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1,25-$(OH)_2$-vitamin D_3 prevents activation of hepatic stellate cells *in vitro* and ameliorates inflammatory liver damage but not fibrosis in the Abcb4$^{-/-}$ model

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ABSTRACT

Background/Purpose of the study: Vitamin D_3-deficiency is common in patients with chronic liver-disease and may promote disease progression. Vitamin D_3-administration has thus been proposed as a therapeutic approach. Vitamin D_3 has immunomodulatory effects and may modulate autoimmune liver-disease such as primary sclerosing cholangitis. Although various mechanisms of action have been proposed, experimental evidence is limited.

Here we test the hypothesis that active 1,25-$(OH)_2$-vitamin D_3 inhibits activation of hepatic stellate cells (HSC) *in vitro* and modulates liver-injury *in vivo*.

Methods: Proliferation and activation of primary murine HSC were assessed by BrdU- and PicoGreen®-assays, immunoblotting, immunofluorescence-microscopy, quantitative-PCR, and zymography following calcitriol-treatment. Wild-type and ATP-binding cassette transporter b4$^{-/-}$ (Abcb4$^{-/-}$)-mice received calcitriol for 4 weeks. Liver-damage, inflammation, and fibrosis were assessed by serum liver-tests, Sirius-red staining, quantitative-PCR, immunoblotting, immunohistochemistry and hydroxyproline quantification.

Results: *In vitro*, calcitriol inhibited activation and proliferation of murine HSC as shown by reduced α-smooth muscle actin and platelet-derived growth factor-receptor-β-protein-levels, BrdU and PicoGreen®-assays. Furthermore, mRNA-levels and activity of matrix metalloproteinase 13 were profoundly increased. In vivo, calcitriol ameliorated inflammatory liver-injury reflected by reduced levels of alanine aminotransferase in Abcb4$^{-/-}$-mice. In accordance, their livers had lower mRNA-levels of F4/80, tumor necrosis factor-receptor 1 and a lower count of portal CD11b positive cells. In contrast, no effect on overall fibrosis was observed.

Conclusion: Calcitriol inhibits activation and proliferation of HSCs *in vitro*. In Abcb4$^{-/-}$-mice, administration of calcitriol ameliorates inflammatory liver-damage but has no effect on biliary fibrosis after 4 weeks of treatment.

1. Introduction

Chronic cholestatic liver-diseases such as primary sclerosing cholangitis (PSC) are associated with fibrosis and cirrhosis of the liver. PSC usually affects middle-aged men. Their estimated median survival without liver-transplantation is reported with approximately 12 years [1]. Unfortunately, none of the available

Abbreviations: α-SMA, α-smooth muscle actin; Abcb4, ATP-binding cassette transporter b4; BrdU, bromodeoxyuridine; HSC, hepatic stellate cells; Mdr2, multidrug resistance protein 2; MMP, matrix metalloproteinase; IL-1R, interleukin-1 receptor; PDGF-R, Platelet-derived growth factor-receptor-β; TIMP, tissue inhibitor of metalloproteinase; TGF-β, tissue growth factor−β; TNF-α, tumor necrosis factor-α; TNF-R, tumor necrosis factor-receptor 1; UDCA, ursodeoxycholic acid.

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therapeutics has been proven to halt disease progression and affect prognosis. Therefore, new therapeutic approaches are urgently needed [2].

Hepatic stellate cells (HSC) are the primary source of extracellular matrix proteins deposited in liver-fibrosis [3]. Prevention of HSC-activation and inhibition of HSC-activity may be promising therapeutic approaches to effectively obviate fibrogenesis, even independently of its underlying origin.

1,25-$(OH)_2$-vitamin D_3 (calcitriol) has been described as an antifibrotic agent, possibly via inhibition of HSC. Calcitriol inhibited the expression of profibrogenic markers in primary rat HSC *in vitro* and had antifibrotic effects *in vivo*, ameliorating liver-fibrosis in thioacetamide (TAA)-treated rats, a model of hepatic fibrosis [4].

Furthermore, Ding et al. [5] observed an amelioration of liver-fibrosis by administration of the vitamin D receptor (VDR)-ligand calcipotriol in carbon tetrachloride-treated (CCL_4) mice, a model of liver-fibrosis with an inflammatory pattern. Noteworthy, vitamin D_3-deficiency is observed in more than 90% of patients suffering from chronic liver-disease [6].

Low vitamin D_3-levels were linked to more severe fibrosis in patients suffering from chronic hepatitis C and more severe histological findings in non-alcoholic fatty liver-disease [7,8]. A role for vitamin D_3-deficiency in autoimmune hepatitis, hepatocellular carcinoma, and cholestatic liver-diseases such as PSC was also discussed [9–11]. Therefore, supplemental administration of vitamin D_3 to patients suffering from chronic liver-disease is broadly debated [12,13]. However, both clinical and experimental evidence to indicate whether vitamin D_3-administration can be utilized to modulate chronic cholestatic liver-disease is limited to date and *in vivo* studies are urgently required to address this question.

The ATP-binding cassette transporter b4 (Abcb4/Mdr2) knockout-mouse lacking the canalicular phospholipid export pump is an established model for biliary liver-fibrosis [14,15]. These mice develop a phenotype mimicking features of human PSC [15,16].

Here, we investigated the potential protective effects of active 1,25-$(OH)_2$vitamin D_3 on HSC-proliferation and activation *in vitro* in primary murine HSC (mHSC) and *in vivo* in Abcb4$^{-/-}$-mice.

2. Material and methods

2.1. Isolation and culture of primary mHSC

Isolation of primary mHSC was performed by pronase-collagenase perfusion followed by density gradient centrifugation in 13.2% Nycodenz (Axis-Shield PoC, Norway) [17]. Purity of preparation was assessed by confirmation of vitamin-A autofluorescence. Cells were stimulated with 2.5 μmol/L calcitriol or 0.1% DMSO (control) at indicated times.

2.2. Immunoblotting

Proteins were loaded in equal amounts and separated by SDS-PAGE and transferred onto PVDF membranes (Merck-Millipore, Germany). Membranes were incubated with monoclonal antibodies against α-smooth muscle actin (SMA) (Sigma-Aldrich, Germany), Platelet-derived growth factor-receptor-β (PDGF-R) (Cell Signaling Technology, USA), or β-actin (Sigma-Aldrich, Germany) as a loading control followed by goat-anti-mouse-IgG-HRP antibody (Bio-Rad, Germany), and visualization by Clarity™ Western ECL Substrate (Bio-Rad, Germany) and detected with the ChemoCam (INTAS, Germany).

2.3. Immunofluorescence-microscopy

MHSCs were incubated with a monoclonal antibody against α-SMA (Sigma-Aldrich, Germany) and detected with an AlexaFluor 488 labeled anti-mouse-IgG (Molecular Probes, USA). Nuclei were counterstained with Hoechst-33342 (Sigma-Aldrich, Germany).

2.4. Proliferation-assays

MHSCs were incubated with PicoGreen® and fluorescence-signals were normalized to control values. Proliferation of primary mHSCs was measured using a BrdU-assay kit (Roche, Germany) according to the manufacturer's instructions.

2.5. Real-time PCR

Real-time PCR was performed in a Sybr® green system (Realplex 4, Eppendorf, Germany). Expression was calculated according the ΔΔCt method with GAPDH as housekeeping gene and normalized against the means of controls.

2.6. Zymography

Cell culture media were collected at day 7, 10, 12, and 14, and separated on a gelatine-acrylamide gel. Gels were developed for 18 h in incubation-buffer and stained with Coomassie Brilliant Blue R (Carl Roth, Germany). Optical densities of specific proteinase-activities illustrated by inverse band intensity were determined.

2.7. Animals and in vivo experiments

Abcb4$^{-/-}$-mice were obtained from Jackson Laboratory (USA) and bred by Charles River (Germany). Wild type-mice (Wt; FVB/N) were from Charles River. The animals were housed at a 12/12 h light/dark-cycle and were fed ad libitum. Animals were kept according to local regulations. The experiments were approved by local authorities. All institutional and national guidelines for the care and use of laboratory animals were followed.

8-week old Abcb4$^{-/-}$- and wt-mice received i.p.-injections of calcitriol (0.5 μg/100 g body-weight twice weekly) or vehicle (ethanol 0.16 mg/100 g body-weight) as control for 4 weeks, a time span previously shown to be sufficient to identify modulation of liver-fibrosis [18]. After narcotization with isoflurane (Abbott GmbH, Germany) animals were sacrificed by cervical dislocation.

2.8. Serum-biochemistry

Bilirubin, alkaline phosphatase (ALP), and alanine transaminase (ALT) were analyzed using a Cobas Integra 800-analyzer (Roche Diagnostics, Germany). Calcium was determined with a respons® 910-analyzer (Diasys, Germany).

2.9. Quantification of hydroxyproline

Hydroxyproline-content was determined according to the method of Edwards et al. [19].

2.10. Sirius-red staining

Liver-samples were fixed using 4% formaldehyde. After embedding in paraffin, 4 μm sections were stained with Sirius-red.

2.11. Immunohistochemistry

Immunohistochemistry of CD11b was performed on paraffin-sections (4 μm) with a polyclonal rabbit antibody against mouse CD11b (Abcam, UK). Antibody binding was measured with the

Super Sensitive™ Link-Label IHC Detection system from Biogenex (USA). CD11b positive cells were counted in 20 portal-fields per mouse under blinded conditions.

2.12. Statistical analysis

Statistical calculations were done with the SPSS 22 software-package (IBM, USA) using the Mann-Whitney-U-test or t-test, where appropriate. Results are reported as means ± standard-deviation. P-values lower than 0.05 were considered as statistically significant.

3. Results

3.1. Calcitriol inhibits activation and proliferation of HSC and induces expression and secretion of the antifibrotic matrix metalloproteinase 13 in vitro

α-SMA- and PDGF-R-protein-expression were assessed by immunoblotting as markers of HSC-activation. α-SMA-expression continuously increased during the observation period of 14 days after isolation (data not shown). When compared with controls, α-SMA-expression was reduced by 81% by calcitriol-treatment (n = 9, p < .01, Mann-Whitney-U-test) at 14 days, reflecting impaired activation (Fig. 1A and B).

Furthermore we observed in calcitriol-treated HSC a 38% reduction (n = 10; p < .01, Mann-Whitney-U-test) of PDGF-R-expression after 14 days of treatment when compared with controls (Fig. 1C).

In line with these findings immunofluorescence-microscopy of α-SMA in mHSC revealed diminished α-SMA-staining under calcitriol-treatment for 7 days (Fig. 1D).

Regarding proliferation PicoGreen®-assays identified a 34% reduction in cell-number after 14 days of calcitriol-treatment (n = 7, p < .01, Mann-Whitney-U-test) (Fig. 1E). The anti-proliferative properties of calcitriol on mHSC were further confirmed by BrdU-assays. After 7 days of calcitriol-treatment, cells presented with a 28% reduction of proliferation when compared with controls (n = 5, p < .01, Mann-Whitney-U-test) (Fig. 1F).

Treatment with calcitriol profoundly increased mRNA-expression of the antifibrotic matrix metalloproteinase (MMP) 13 by 56.1-fold when compared with controls (n = 5 p < .05, t-test) after 14 days of treatment (Fig. 1G). In contrast, no differences were observed for collagen 1α1, collagen 1α2, MMP2, MMP9, tissue growth factor−β (TGF-β), and tissue inhibitor of metalloproteinase (TIMP) mRNA-levels (Supplemental Fig. 1).

The increase of MMP13 was confirmed on the functional-level, applying zymography-assays. We detected higher MMP13-activity in cell-supernatants of treated HSC when compared with controls. This effect was most pronounced at day 10, when MMP13-activity was up-regulated 6.7-fold (n = 7, p < .05, t-test) (Fig. 1H).

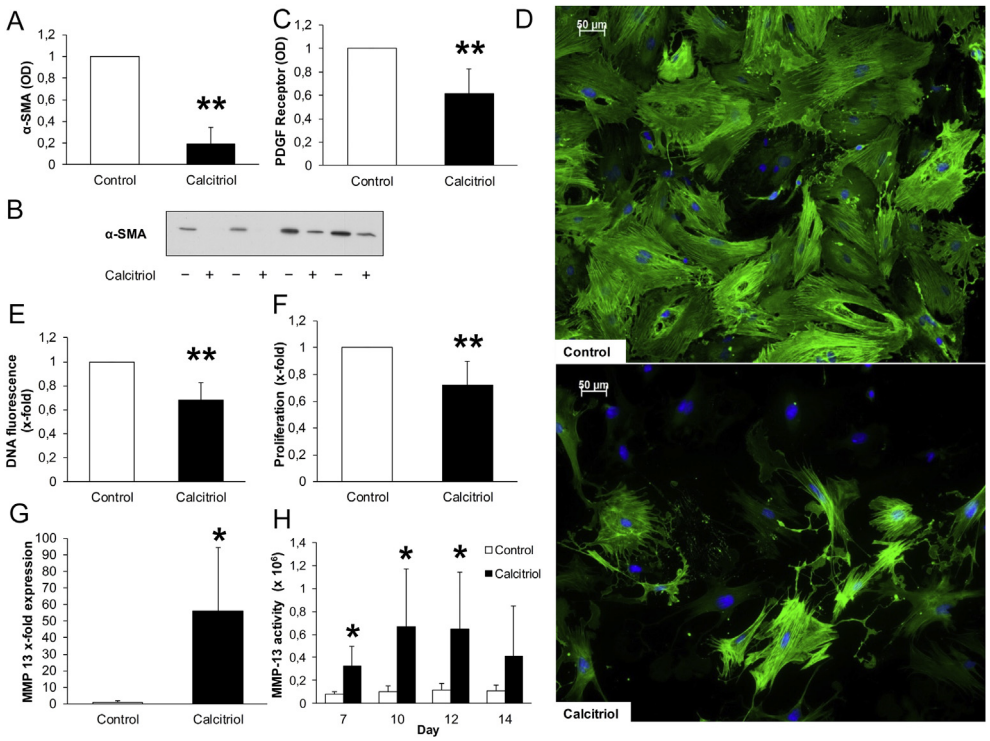

Fig. 1. Calcitriol has anti-fibrotic properties *in vitro* MHSC were treated with calcitriol or DMSO as control. Activation was determined by measuring expression of α-SMA in primary mHSC by immunobloting (A,B). (A) Densitometry data of α-SMA (OD) at 14 days of treatment with calcitriol are shown normalized to controls (n = 9; **, p < .01; Mann-Whitney-U-test). Calcitriol-treatment significantly reduced activation of mHSC. (B) The hepatic α-SMA-levels are illustrated by a representative blot. (C) Immunoblotting of PDGF-receptor (PDGF-R) in mHSC showed significantly reduced levels under calcitriol-treatment. Densitometry data of PDGF-R (OD) at 14 days of treatment are indicated normalized to controls (n = 10; **, p < .01, Mann-Whitney-U-test). (D) mHSC were labeled with α-SMA (green). Nuclei (blue) were stained with Hoechst 33342. Calcitriol-treatment of mHSC diminished α-SMA-expression as illustrated. (E) mHSCs were stained with PicoGreen®. Fluorescence was measured at day 14 of treatment and was normalized to controls (n = 7; **, p < .01; Mann-Whitney-U-test). (F) mHSCs-proliferation was assessed by BrdU assay after 7 days of treatment and normalized on controls (n = 5; **, p < .01; Mann-Whitney-U-test). Calcitriol-treatment reduced mHSC proliferation as shown in 1e and 1f. (G) Real-time PCR of MMP13 in calcitriol-treated mHSCs after 14 days to a reference of controls (n = 5; *, p < .05; t-test). (H) Densitometry data (OD) of MMP13-activity (zymography) was measured in cell supernatants after 7, 10, 12, and 14 days and compared to controls. (n = 7; *, p < .05; Mann-Whitney-U-test). Calcitriol-treatment significantly induced the anti-fibrotic enzyme MMP13 *in vitro*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Taken together calcitriol inhibited activation and proliferation and exerts antifibrotic properties *in vitro* by up-regulation of the antifibrotic enzyme MMP13 in mHSC.

3.2. Calcitriol-treatment ameliorates inflammatory hepatic injury in Abcb4$^{-/-}$-mice

To identify beneficial effects of calcitriol-administration on cholestatic liver-injury, we performed serum-biochemistry of bilirubin and relevant liver-enzymes (ALT, ALP). Calcitriol significantly reduced hepatic-injury in Abcb4$^{-/-}$-mice as quantified by serum ALT-levels (470 ± 118 U/l in calcitriol-treated Abcb4$^{-/-}$-mice vs. 668 ± 214 U/l in untreated Abcb4$^{-/-}$-mice; n = 6–11; p < .05; t-test, Fig. 2A). In contrast, there were no relevant alterations of serum-markers of cholestasis in Abcb4$^{-/-}$-animals, while mild effects were observed in wt-mice but not in cholestatic Abcb4$^{-/-}$-mice (Fig. 2A). Application of calcitriol in the above-mentioned dosage did not induce apparent toxic effects in wt- and Abcb4$^{-/-}$-mice and serum calcium-levels were unaffected (n = 3–6, not significant, t-test, Fig. 2A).

Quantitative PCR of inflammation-related genes identified a 36% reduction of hepatic TNF-R-mRNA in calcitriol-treated Abcb4$^{-/-}$-mice when compared with controls (n = 9–10, p < .05, Mann-Whitney-U-test). Furthermore, F4/80-mRNA was down-regulated by 35% in calcitriol-treated Abcb4$^{-/-}$-mice (n = 9–10, p < .05, Mann-Whitney-U-test) (Fig. 2B).

Calcitriol-treated Abcb4$^{-/-}$-animals showed a lower amount of portal neutrophil-infiltration than controls as indicated by the

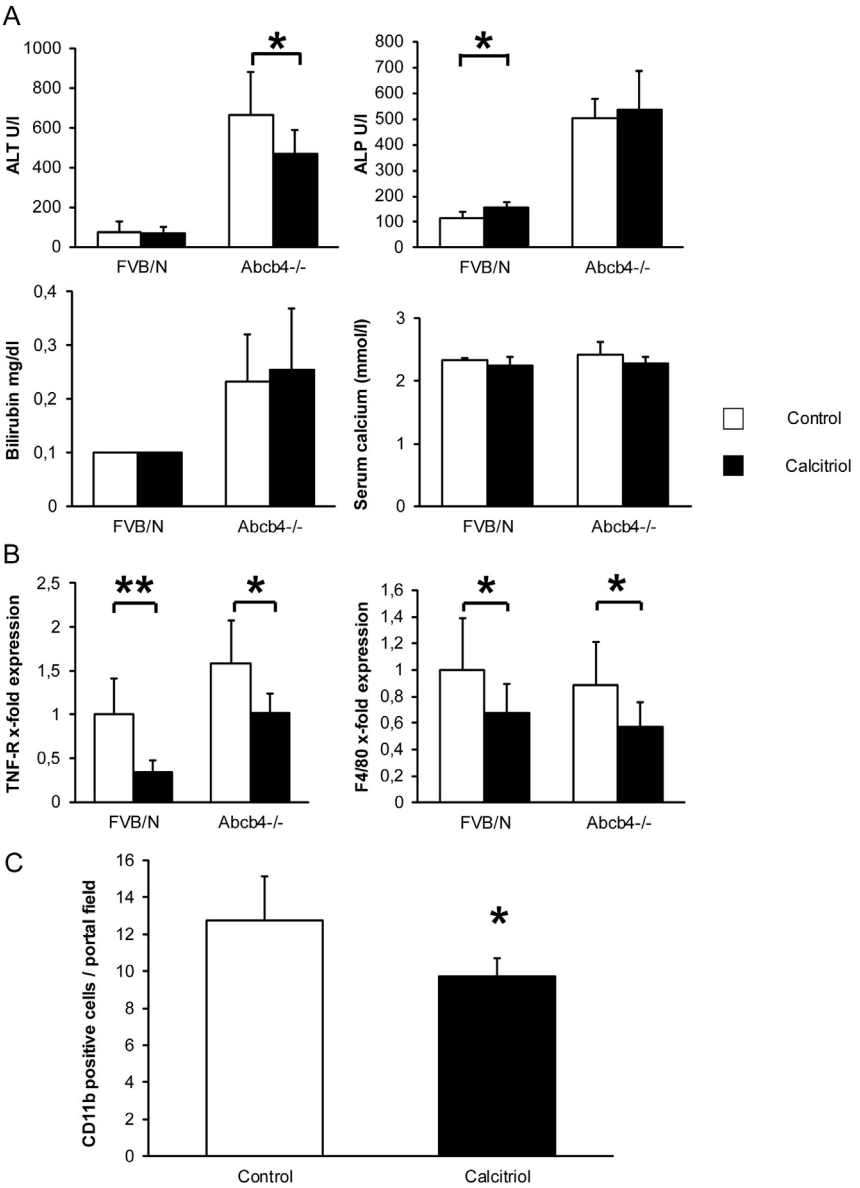

Fig. 2. Treatment with calcitriol has anti-inflammatory effects *in vivo* 8 weeks old wildtype (FVB/N) and Abcb4$^{-/-}$-animals were treated with ethanol (control) as vehicle and calcitriol for 4 weeks, respectively. (A) Alanine aminotransferase (ALT), alkaline phosphatase (ALP) and bilirubin were determined (n = 6–11; *, p < .05; t-test). Calcitriol significantly reduced hepatic-damage in Abcb4$^{-/-}$ – animals as illustrated by ALT-levels. A minor elevation of ALP was observed in FVB/N-mice treated with calcitriol. Calcitriol-treatment did not affect serum calcium-levels when compared to controls (n = 3–6; not significant; t-test). (B) Pro-inflammatory genes were assessed via quantitative real-time PCR. (n=9–10 **, p < .01, *p < .05; Mann-Whitney-U-test). Calcitriol-treatment resulted in a significant reduction of the pro-inflammatory genes F4/80 and TNF-R. (C) Immunohistochemistry of hepatic CD11b showed a significant lower CD11b-expression in calcitriol-treated Abcb4$^{-/-}$-mice than in control treated Abcb4$^{-/-}$-mice. Positive cells for CD11b were counted in 10 (calcitriol) vs. 6 (control) mice per group in 20 portal–fields per animal (n = 6–10, *p < .05; t-test).

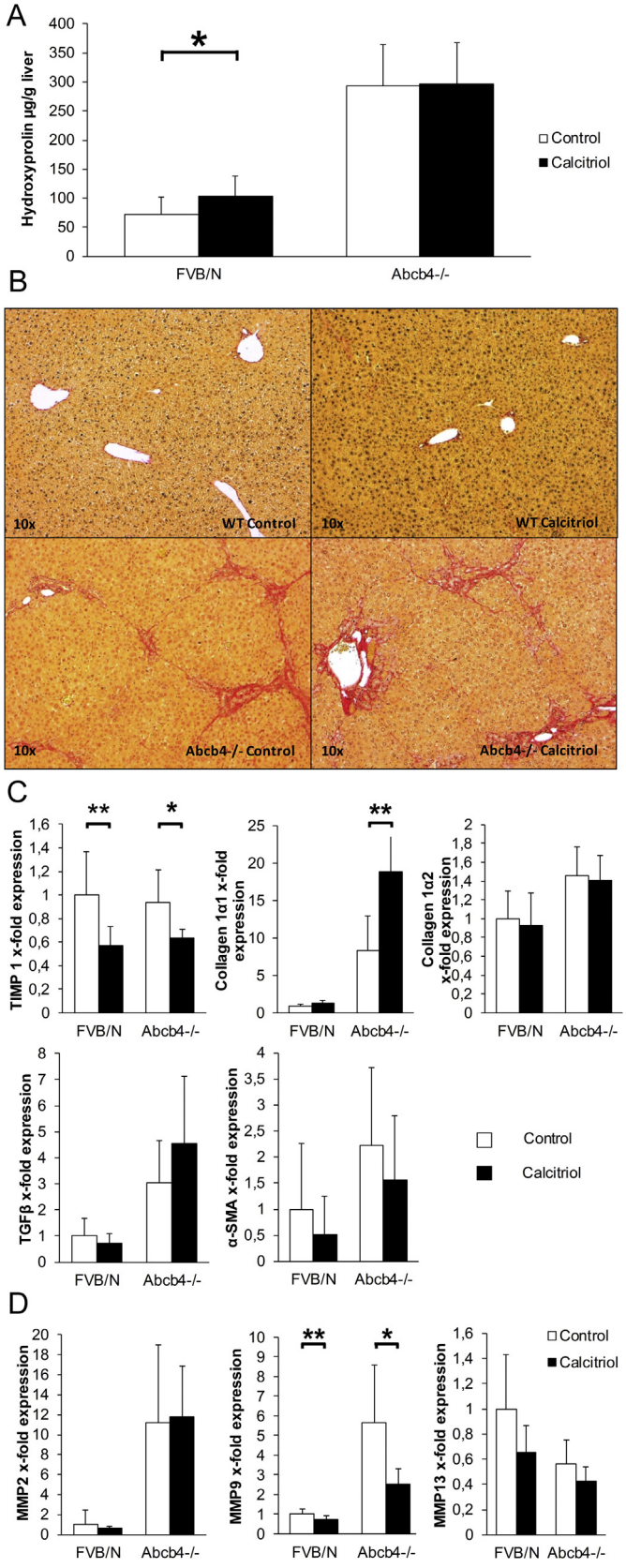

Fig. 3. Calcitriol-treatment does not ameliorate fibrosis in Abcb4$^{-/-}$-mice FVB/N and Abcb4$^{-/-}$-mice were assessed by hydroxyproline measurement and genetic profiling of fibrosis relevant genes using quantitative real-time PCR and illustrated by sirius-red staining at 12 weeks of age. (A) Hydroxyproline-content of liver-homogenates (µg/g liver) was measured (n = 9–11; *, p < .05; t-test). No differences between calcitriol and control treated Abcb4$^{-/-}$-animals were found. Wt-animals revealed a minor increase of hepatic hydroxyproline-content under calcitriol-treatment when compared with untreated controls. (B) Sirius-red staining illustrates more fibrosis in Abcb4$^{-/-}$-animals when compared to FVB/N-mice. No reduction of fibrosis was observed in calcitriol-treated Abcb4$^{-/-}$-animals (original magnification, 10×) as illustrated. (C) Pro-fibrotic genes and (D) anti-fibrotic genes were assessed via quantitative real-time PCR. (n = 7–11; **, p < .01, *p < .05; Mann-Whitney-U-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

results of CD11b-immunohistochemistry. The average-count of CD11b positive cells of 20 portal–fields per mouse decreased by 24% (n = 10 vs. 6, p < .05, t-test) (Fig. 2C).

No changes were observed for TNF-α, interleukin-1β, interleukin-6, interleukin-1-receptor, CD4, CD8 and CD11b (Supplemental Fig. 2).

In summary, calcitriol-treatment diminished hepatic inflammatory injury in Abcb4$^{-/-}$-mice but had no effect on serum-parameters of cholestasis.

3.3. Calcitriol does not prevent liver-fibrosis in Abcb4$^{-/-}$-mice

Despite reduced inflammatory activity, no effect of calcitriol-treatment was seen on hepatic hydroxyproline-content. Wt-, but not cholestatic Abcb4$^{-/-}$-animals revealed a minimal increase of hepatic hydroxyproline-content under calcitriol-treatment when compared with untreated controls (103.2 ± 34.9 vs. 72.4 ± 29.7 µg/g liver, n = 9–11; p < .05; t-test) (Fig. 3A).

In Abcb4$^{-/-}$-animals fibrosis appeared more pronounced in Sirius-red staining than in wt-animals, but no differences appeared between calcitriol-treated mice and control-treated animals as illustrated (Fig. 3B).

In line with absent biochemical alterations of cholestatic markers in calcitriol-treated Abcb4$^{-/-}$-mice, only minor alterations were found on mRNA-level. While some profibrotic genes were slightly down-regulated (e.g. 32% down-regulation of TIMP, n = 9–10, p < .05, Mann-Whitney-U-test, Fig. 3C), some profibrotic alterations were detected (e.g. 2.3-fold increase in hepatic collagen 1α1 mRNA, n = 7–8, p < .01, Mann-Whitney-U-test). Most genes tested were unaltered, such as collagen 1α2, TGF-β, α-SMA, MMP2, SMAD3, SMAD7 and MMP13-mRNA (Fig. 3C, D and Supplemental Fig. 2).

Taken together, calcitriol-treatment does not exert antifbrotic effects in Abcb4$^{-/-}$-mice after 4 weeks of treatment.

4. Discussion

Chronic liver-diseases often progress to liver-fibrosis and eventually liver-cirrhosis, a condition that is associated with high morbidity and mortality. At the moment, no genuine antifibrotic agents are available to halt progression of disease. However, such agents are urgently needed for liver-diseases where no causative treatment is available, as is the case in PSC.

Vitamin D_3-administration has been proposed to be such an alternative therapeutic agent. There is some but limited experimental evidence available supporting this approach [4,5] in general, while no data were available in the setting of cholestasis until very recently [20,21].

Our study characterizes antifibrotic mechanisms of calcitriol *in vitro*, namely an inhibition of activation and proliferation of mHSC and the profound induction of the antifibrotic enzyme MMP13. *In vivo* in a murine model of chronic cholestasis inflammatory liver-damage as illustrated by an improvement of serum-biochemistry was ameliorated, while no effect on liver-fibrosis was found. Liver-fibrosis is associated with an increase in α-SMA

positive cells [22]. We found an inhibition of activation and proliferation of mHSC during calcitriol-treatment. The reduced α-SMA -levels in the calcitriol-groups seem analogous to findings in TGF-β stimulated primary human HSC under vitamin D_3-treatment [23]. This supports the validity of comparability between murine and human HSC. Interestingly, calcitriol showed significant anti-proliferative effects on these cells under spontaneous activation which might be due to the down-regulation of PDGF-R. Calcitriol-dependent inhibition of activation and proliferation of HSC could thus be relevant mechanisms to prevent fibrotic disease *in vivo*.

As an additional antifibrotic action of calcitriol, we found a marked up-regulation of MMP13 *in vitro*. MMP13 is involved in the degradation of extracellular matrix *in vivo* [24], and induction of MMP13 has been investigated as a therapeutic strategy: Administration of hyaluronic acid-shielded MMP13-encoding plasmid DNA led to reduction of collagen deposition and amelioration of aspartate transaminase-levels in CCL_4-induced liver-fibrosis [25]. These findings underline the role of MMP13 as an anti-fibrotic enzyme, which was prominently up-regulated following calcitriol-treatment *in vitro* on mRNA- but also functional-level in our study.

To our best knowledge this is the first report on long-term effects of active vitamin D_3 in primary cultured stellate cells. Taken together, our *in vitro* findings underline the potential of calcitriol as an anti-proliferative and anti-fibrotic stimulus and provide new mechanistic insights.

To test beneficial effects of calcitriol on cholestatic fibrosis *in vivo*, we chose the Abcb4$^{-/-}$-mouse, which also mimics features of human PSC [14,15,18]. Interestingly, Abcb4$^{-/-}$-mice were found to be spontaneously 25-(OH)-vitamin D_3-deficient, mimicking human cholestatic disease also in this respect [20].

We detected profound anti-inflammatory effects of calcitriol-treatment in Abcb4$^{-/-}$-animals as illustrated by the reduction of ALT serum-levels. In line with this finding, we found a repression of gene-expression of the pro-inflammatory mediators TNF-R and F4/80 in liver-tissue as well as a reduction of neutrophil-accumulation in portal-fields of calcitriol-treated Abcb4$^{-/-}$-animals. The TNF-R is part of a well-established signalling cascade mediating liver-damage, as characterized in acetaminophen- and CCL_4-induced liver-toxicity [26,27]. The reduction of F4/80-mRNA and of portal neutrophil-accumulation in liver-tissue might indicate a reduction of immune-cell infiltration by calcitriol-treatment.

While an anti-inflammatory effect of calcitriol-treatment was documented by reduction of serum ALT-levels, we found no dominant antifibrotic effects in Abcb4$^{-/-}$-mice, as indicated by unaltered hydroxyproline-levels and Sirius red staining. Minimal effects were seen in wt-mice. Alkaline phosphatase and hydroxyproline-levels were slightly increased following calcitriol-treatment, albeit the increase of hydroxyproline did not reach pathologic-levels when compared with other studies [14,18]. Induction of intestinal ALP by vitamin D_3 has been described earlier [28] and has been implicated in endotoxin-binding and improvement of intestinal barrier function [29]. Both findings appeared to have no negative effects.

On mRNA-level, we detected a reduction of TIMP in Abcb4$^{-/-}$-mice following calcitriol-treatment. This is in line with former studies, which described reduced expression of TIMP as an anti-fibrotic mechanism of vitamin D_3 in rat HSC [4,30].

Despite reduced expression of TIMP, calcitriol failed to ameliorate the amount of fibrosis in Abcb4$^{-/-}$-mice. The lacking anti-fibrotic effect of TIMP reduction on fibrotic endpoints such as hydroxyprolin-levels may be attributed to simultaneous profibrogenic alterations in MMP9 and collagen 1α1 mRNA-expression. This may result in an equilibrium of anti- and pro-fibrogenic genes and prevent antifibrotic effects.

Interestingly, the observed anti-inflammatory potency but missing anti-fibrotic property of vitamin D_3-supplementation in cholestatic fibrosis is in accordance with a recent study by Hochrath et al. [20]. In line with our results, they found that vitamin D_3-supplementation has anti-inflammatory effects in the Abcb4$^{-/-}$-mouse as illustrated by reduced aminotransferase-levels but does not yield antifibrotic effects after 12 weeks of treatment. In an elegant study design, a role for vitamin D_3 in this model of biliary fibrosis was indirectly supported, as introduction of a vitamin D_3-deficient diet aggravated hepatic fibrosis. The work of Hochrath et al. and the current study reported here nicely complement each other. Hochrath et al. report on long term oral administration of the inactive calcitriol precursor vitamin D in young animals (4 weeks) without a fully developed phenotype, which in summary may be considered a preventive treatment strategy. In contrast, we performed our study in 8 week old mice with an established fibrotic phenotype, applying the active form of vitamin D_3, calcitriol, which aimed to unravel therapeutic effects. Furthermore, by choosing an intraperitoneal administration-route, alterations in pharmacokinetics due to different degrees of liver-disease were compensated. Another study that investigated the effects of vitamin D_3 on cholestatic fibrosis was performed in bile-duct ligated rats [21]. Of note these animals showed a high mortality after a short period probably due to hypercalcemia. Therefore this approach cannot serve as a model for studying long-term-effects of vitamin D_3 on cholestatic fibrosis, as it was addressed in our study.

However as discussed earlier, both calcitriol and a synthetic vitamin D_3-ligand named calcipotriol showed antifibrotic effects in liver-fibrosis induced by TAA and CCL_4 [4,5,21]. This raises the question whether vitamin D_3 has the potency to ameliorate certain entities of liver-fibrosis with a distinct inflammatory profile while it does not in models of cholestatic fibrosis. One might speculate that long term preventive administration of active vitamin D_3 may still hold antifibrotic properties also in cholestatic fibrosis.

Taken together, vitamin D3-deficiency is common in patients suffering from chronic liver-diseases [6]. Vitamin D_3 plays multiple important roles in non-skeletal functions [31] as well as in the pathophysiology of osteoporosis in chronic liver-diseases [32].

This study identifies vitamin D_3 in addition to its established roles as prospective anti-fibrotic agent in liver-fibrosis and as a potential anti-inflammatory hepatoprotective agent in PSC. Further studies have to clarify whether the anti-inflammatory potency of calcitriol can be also beneficial for cholestatic fibrosis and PSC or only for certain entities of liver-fibrosis with a distinct inflammatory profile.

Conflict of interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.074.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.074.

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