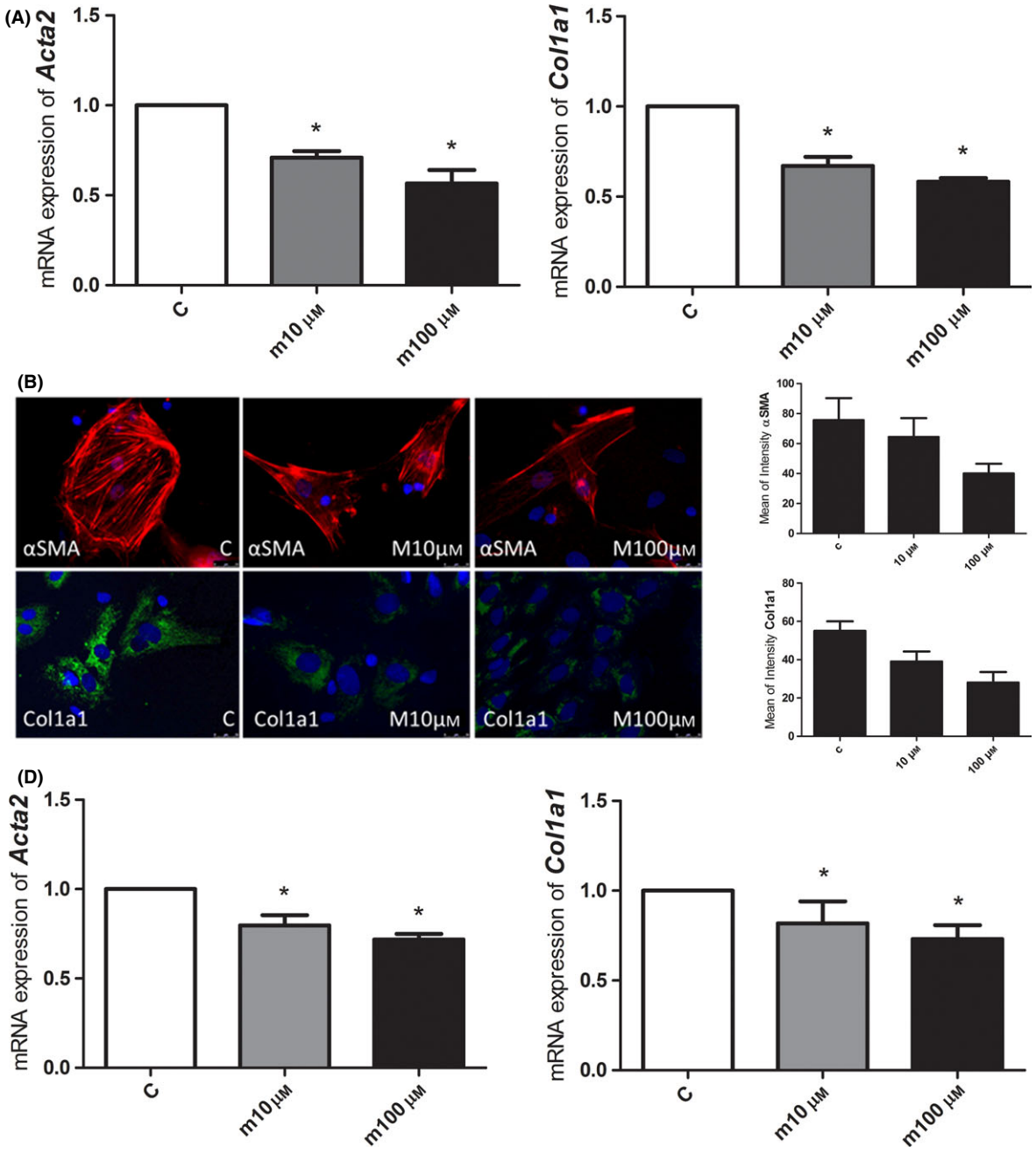

Fig. 1. Melatonin suppresses HSC activation. (A,B) Freshly isolated primary rat HSC were culture-activated in the absence and presence of melatonin (10 or 100 μM) for 7 days and analyzed for expression of *Acta2*/αSMA and *Col1α1*/COL1A1 at the mRNA (A) and protein (B) level. Melatonin dose-dependently suppressed expression of both HSC activation markers. Graphs in B show the mean of the immunofluorescence signal of 20 individual cells for each condition. Magnification 40×, scale bars 25 μm. (C) Fully activated HSC (7 days in culture) were treated for 3 days with various concentrations melatonin, after which mRNA levels of *Acta2* and *Col1a1* were quantified. Melatonin dose-dependently suppressed expression of these activation markers in fully activated HSC. *Indicates $P < 0.05$.

reduced the mRNA levels of *Acta2* (−30% and −44% at 10 and 100 μM melatonin, respectively) and *Col1a1* (−33% and −42% at 10 and 100 μM melatonin, respectively). A similar dose-dependent reduction was detected for cellular αSMA and COL1A1 protein levels 7 days after melatonin treatment, as determined by (quantitative) immunofluorescence microscopy (Fig. 1B). These results show that melatonin suppresses the HSC activation process and thus may prevent the development of liver fibrosis. In a next set of experiments, we first allowed full activation of the HSC during 7-day culturing, which was followed by a 3-day treatment with melatonin to determine whether melatonin can also reverse the HSC transdifferentiation process. Fig. 1C shows that melatonin also caused a dose-dependent decrease in *Acta2* (−21% and −29% at 10 and 100 μM melatonin, respectively) and *Col1a1* (−19% and −27% at 10 and 100 μM melatonin, respectively) mRNA levels when fully activated HSC were

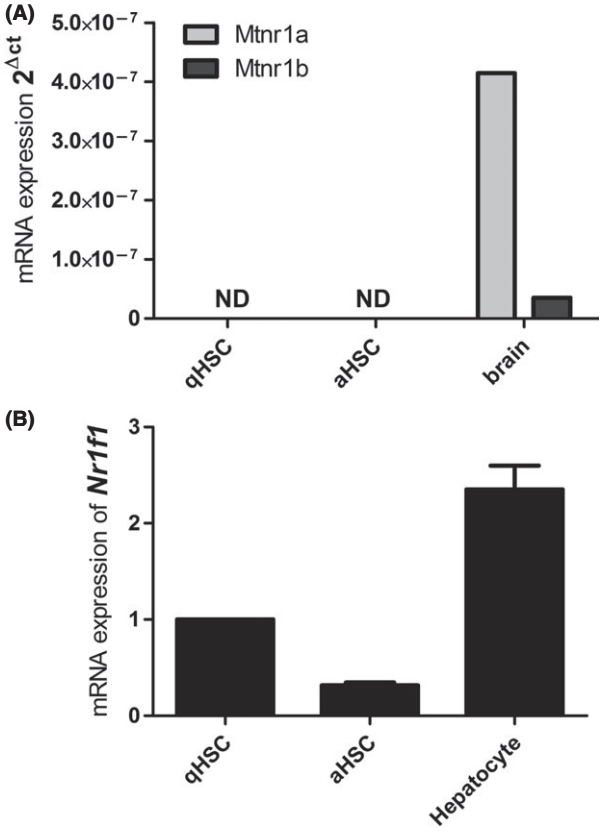

Fig. 2. HSC express nuclear melatonin receptor and do not express membranous melatonin receptor. Expression of the membranous (*Mtnr1*a and *Mtnr1b*; A) and nuclear (*Nr1f1*; B) in freshly isolated primary rat HSC (quiescent HSC; qHSC) and culture-activated HSC (aHSC) were quantified by qPCR. Rat brain tissue served as control for expression of *Mtnr1*a/*b* (A). *Nr1f1* expression was also quantified in purified rat hepatocytes (B). *Mtnr1*a and *Mtnr1b* were undetectable in qHSC and aHSC (A), while *Nr1f1* expression was readily detectable in qHSC and aHSC, with approximately 2.5-fold higher levels in qHSC (B).

exposed to melatonin for 3 days. Taken together, these data show that melatonin both suppresses and reverses the activation of hepatic stellate cells in vitro.

To explore potential mechanisms that underlie the antifibrotic action of melatonin, we analyzed the expression of the membranous (MTNRa1, MTNRb1) and nuclear (retinoic acid receptor-related orphan receptor-alpha; RORα/*Nr1f1*) melatonin receptors. We were unable to detect any significant amounts of *Mtnra1* and *Mtnrb1* mRNA in quiescent HSC (qHSC) or activated HSC (aHSC), while both were readily detectable in rat brain tissue taken as a positive control (Fig. 2A). In contrast, *Nr1f1* mRNA levels are adequately expressed in both qHSC and aHSC, with almost 3.3-fold higher levels in qHSC compared to aHSC. Hepatic RORα expression is not unique for HSC as rat hepatocytes also contain high *Nr1f1* mRNA levels (2.3-fold higher compared to qHSC) (Fig. 2B).

Next, we exposed activating HSC to equimolar concentrations (10 μM) of melatonin, the RORα agonist SR1078, or the RORα antagonist SR1001 and analyzed the mRNA levels of *Acta2* and *Col1a1* after 1, 3, and 5 days. As observed before, 10 μM melatonin gave a minor, but significant reduction in both *Acta2* and *Col1a1* mRNA levels in HSC after a 5-day exposure (Fig. 3A,B). In contrast, 10 μM SR1078 completely abrogated the induction of *Acta2* (Fig. 3A), while also strongly suppressing the induction of *Col1a1* mRNA expression (−69% at day 5 compared to control HSC; Fig. 3B). Exposure to the RORα antagonist SR1001 on the other hand did not affect the induction of *Acta2* and *Col1a1* expression at any time point compared to control HSC. Both melatonin and SR1078 give rise to a minor induction of *Nr1f1* mRNA levels (at days 3 and 5) as well as the RORα downstream target *Plin2* (encoding adipose differentiation-related protein ADFP) (at days 1 and 3), while no effect was observed of SR1001 on expression of these genes (Fig. 3C,D). In accordance with a suppression of the activation process, melatonin and SR1078 also delayed the loss of lipid droplets from HSC, which was particularly evident 3 days after the start of the treatment (Fig. 4). At later time points (day 5), HSC were clearly smaller in size when treated with SR1078, and to a lesser extent with melatonin, compared to control or SR1001-treated HSC (Fig. 4, compare HSC in insets).

To further analyze a potential effect of melatonin and RORα on HSC proliferation, we performed a real-time cell-monitoring experiment during 5 days starting with freshly isolated qHSC (Fig. 5A). After initial seeding, the cell index of control HSC starts increasing only after 2–2.5 days, indicating a relative long period where HSC first lose their lipid content and transdifferentiate into myofibroblasts. In the following 2–3 days, a steady increase in cell index (up to 7 AU) is observed for control HSC. Melatonin both prolongs the initial lag period as well as the speed of the increase in cell index during the second (day 3–5) period. SR1078 almost completely prevented the increase in cell index over the 5-day period. The cell index represents the cellular coverage of the culture well, and for HSC, this is a cumulative readout of cell stretching (activation) and cell division (proliferation). To determine whether melatonin and RORα directly affect HSC proliferation, we performed a bromodeoxyuridine (BrdU) incorporation assay at day 5 (Fig. 5B), which revealed a clear reduction in BrdU incorporation when HSC were treated with melatonin (−37% compared to control HSC) or SR1078 (−63% compared to control HSC).

To confirm that melatonin is acting via RORα in suppressing HSC activation, we treated HSC with 10 μM melatonin in the presence or absence of 10 μM SR1001, the RORα antagonist. As shown in Fig. 6, mRNA levels of *Acta2* and *Col1a1* were significantly increased in melatonin/SR1001-cotreated HSC compared to HSC treated with melatonin alone, while no significant difference was detected between control, SR1001-treated, and melatonin/SR1001-cotreated HSC.

RORα suppresses the expression of 5-lipoxygenase (5-LO) that converts arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) as the first step in leukotriene synthesis [24]. 5-LO has been shown to promote liver fibrosis, but only in relation to its expression in Kupffer cells, the liver-resident macrophages [16, 18]. Anecdotal evidence suggests that 5-LO is also expressed by HSC and

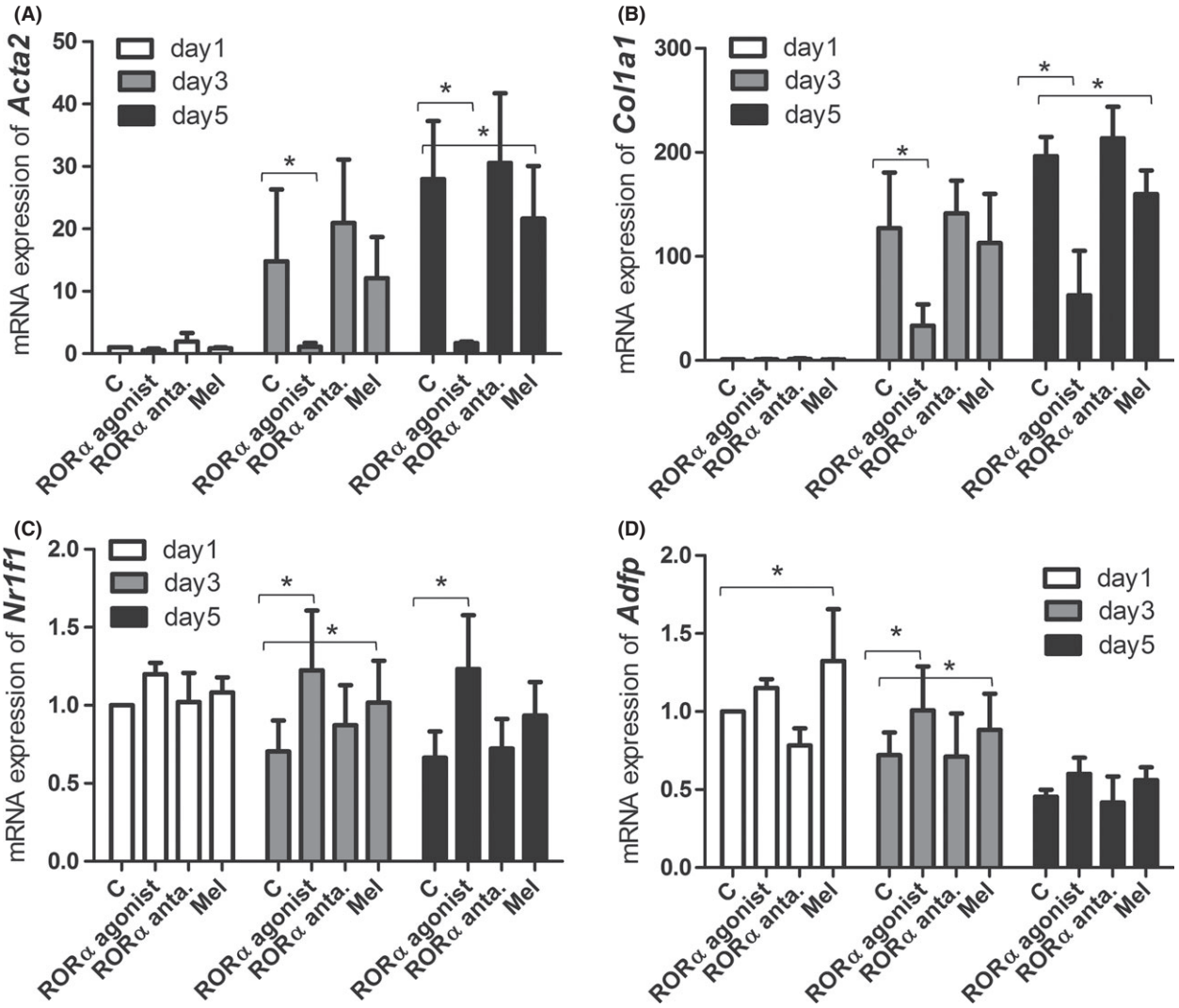

Fig. 3. The RORα agonist SR1078 potently suppresses HSC activation. Freshly isolated HSC were culture-activated in the absence or presence of 10 μM melatonin, 10 μM SR10878 (RORα agonist), or 10 μM SR1001 (RORα antagonist) for 1, 3, or 5 days and analyzed by qPCR for mRNA expression of *Acta2* (A), *Col1α1* (B), *Nr1f1* (C), and the RORα downstream target gene *Adfp* (D). The RORα agonist S1078 potently suppressed *Acta2* and *Col1a1* expression in activating HSC, while the RORα antagonist SR1001 did not affect expression of these HSC activation markers (A,B). The RORα agonist caused a modest and transient induction of its own expression and that of *Adfp* (C,D). *Indicates $P < 0.05$.

produces cysteinyl leukotrienes. In accordance, we detected significant *Alox5* mRNA expression in rat HSC (Fig. 7A–C), which was induced upon HSC activation (Fig. 7B) and was dose-dependently suppressed by melatonin (Fig. 7A) and SR1078 (Fig. 7B). The RORα antagonist SR1001 prevented the melatonin-induced suppression of *Alox5* expression (Fig. 7C). Finally, we treated aHSC for 24 hr with the 5-LO inhibitor AA861 and found that it suppressed expression of *Col1a1* and *Acta2* (Fig. 7D). Taken together, these data show that melatonin directly suppresses HSC proliferation and activation through RORα-mediated suppression of *Alox5* expression.

Discussion

In this study, we show that melatonin directly suppresses proliferation and activation of HCS, which is mediated via the nuclear receptor RORα and its downstream target gene, *Alox5*, which encodes 5-lipoxygenase (5-LO). A high-affinity agonist of RORα, as well as an antagonist of 5-LO, showed potent antifibrotic effects on HSC, further highlighting their potential as therapeutic target for antifibrotic therapy in liver disease.

Prior in vivo studies using laboratory animals have convincingly showed that melatonin has potent hepatoprotective properties in a wide array of acute and chronic liver disease models, including viral hepatitis, CCl_4 toxicity, and obstructive cholestatic-, drug-, or diet-induced liver injury [8, 14, 15, 25]. In most cases, the therapeutic effects of melatonin have been attributed to its strong antioxidative capacity and/or the stimulation of antioxidant enzymes in the liver, in particular protecting the functional liver cells, the hepatocytes [7, 26–28]. Chronic injury to these hepatocytes leads to liver fibrosis. Excessive deposition of extra-

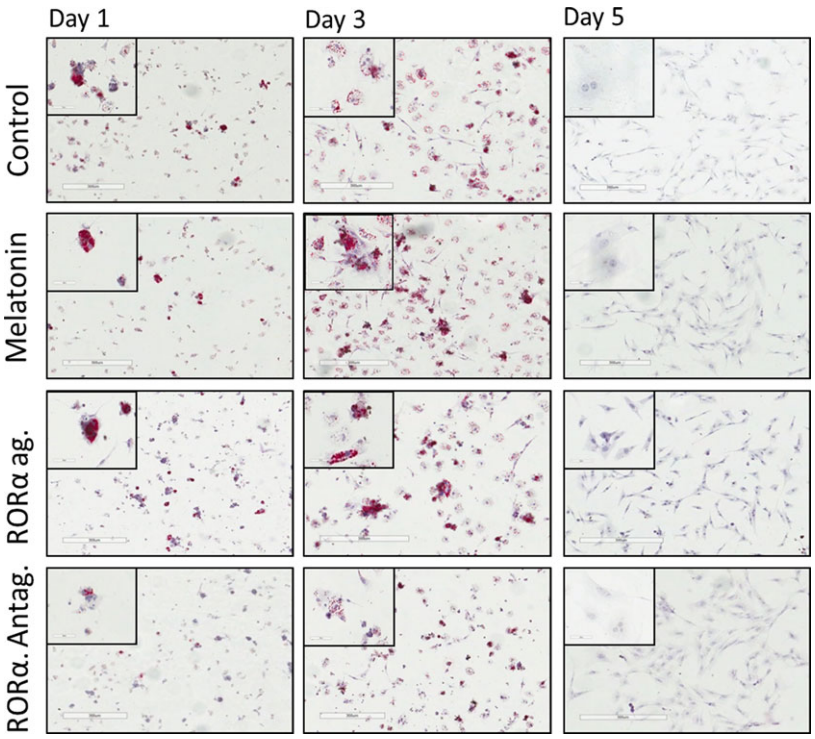

Fig. 4. Melatonin and SR1078 (RORα agonist) slow down lipid depletion in HSC activation. Freshly isolated HSC were culture-activated in the absence or presence of 10 μM melatonin, 10 μM SR10878 (RORα agonist), or 10 μM SR1001 (RORα antagonist) for 1, 3, or 5 days and analyzed by Oil red O staining. Bigger and more intensely stained lipid droplets are particularly evident after 3-day culture in the presence of melatonin and the RORα agonist compared to the untreated control or RORα antagonist. HSC treated with the RORα agonist are less stretched at day 5. Magnification 40×, scale bars 300 μm.

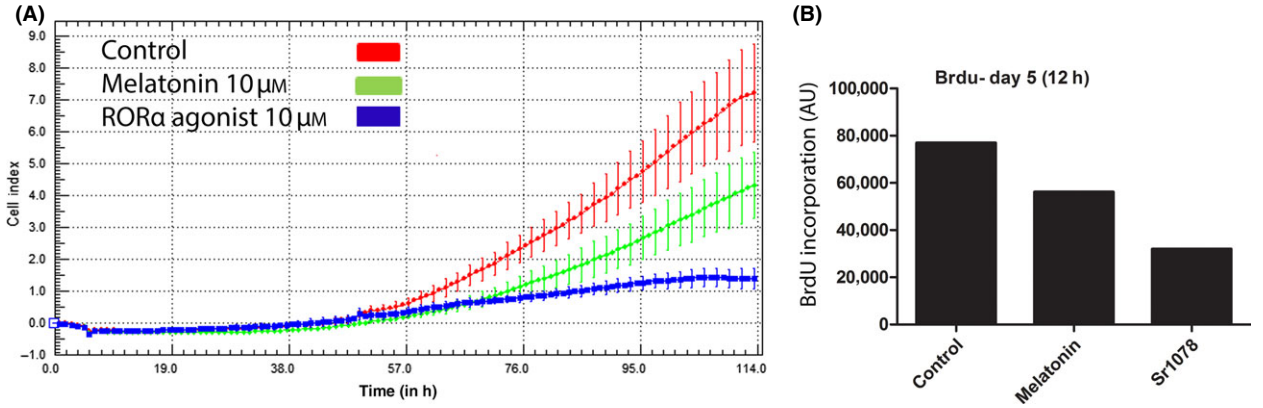

Fig. 5. Melatonin and SR1078 (RORα agonist) suppress HSC proliferation. Freshly isolated HSC were culture-activated in the absence or presence of 10 μM melatonin or 10 μM SR10878 (RORα agonist) and analyzed by real-time cell proliferation/stretching monitoring (xCELLigence) during 115 hr (A). Cell proliferation was analyzed by BrdU incorporation for 12 hr on day 5 (B). Melatonin and SR1078 both suppressed cellular resistance (A) and BrdU incorporation (B) with SR1078 showing the most potent effect.

cellular matrix proteins disturbs the functional architecture of the liver and thereby seriously compromises its function. Moreover, liver fibrosis may progress to irreversible cirrhosis and liver cancer. Although melatonin has been shown to inhibit liver fibrosis in chronic liver injury models, it remained to be determined whether this is a result of reduced hepatocyte damage and thereby indirectly preventing HSC activation, or whether melatonin acts directly on HSC.

Using culture-activated primary rat HSC, we show in this study that melatonin suppresses HSC proliferation and activation directly. The antiproliferative action of melatonin has been intensively studied for cancer cells, in particular that of the colon [29], liver [30, 31], breast [32], ovary [33], prostate [34], and pancreas [35]. There is no universal mechanism that controls the oncostatic function of melatonin. The membranous melatonin receptors may be involved, in particular MTNR1a [36–38], but also MTNR1a/b-independent mechanisms have been described [39–41]. The latter suggests the involvement of the nuclear receptor RORα [42] or the potent antioxidant properties of melatonin [43, 44]. Interestingly, 5-LO has been shown to promote cancer cell viability, proliferation, and metastasis and 5-LO antagonist is considered for cancer chemotherapy [45]. It is, however, unknown whether 5-LO expression is under control of RORα in cancer cells; thus, it remains to be determined whether this participates in the oncostatic function of melatonin. Apart from a direct

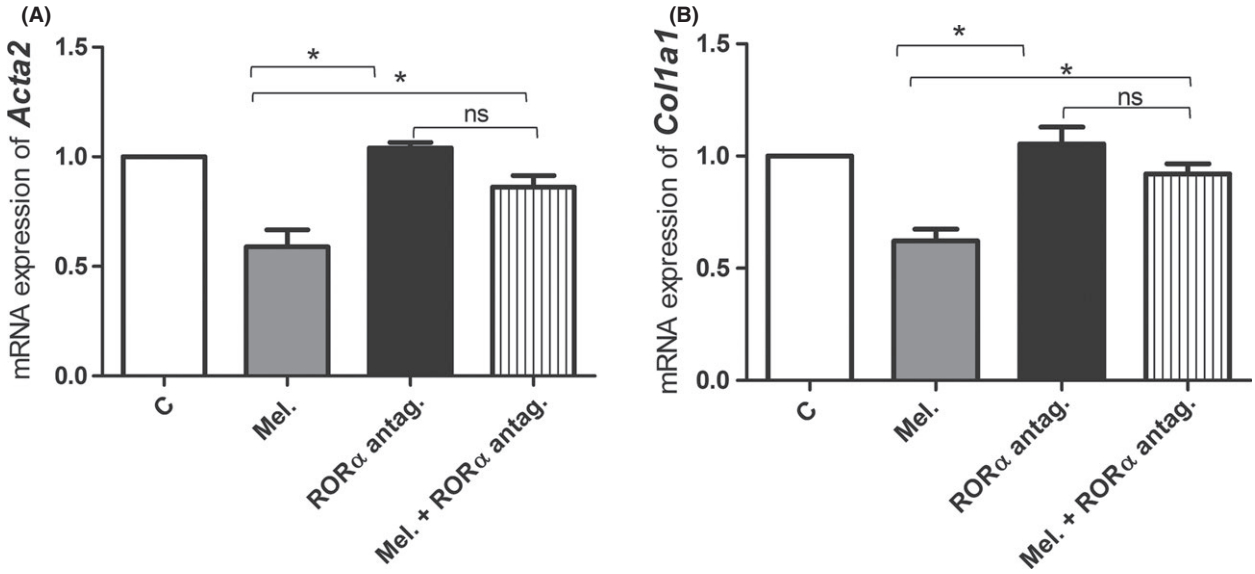

Fig. 6. Melatonin-mediated suppression of HSC activation acts via RORα. Freshly isolated HSC were culture-activated for 5 days in the absence or presence of 10 μM melatonin, 10 μM SR1001 (RORα antagonist), or both melatonin and SR1001 and analyzed by qPCR for mRNA expression of *Acta2* and *Col1α1*. SR1001 completely blocked the suppressive effect of melatonin on *Acta2* and *Col1a1* expression. *Indicates $P < 0.05$.

effect of melatonin on cancer cells, it may also indirectly suppress tumor development in vivo through modulation of the endocrine and/or immune system [46].

Hepatic MTNR1a and MTNR1b are predominantly expressed in bile duct epithelial cells (cholangiocytes), and melatonin has been shown to suppress cholangiocyte hyperplasia in obstructive cholestasis in bile duct-ligated (BDL) rats, predominantly through an MT1-dependent mechanism [47]. Cholangiocytes actually produce melatonin themselves, which is secreted into bile and provides autocrine suppression of bile duct proliferation. Hepatic melatonin production is strongly suppressed in cholangiocarcinoma cells, releasing the inhibitory action of melatonin and promoting tumor growth [48, 49]. Interestingly, BDL-associated liver fibrosis is strongly suppressed in rats that are kept continuously in the dark and show enhanced pineal melatonin synthesis and serum melatonin levels [50].

We were unable to detect any significant expression of either *Mtnr1*a or *Mtnr1b* in quiescent or activated HSC, ruling out a direct antifibrotic function of these membranous melatonin receptors. Melatonin has been shown to prevent hydrogen peroxide (H_2O_2)-induced superactivation of HSC in vitro [51], which is likely due to its potent antioxidant properties. However, oxidative stress does not seem to be the primary mechanism that causes activation of cultured HSC as activated HSC contain strongly enhanced cellular (reduced) glutathione levels [52]. In line, it is unlikely that the antioxidant properties of melatonin will have a significant effect on culture activation of HSC. This is why we focused on the potential role of the nuclear melatonin receptor, RORα (*Nr1f1*). There is recent evidence that RORα can suppress cell proliferation [53]. Besides melatonin, cholesterol sulfate (CS) is another endogenous activator of RORα and it was shown that CS-activated RORα modulates the expression of cell-cycle-regulating factors, such as p53, p27, and cyclin D in vascular smooth muscle cells (vSMCs). Consistent with this, RORα overexpression or CS treatment suppressed the proliferation of human aortic SMCs. In addition, RORα inhibited the migration kinetics of rat A7r5 cells, suggesting an effect on cytoskeletal proteins, such as αSMA, like we observed for HSC. Similarly, RORα has also recently been assigned oncostatic activity, as it suppresses proliferation of hepatoma cells via reprogramming of glucose metabolism and also in colon cancer by regulating of Wnt/b-catenin target genes [54, 55].

The first RORα target gene that was identified was *ALOX5*, encoding 5-LO [25]. Upon activation by melatonin, 5-LO expression was strongly suppressed. 5-LO is known to promote liver fibrosis [20]. However, these studies focused on 5-LO expression in Kupffer cells and revealed that inhibition of 5-LO led to cell cycle arrest of these liver-resident macrophages. There is one earlier study that reported 5-LO expression in mouse HSC [56], and we confirmed the expression of 5-LO in primary rat HSC and that both melatonin and SR1078 suppressed its expression in these cells. Moreover, pharmacological inhibition of 5-LO by AA861 suppressed HSC activation, thus providing insight in the downstream molecular mechanisms of the antifibrotic effect of melatonin. It is unknown whether Kupffer cells express RORα and may also be a target for melatonin in suppressing 5-LO and thereby hepatic inflammation and/or fibrosis.

Rat hepatocytes express RORα even to higher levels than HSC. In case of liver regeneration after chronic injury, hepatocytes need to divide and reconstitute the lost tissue. Melatonin may theoretically suppress proliferation of hepatocytes also and thereby impair proper liver repair. In models of partial hepatectomy, however, melatonin

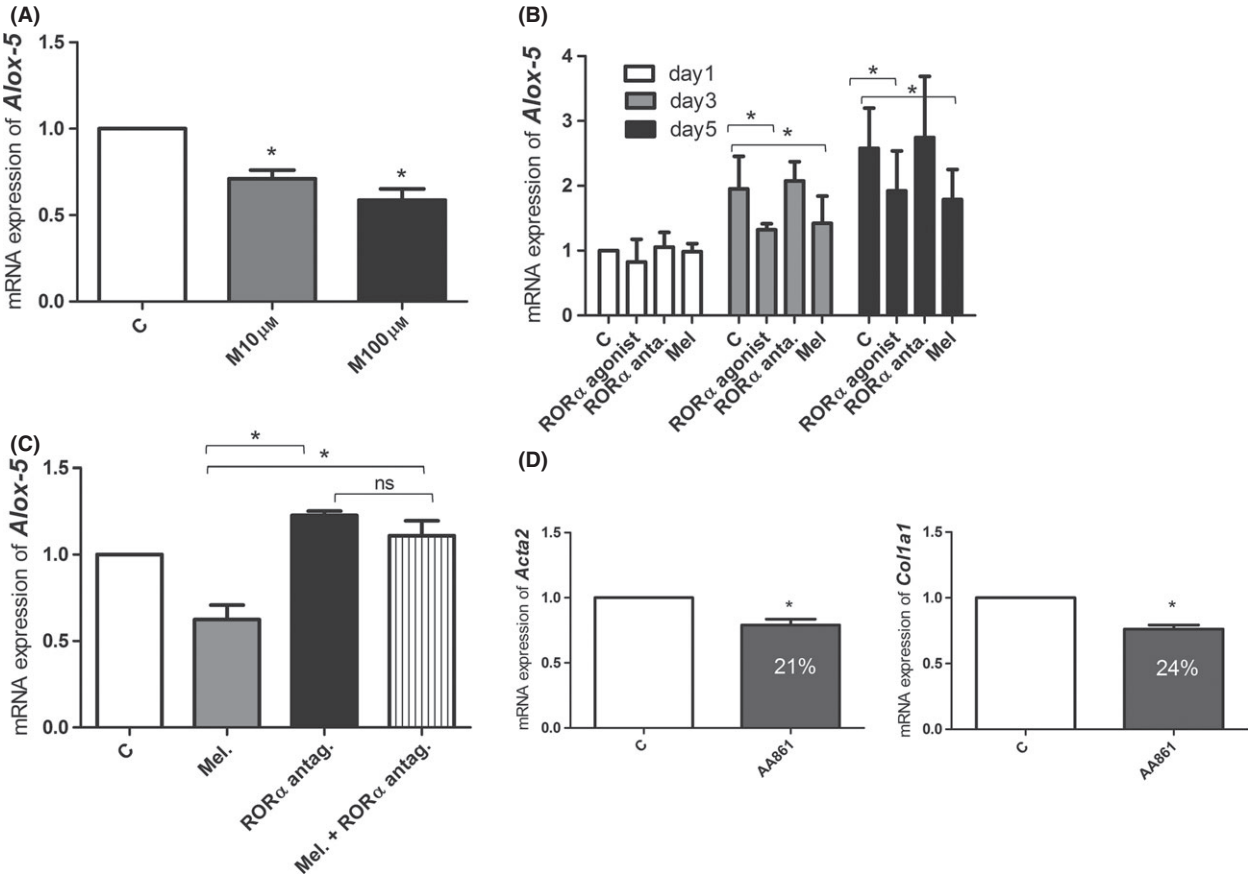

Fig. 7. Melatonin suppresses HSC activation through RORα-mediated reduction in *Alox5* expression. Freshly isolated HSC were culture-activated for 7 days (A), 1–5 days (B), or 5 days (C) in the absence or presence of 10 μM melatonin, 10 μM SR1078, 10 μM SR1001, or melatonin+SR1001 (as indicated) and analyzed by qPCR for mRNA expression of *Alox5*. *Alox5* mRNA levels are suppressed by melatonin (A–C) and SR1078 (B), and the effect of melatonin is blocked by SR1001 (C). (D) Fully activated HSC were treated for 24 hr with 10 μM of the 5-LO inhibitor AA861, which decreased mRNA expression of both *Acta2* and *Col1α1*. *Indicates $P < 0.05$.

treatment has not been shown to resort in adverse effects. In fact, its potent antioxidant properties prevent loss of hepatocytes during liver regeneration [26]. Moreover, inhibition of 5-LO by AA861 appeared to stimulate liver regeneration after partial hepatectomy in rats [57]. In line, the first clinical application of a large single dose of melatonin prior to major liver resection was well tolerated by patients and was associated with a reduced stay at the ICU as well as total hospital stay [58].

Theoretically, melatonin-mediated effects observed on RORα could be mediated via the membrane receptors [59]. However, as these membrane receptors are absent in HSC, melatonin most likely acts directly on RORα. Such direct physical interaction between melatonin and RORα was shown in T lymphocytes [60]. Moreover, we show that the RORα antagonist SR1001 blocks the antifibrotic action of melatonin, providing strong evidence that melatonin acts directly on the nuclear receptor to suppress HSC activation.

Taken together, our results show that melatonin prevents and reverses HSC proliferation and activation and therefore is a suitable drug to treat liver fibrosis, independent of its etiology. Given its strong antioxidant and oncostatic properties, melatonin in addition prevents loss of functional liver tissue and inhibits progression to liver cancer. The identification of RORα and 5-LO as the key factors involved in the antifibrotic potential of melatonin may further allow the development of even more potent and/or selective antifibrotic drugs.

Author contributions

Shiva Shajari participated in concept/design, acquisition of data, data analysis/interpretation, drafting of the manuscript, and finalizing the manuscript after all suggestions from co-authors. Almudena Laliena participated in concept/design, acquisition of data, data analysis/interpretation, and critical revision of the manuscript. Janette Heegsma participated in acquisition of data, data analysis/interpretation, and critical revision of the manuscript. María Jesús Tuñón participated in concept/design, data analysis/interpretation, and critical revision of the manuscript. Han Moshage participated in concept/design, data analysis/interpretation, and critical revision of the manuscript. Klaas Nico Faber participated in concept/design, data analysis/interpretation, drafting of the manuscript, and finalizing the manuscript after all suggestions from co-authors and supervisor of whole project.

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