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RESEARCH ARTICLE

Hydrophilic Bile Acid, Ursodeoxycholic Acid Attenuates the Effect of TGF-β1 On Human Tenon's Fibroblast

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Abstract Introduction: Trabeculectomy is performed to lower IOP in glaucoma. Excessive scarring of the filtering bleb was reported post-surgery and thus lead to failed trabeculectomy. The excessive scarring occurs due to elevated level of TGF-β1 and thus leading to differentiation of tenon's fibroblast to myofibroblast. Ursodeoxycholic acid (UDCA), the most hydrophilic bile acid, reported as beneficial agent for the treatment of ocular diseases. This study aimed to investigate whether UDCA is able to reduce the differentiation of fibroblast to myofibroblast in TGF-β1-induced human tenons fibroblast (HTF). Material and Methods: Primary HTFs were obtained and divided into untreated group, TGF-β1 treated group (20 ng/ml), UDCA treated group (100 μM) and TGF-β1 co-treated with UDCA (combination) group. All cells were subjected for immunostaining (α-SMA and F-actin expression), PCR array (downstream target of TGF-β1: Nf-κβ1, JUN, TIMP1, SMAD3, SMAD2) and western blot (F-actin expression). The statistically significance was set to $p < 0.05$ and 4 biological replicates were used. Results: The expression of α-SMA and F-actin suggest that UDCA inhibits the fibrous structure of striated actin induces by TGF-β1 as this actin were highly expressed in TGF-β only group. No significance changes in downstream target genes of TGF-β1 except for Nf-κβ1. Interestingly, protein expression of ERK 1/2 and gene expression of Nf-κβ1 were significantly decreased in combination group (TGF-β1 with UDCA) compared to TGF-β1 only group. Conclusion: UDCA attenuates the effect of TGF-β1 on human Tenon's fibroblast by downregulating the expression of α-SMA and F-actin expression which could be mediated by ERK1/2 and NF-κβ1.

Keywords: Glaucoma, Ursodeoxycholic acid, Human Tenon's Fibroblast

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Introduction

Glaucoma is a multifactorial disease which occurs due to prolong damage of the nerve in optic disc and subsequently lead to impair visual field. Globally, glaucoma is reported as the second most disease that lead to blindness, after cataract. Raised intraocular pressure (IOP) widely known as the most common

factor leading to the development glaucoma. In research, raised IOP has successfully induced glaucoma in animal model (1). Trabeculectomy (glaucoma filtration surgery) is a procedure done to reduce the IOP in patients. The major reason of the unsuccessful trabeculectomy is excessive scarring of post-surgery (1) and complication associated with the post-surgery such as blindness has been reported (2). Currently, mitomycin C (MMC) and 5-fluorouracil (5-FU) are given intraoperative to combat fibrosis and improve the outcome of trabeculectomy. Despite the improvement of procedure in glaucoma filtration surgery, it is still fails to improve the eyesight of patients.

The main component of scar tissue known as Tenon's fibroblasts are reported in glaucoma surgery. Majority of fibroblast will differentiate into myofibroblast and become the prominent cells which are responsible for the wound contraction. Studies showed that myofibroblast exhibits similar characteristics to smooth muscle cells which explains why myofibroblast can contract the wound (2), since it contains the identical form of actin institute in the smooth muscle cells, (alpha smooth muscle actin; αSMA) (4). However, myofibroblast and smooth muscle cells possess distinct transcriptional control mechanism to regulate its expression (5). Myofibroblast contracts the edges of the wound by the smooth muscle type actin-myosin complex and shown to secrete and remodel extracellular matrix (ECM) proteins (6). The contraction stage ends as myofibroblast are lost by apoptosis. If apoptosis mechanism does not function, the proliferation of myofibroblast will persist. Persistence proliferation eventually leads to increase production of ECM and extreme contraction leading to pathological states like hypertrophic scars and liver cirrhosis (7,8). Myofibroblasts express smooth muscle contractile proteins for instance desmin, αSMA, vimentin and F-actin. Myofibroblast transformation is commonly induced by treating fibroblast with transforming growth factor-β (TGF-β) (9). Among the three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3), numerous studies have shown TGF-β1 is the main cause for the differentiation of fibroblast to myofibroblast (10).

Bile acids are a product generated from cholesterol metabolism which occurs in the liver. The synthesized bile acids are stored in the gallbladder. In human, the major types of bile acids are cholic acid and chenodeoxycholic acid. Meanwhile, the most hydrophilic bile acid, ursodeoxycholic acid (UDCA) is found less than 2 % of total bile acid composition. Since 618 AD, UDCA is known as a beneficial agent to treat liver diseases and other liver-related disorders. It has been used effectively to treat a spectrum of liver diseases such as primary biliary cirrhosis (11).Interestingly, UDCA has been reported to have potential in treating non-liver diseases such as neurodegenerative (12,13), heart (14) and ocular diseases (15). Bile acids have been shown as an anti-scarring agent as they are able to reduce fibroblast proliferation and viability (16), which subsequently attenuates fibrosis. Bile acids haves been shown to reduce fibroblast proliferation and viability in human Tenon's fibroblasts (17). UDCA has been reported to attenuate the dermal fibroblast proliferation (18).

This study was done to investigate the anti-scarring property of ursodeoxycholic acid (UDCA) in TGFβ1-induced transdifferentiation of human Tenon's fibroblast to myofibroblast. By understanding its anti-scarring mechanism in fibrosis, we hope to propose UDCA as a potential anti-scarring agent, thus a safer therapy for a better post-operative prognosis for trabeculectomy.

EXPERIMENTAL

Materials

For this study, cell culture media were from Gibco Life Technologies, all other chemicals were from Sigma-Aldrich Company Ltd. and for western blot, chemicals and antibodies were from Thermo Scientific, unless stated otherwise.

Cell Culture of Human Tenon's Fibroblasts (HTFs)

This research has been approved by Ministry of Health Malaysia Medical Research and Ethics Committee. Tenon's capsule explants were collected with consent from patients who undergoing trabeculectomy as reported previously (19). All procedures were conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). For this study, all cells were only used at passages 3 to 6. All experiments were performed at least three times with similar results. In this study, HTFs were divided into untreated group (control), TGF-β1 only group (20 ng/ml) for 24 hours and TGF-β1 with 100 μM UDCA group for 24 hours.

Immunocytofluorescence Staining

The HTFs primary cells were treated with 20 ng/ml TGF-β1, 100 μM UDCA and 100 μM UDCA with 20 ng/ml TGF-β1. Immunocytofluorescence staining was employed to investigate the expression levels of

F-actin using Alexa Fluor 635-Phalloidin (R&D System, USA) and α- smooth muscle actin (Invitrogen, USA). HTFs were seeded in chamber slides with about 1.5x105 cells per well and divided into; untreated, TGF-β1 only, UDCA only and TGF-β1 with UDCA. The cells were maintained in maintainance media until end of treatment. At the end of treatment, cells were rinsed with Phosphate Buffered Saline (PBS) and fixed with 4% paraformaldehyde. Cells were blocked for 30 minutes with Bovine Serum Albumin (BSA) at concentration 10 % minutes and incubated with Alexa Fluor 635-Phalloidin for 24 hours. The cells were washed after an overnight incubation and mounted to be viewed under Confocal Microscope (Leica Microsystems, UK).

PCR Array for gene expression

Cells were seeded at the density of 3 x 10^5 cells in each 6-well plate and incubated for 24 hours in humidified environment at 370C with 5% CO_2. Subsequently, the HTFs monolayers were introduced to the same treatment as performed in immunocytofluorescence staining. Total RNAs were extracted from all groups of HTFs using RNeasy mini kit (Qiagen). Concentrations of RNA were measured using a nanodrop and RNAs were stored at -80^{o}C for further processing. 0.5 ug of total RNA were reversely transcribed into cDNA using RT2 First Strand Kit (Qiagen, Netherlands). Then cDNAs were mixed with SYBR green mastermix (Qiagen). The cocktail was pipetted into each well of 96 well plates of RT2 Profiler PCR Arrays plate pre-coated (PCR array, SAbiosciences) with targeted genes (Nf-κβ, JUN, TIMP1, SMAD3 and SMAD2) and housekeeping genes (GAPDH, HPRT, RPLP). Plate was run on real time cycler CFX96 Real-Time PCR (Bio-rad, USA). Data were analyzed and transcript expressions were measured as fold change over the value of control group.

Western Blotting

For this part, cells were seeded at the density of 2.5 x 10^5 cells in each 6-well plate. After treatment, all well were washed with cold-PBS and cells were lysed using RIPA buffer with protease inhibitors (Pierce, USA). The protein lysates were sonicated and centrifuged for 15 minutes at 14000 rpm to obtain the protein pellet. The total protein was quantified and 30μg of each protein were separated using SDS-PAGE and transferred into nitrocellulose membrane. The membrane was blocked and incubated with phosphorylated and total ERK 1/2 (Santa Cruz Biotechnology, USA; 1: 1000), F-actin (R&D System, USA; 1: 1000) and β-actin (Santa Cruz Biotechnology, USA; 1: 1000) antibodies overnight. The membranes were then incubated with secondary antibody (DAKO, Denmark, 1:500) and washed. Lastly, the membranes were incubated with Super Signal West Pico Chemiluminescent Substrate and analysed by normalisation with β-actin.

Statistical analysis

All the data in here were expressed as mean ± SEM generated from n ≥ 3. Normality was checked and then analyzed using Student's T-test analysis using SPSS ver. 20. The data is considered statistically significant at $p < 0.05$. For experimental details see each figure legend.

Results and discussion

UDCA downregulates the expression of F-actin and α-SMA in TGF-β1 treated human Tenon's fibroblast

Western blotting was used to investigate the effect of TGF- β1 with ursodeoxycholic acid (UDCA) on F-actin expression of HTF. Cell lysates were immunoblotted with F-actin (Figure 1A). When compared with the control HTFs, the ratio of F-actin in TGF-β1 treated-HTF was significantly upregulated (p value < 0.05) compared to control and UDCA only. In UDCA treated HTF, the ratio was slightly decreased when compared to control group. Interestingly, in HTF treated with UDCA and TGF- β1, the expression ratio was decreased in comparison to the TGF- β1 only cells. To demonstrate the expression of F-actin and alpha smooth muscle actin (α-SMA) in human Tenon's fibroblast (HTF), HTF were stained with Fluorescein (green; α-SMA) (Figure 1B (i)) and Alexa Fluor 635-Phalloidin (red; F-actin) (Figure 1B (ii)). The positive striated actin was observed in control and UDCA treated HTF. However, in TGF-β1 treated HTF, an elongation of striated actin structure was observed. Meanwhile, in cells treated with UDCA and TGF-β1, the expression of F-actin and α-SMA were similar as seen in control and UDCA only cells.

Wound healing is a complex process following injury of tissues. In the Tenon's capsule, wound healing occurs due to production of extracellular matrix (ECM) components as well as and collagen contraction of Tenon's fibroblast cells (20). In the course of tissue restoration, fibroblasts phenotype is altered from quiescent state to actively proliferate and contract state which is known as myofibroblasts. The myofibroblasts demonstrated to have similar phenotypic appearances of smooth muscle cells and

contractile state when grown in vitroly. Generally, myofibroblasts are known to be found during tissue repair or response to damage occurs in other tissues where fibroblasts are highly expressed such as lung. Myofibroblasts are rich in double-stranded filamentous polymer known as F-actin (21). In here, the human Tenon's fibroblast showed expression of one continuous line of F-actin expression. In quiescent phenotypes, fibroblast cells expressed the actin in single continuous line (22) which play a role during motility processes (23). In endocytosis and phagocytosis, actin helps cells membrane to form cavity (24) that leads to activation of proteins involved in regulation of actin filament organisation (23).

Wound healing which occurs without the formation of scar could occur as a result of a successful healing process known as regenerative healing. Regenerative healing is characterized into three phases. In the initial phase, fibroblasts migrate into the site of wound and secrete ECM, proteases and growth factors. In the next phase, fibroblasts differentiate into myofibroblast that secrete proteases for remodelling of ECM (6,25, and 26). In the last phase, myofibroblasts typically dissolve by apoptosis after wound closure (27). In certain conditions such as hypertrophic scars and liver cirrhosis, However, myofibroblasts may become persistence due to their overproduction of ECM and by excessive contraction (27). In summary, myofibroblasts are crucial in formation and remodelling of scar tissue after the occurrence of tissue injury. Myofibroblasts have been reported to express α-SMA which is critical for their functions in wound healing such as wound contraction. HTFs were plated on cell culture dishes and stimulated with TGF-β1 for up to 24 hours (Fig. 1). In control cells we did not observe relevant α-SMA expression. In cells incubated with TGF-β1 increased α-SMA expression was observed. Interestingly, HTFs of combination treatment with UDCA and TGF-β1, we did not observe relevance expression of α-SMA.

Involvement of SMAD genes and activity of ERK in UDCA protection against TGF- β1 effects on human Tenon's fibroblast

The members of the SMAD (Sma and Mad Related Family) family of signal transducers such as SMAD2 and SMAD3 are among the downstream targets of TGF- β1 signals. Therefore, in here we measured the expression of SMAD2 and SMAD3 using PCR array (Figure 2). The gene expression of SMAD3 and SMAD2 are shown in Figure 2. There was no significance different between control, TGF- β1 only, UDCA only and combination group in SMAD3 and SMAD2 expression.

The expression and phosphorylation of ERK 1/2 are shown in Figure 3. The HTFs primary cells were treated with 20 ng/ml TGF-β1, 100 μm UDCA, and 100 μM UDCA with 20 ng/ml TGF-β1. Cell lysates were immunoblotted with anti-phophorylated and anti-total antibodies to ERK 1/2. When compared with the control group, the ratio of phosphorylated ERK (p-ERK) to total ERK 1/2 (T-ERK) in TGF-β1 treated HTF was significantly increased ($p<0.05$). Meanwhile, in UDCA only group, the ratio was slightly increased when compared to control group. However, in UDCA with TGF- β1 group HTF, the ratio was similar to the control HTF. Moreover, significant decreased in p-ERK was observed in UDCA with TGF-β1 HTF compared to TGF- β1 only HTF.

Involvement of transcription factors (Nf-κβ1 and JUN) and Tissue inhibitor of metallopeptidases (TIMP) in UDCA protection against TGF-β1 effects on human Tenon's fibroblast

To investigate the involvement of non-SMAD downstream signalLing, we measured the expression of Nf-κβ1, JUN and TIMP2 genes using PCR array. In Nf-κB1 gene, upregulation of expression was observed in TGF- β1 treated HTF (Figure 4). Interestingly, in combination treatment, the effects on TGF-β1 on Nf-κβ1 gene expression was not observed ($p<0.05$). In JUN gene, no significant differences were observed in all HTFs (Figure 4). In Figure 5, no significant differences in TIMP2 expression were observed in all groups.

It is widely described how TGF regulates the activity of myofiboblasts. Most importantly, TGF-β, a fibrogenic peptide growth factor, is known for its regulation in fibroblast differentiation to myofibroblast mediated by α-SMA. Classically, TGF-β initiates signals in cells through SMAD pathways. In this present study we did see significant changes of SMAD2 and SMAD3 gene expression between control, TGF-β1 treated cells and combination groups. The non-SMAD pathway such as members of the MAPK signaling cascades,ERK/12 and JUN, were reported to be relevant for TGF-β1-induced fibroblasts differentiation to myofibroblasts. Therefore, we examined the expression of ERK 1/2 and JUN in here. We observed no significant changes in JUN expression between all groups of HTF. For ERK 1/2, significant decreased in phosphorylation was observed in UDCA with TGF- β1 HTF compared to TGF- β1 only HTF. A rapid phosphorylation of ERK 1/2 has been observed in response to TGF-β treated fibroblasts (28), Inhibitors of ERK 1/2 known to inhibit the expression of α-SMA in TGF-β1 treated fibroblasts (29).

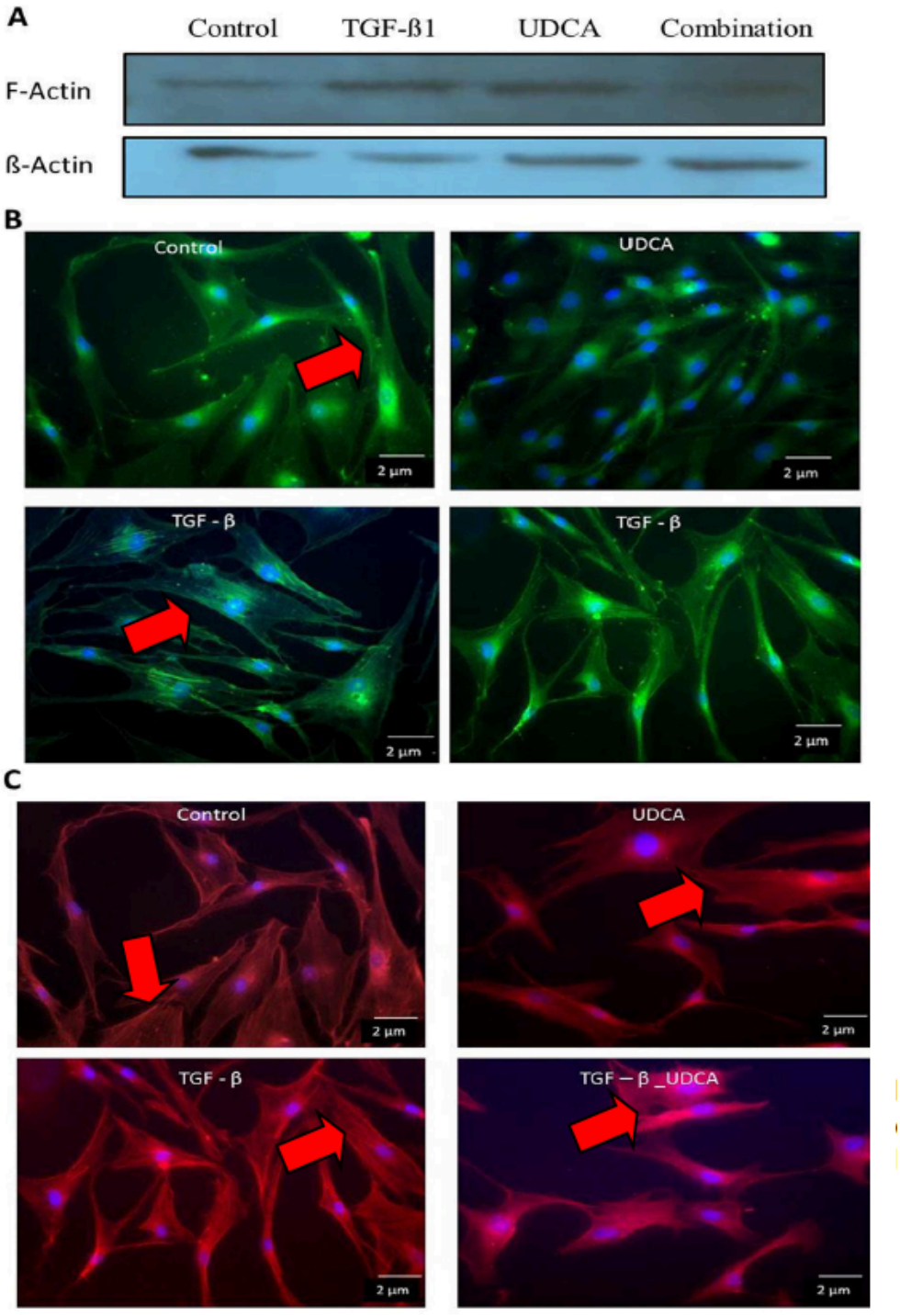

Fig. 1 Expression of F-actin and α-smooth muscle actin in TGF-β treated HTF cells. A; Western blotting was performed to assess the expression of F-actin in TGF-β treated cells and TGF-β with UDCA treated cells (combination). B; Immunofluorescence staining was performed to detect α-smooth muscle actin and C; F-actin expression in control, TGF-β treated cell and in the present of UDCA. n≥3, *p< 0.05. The red arrow showed the positive striated actin structure with magnification x10.

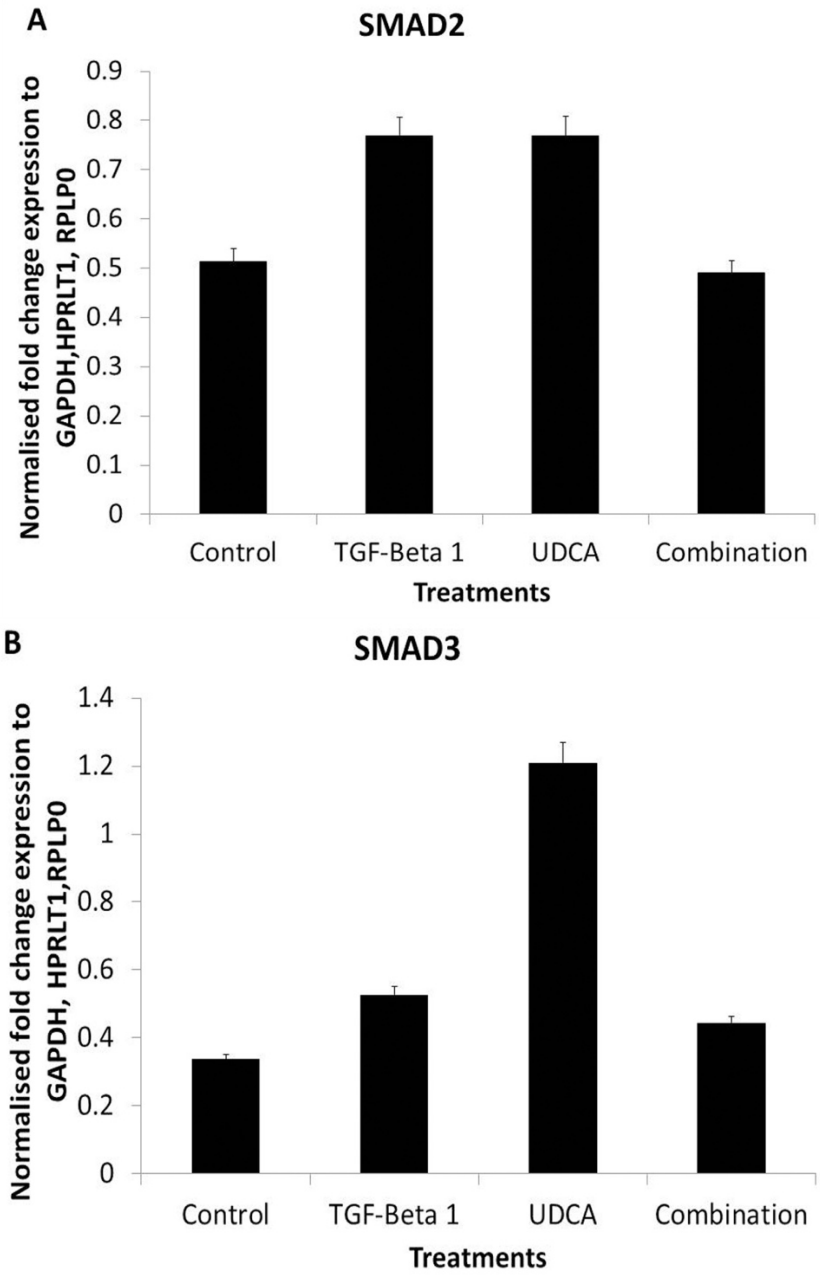

Fig. 2 Expression of SMAD3 and SMAD2 genes in TGF-β treated HTF cells. There was no significance different between control and treated HTF cells. n≥3, *p < 0.05.

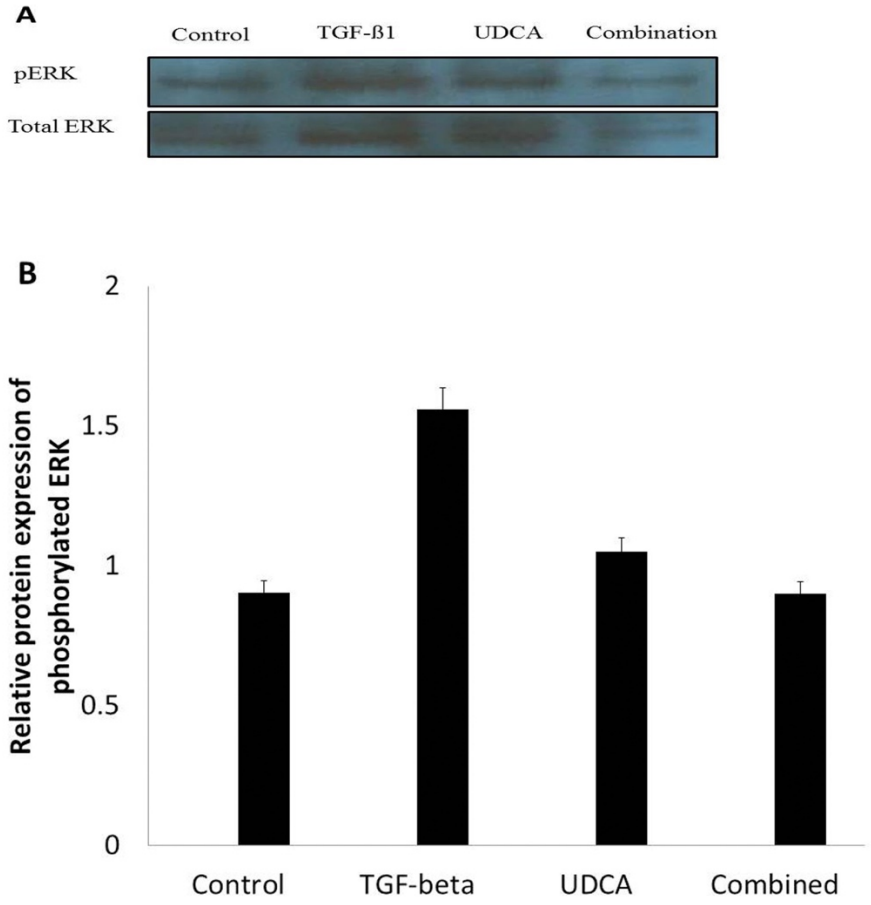

Fig. 3 Ursodeoxycholic acid decreases ERK 1/2 phosphorylation in TGF-β treated HTF cells. The expressions and phosphorylation of ERK are shown. The ratio of phosphorylated ERK/ total ERK was significantly increased in TGF-β treated HTF cells compared to control. However, the effect of TGF-β on ERK phosphorylation was not observed in the presence of UDCA. n≥3, * $p < 0.05$.

Previously, UDCA is commonly used to treat liver diseases and other diseases related to liver such as sclerosing. Nowadays, UDCA has been widely reported as a drug with dual actions; anti-apoptotic and pro-apoptotic signalling (30). UDCA is reported to have an anti-apoptotic effect in cells such as, hepatocytes (30), cardiomyocytes (31), cholangiocytes (32), brain (33), and eyes (15). Meanwhile, its anti-apoptotic signalling is reported in cancer cells including breast cancer, prostate and colorectal cancer cells.

In eyes, UDCA has been suggested as a potential drug in the treatment of retinitis pigmentosa, a neurodegenerative disease of retina (34). Fernández-Sánchez and colleagues (35) reported that systemic tauro-conjugated UDCA (TUDCA) treatment preserved retina structure as well as function of P23H-transgenic animal (36). Transgenic P23H albino rat are engineered to be a model of rhodopsin mutation which commonly occur in human. This model develops a dysfunctional rod, and loss of photo receptor which leads to degenerative alteration in the inner retina. P23H rats treated with TUDCA demonstrated a significant reduction in TUNEL labeling which indicates that UDCA suppressed apoptosis. In cataract animal's model, UDCA and TUDCA have been shown to suppress choroidal neovascularation (CNV) formation and subsequently reduce the inflammation in retina (36). Furthermore, TUDCA and UDCA treated cataract rats showed suppression of vascular endothelial growth factor (VEGF) in retina compare to untreated cataract rats. Apart from that, study on the cells and animals shows that UDCA prevents selenite-induced cataract in lenses by maintaining antioxidant status; GSH level, inhibit peroxidation and decreased MDA levels (37).

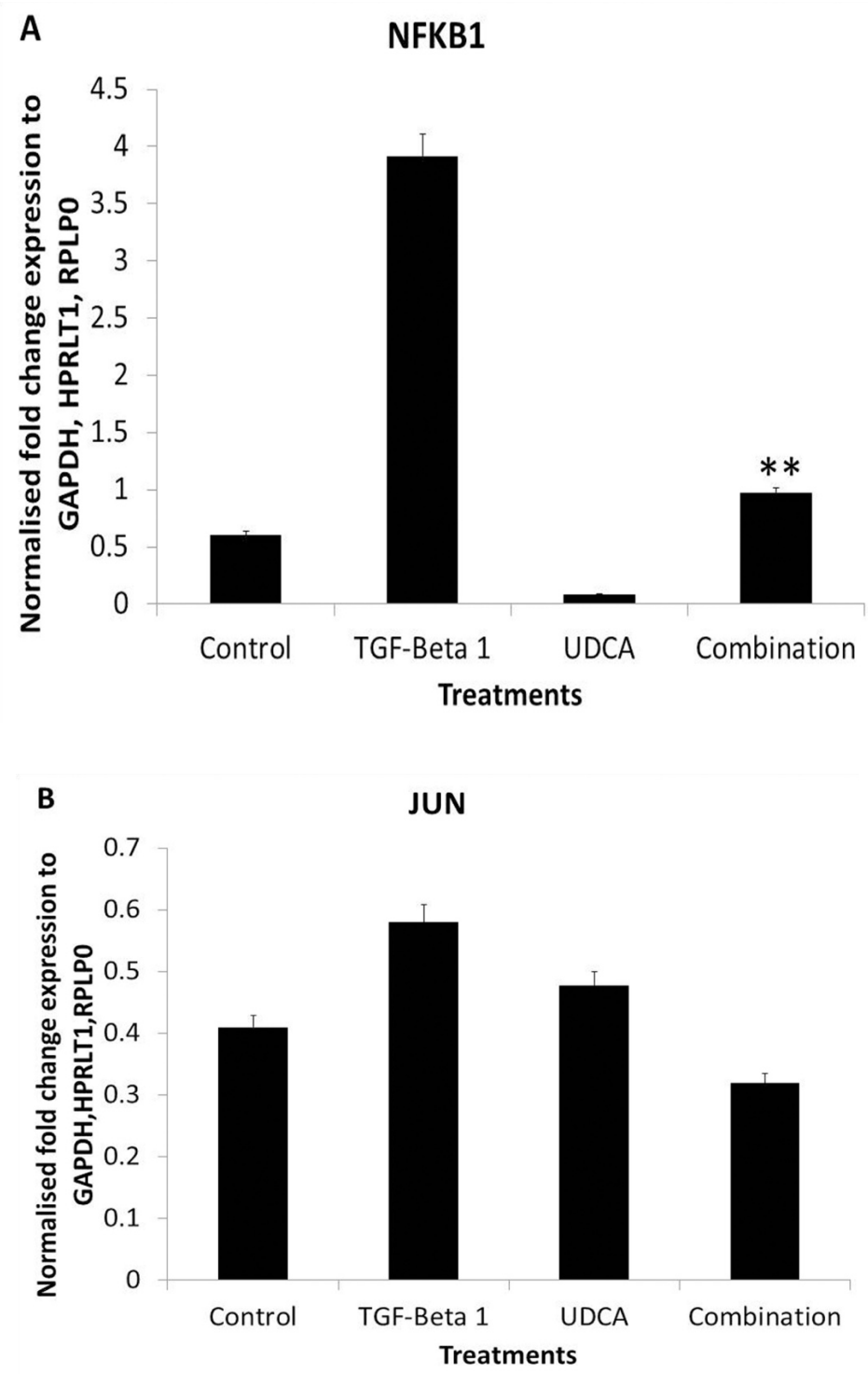

Fig. 4 Ursodeoxycholic acid downregulates transcription factor Nf-κβ1 gene expression in TGF-β1 treated HTF cells. Meanwhile, there were no significant difference in transcription factor JUN gene expression between control and all treated HTF cells. n≥3, **p < 0.05, TGF-β1 versus combination.

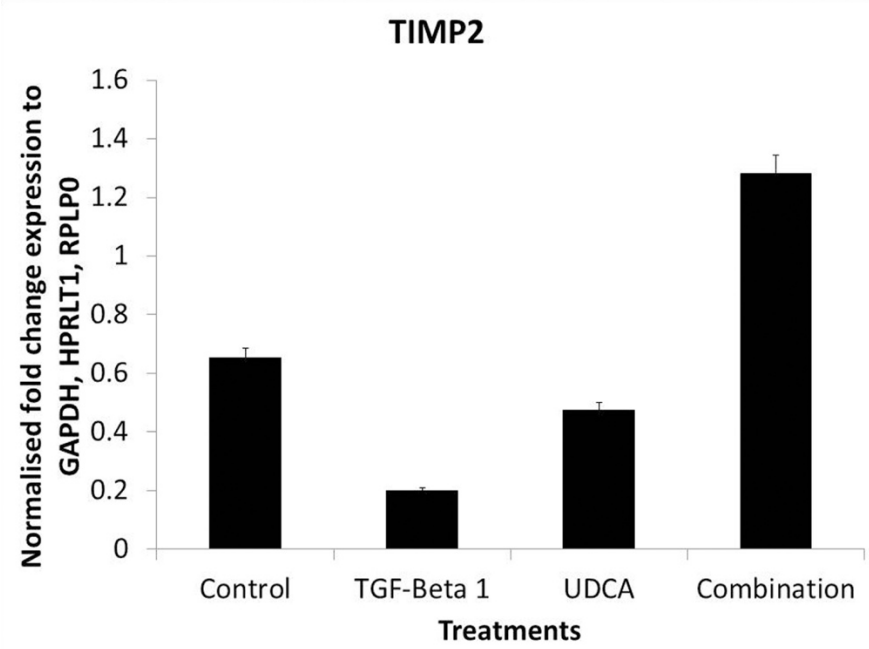

Fig. 5 Ursodeoxycholic acid affects the expression of tissue inhibitor of metalloproteinase (TIMP2) gene expression of TGF-β1 treated HTF cells. Upregulation of TIMP2 was observed in HTFs treated with TGF-β1 and UDCA compared to TGF-β1 only. However, no significant difference changes were observed. n≥3, p< 0.05.downregulates transcription factor Nf-κβ1 gene expression in TGF-β1 treated HTF cells. Meanwhile, there were no significant difference in transcription factor JUN gene expression between control and all treated HTF cells. n≥3, **p < 0.05, TGF-β1 versus combination.

Although UDCA has been reported by numerous studies as an anti-apoptotic agent, however in some cells and especially cancer cells, UDCA is reported as pro-apoptotic agent. Due to that, researchers mostly suggested UDCA effects are cell specific. In gastrointestinal cancer cell lines (SNU601) UDCA is reported to activate ERK 1/2 and caspases (-8, -3 and -6) and subsequently induced apoptosis (38). In another studies UDCA was suggested as anticancer agent which offers elimination of the apoptotic-sensitive and resistant cancer cells (39). Apart from that, UDCA shows to induced apoptosis in prostate cancer cells with the interaction of TGR5 receptor on the cell membrane. In clinical trial, UDCA treatment reduced flutamide-induced hepatopathy incidence in prostate cancer patients (40).

Conclusion

For the first time in here, we showed UDCA attenuates the effects of TGF-β1 on the HTFs by downregulating the expression of α-SMA, ERK 1/2 and Nf-κβ1.Understanding of how UDCA is involved in fibroblast differentiation and myofibroblast formation may provide valuable insights into future therapy that can control scarring and perhaps fibrosis. This will further improve the outcome of glaucoma filtration surgery. Further studies are required to investigate the ability of UDCA in modulating fibroblast functions.

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