ORIGINAL ARTICLE

SLIT3 knockdown inhibited TGF-β-induced hepatic stellate cells activation by down-regulating YAP signal

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Abstract

Objective Liver fibrosis is a chronic liver disease caused by a variety of pathophysiological. However, there are no effective treatments to combat it. HSCs are a major source of fibrotic cells and exploring the mechanisms of HSC activation may provide new strategies for the treatment of liver fibrosis.

Objectives To explore the role and underlying mechanism of SLIT3 in HSCs fibrosis.

Results GSE163211 dataset analysis identified aberrant expression of SLIT3 in NASH F1-F4 tissues and SLIT3 expression level was positively correlated with fibrosis-related proteins. In vitro experiments showed that TGF-β induced upregulation of SLIT3 in LX-2 cells. Knockdown of SLIT3 significantly inhibited TGF-β-induced α-SMA, COL1A2, and COL1A1 expression, inhibited excessive cell proliferation and migration, and suppressed YAP activity.

Conclusion Collectively, our findings suggest that SLIT3 deficiency alleviates TGF-β-induced HSCs activation by inhibiting YAP activity.

Keywords Liver fibrosis · Hepatic stellate cells · SLIT3 · TGF-β · YAP

Introduction

Chronic liver disease causes approximately 2 million deaths each year, accounting for 3.5% of all deaths worldwide (Asrani et al. 2019). Liver fibrosis is one of its most common consequences and manifests itself as a wound-healing response to chronic liver injury, which may be caused by viruses, alcohol abuse, steatosis, and autoimmune or genetic disorders (Angulo et al. 2015; Bruschi et al. 2020; Poilil Surendran et al. 2017). Now, it has become a major world health issue (Del Campo et al. 2018), and it is associated with functional organ damage (Del Campo et al. 2018). If left untreated, it will eventually develop into cirrhosis. To date, the only effective treatment for cirrhosis is liver transplantation (Zhao et al. 2019). Therefore, new therapeutic strategies are urgently needed to prevent the development of liver fibrosis.

Liver fibrosis is a dynamic process involving crosstalk between multiple cell types, and hepatic stellate cells (HSCs) have received widespread attention as a major source of fibrotic cells (Tsuchida et al. 2017). Activation-driven HSCs, which undergo phenotypic trans-differentiation following liver injury, can overproduce extracellular matrix (ECM) and accumulate in the subendothelial space, thereby causing their transition to proliferative or contractile myofibroblast-like cells that induce excessive scarring, eventually leading to end-stage liver disease, cirrhosis, and hepatocellular carcinoma (Marcellin et al. 2018; Tsuchida et al. 2017; Yanguas et al. 2016; Zhang et al. 2016; Zhao et al. 2019). This process is activated by various factors, and studies have shown that the fibrotic effect of TGF-β is closely related to changes in ECM composition (Hinz 2015).

Elevated levels and nuclear translocation of the transcriptional regulator YES-associated protein (YAP) have been reported to drive activation of HSCs in vivo/in vitro (Li et al. 2021; Xiang et al. 2020). YAP signaling is essential for liver regeneration. Connective tissue growth factor (CTGF), a target gene of YAP, is associated with the pathological process of tissue fibrosis. CTGF is overexpressed in liver fibrotic tissues and activates HSCs, and induces synthesis and secretion of ECM proteins, and it is also associated with

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cell proliferation, migration and differentiation (Makino et al. 2018; Ramazani et al. 2018). YAP have been reported to interact with TGF-β-induced Smad2/3 and participate in tissue fibrosis (Hiemer et al. 2014; Zhang et al. 2022).

Mammalian slit-guided ligands 1–3 (SLIT1-3) are highly conserved secreted glycoproteins (Plump et al. 2002). Analysis of GEO data (GSE163211) revealed that SLIT3 is up-regulated in liver tissue from patients with fibrosing non-alcoholic steatohepatitis. SLIT3 has been reported to be involved in stress overload-induced cardiac fibrosis and poor remodeling, reducing fibrillar collagen production and regulating YAP transcription and translation (Gong et al. 2020).The role of SLIT3 in liver fibrosis is unclear. This study aimed to explore the role and underlying mechanism of SLIT3 in HSCs fibrosis.

Materials and methods

GEO data processing

A gene expression microarray dataset GSE163211 was obtained from the GEO database, from the GPL29503 platform, representing datasets from hepatic fibrosis, inflammation and steatosis. Normal liver tissue (Normal, $n=6$) and non-alcoholic steatohepatitis with fibrosis stages 1–4 (NASH F1–F4, $n=46$) from diabetic patients were selected for analysis. The GEO online platform was used to explore the differentially expressed genes (DEGs) in Normal and NASH F1–F4 tissues. A p value of < 0.05 and logFC (fold change) of > 0.5 were set as thresholds to screen DEGs.

Cell culture and treatment

Human immortal HSCs cell line LX-2 was bought from Merck Millipore (Beijing, China). LX-2 cells were cultured in DMEM (Gibco, Gaithersburg, MD, USA) with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA). All culture media contained 100 U/ml of penicillin and 100 μg/ml of streptomycin. LX-2 cells were treated with exogenous 5 ng/mL TGF-β (R&D Systems, USA) and incubated for 24 h to be activated.

Transfection with siRNA and overexpression vector

The transfection of vectors into LX-2 cells was subjected to Lipofectamine 2000 (Invitrogen). Short interfering SLIT3 (siSLIT3), negative control siRNAs (siNC), pcDNA3.1-vector (vector) and SLIT3 overexpression vector (SLIT3) were purchased from RioBio (Guangzhou, China) and addgene (USA).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from TGF-β-treated LX-2 cells using RNAiso Plus reagent (Takara; China). Then it was reverse transcribed into complementary DNA using Prime Script RT Kit (Takara; China). qRT-PCR was performed using the SYBR Green PCR kit (Takara, China) according to the manufacturer's protocol. The primer sequences used to amplify SLIT3 and 18S are as follows: SLIT3 (Forward 5'-GTCAGCGTCATCGAGAGAGG-3'; Reverse 5'-TTC GGCGTGCTCTGGAAAA-3'), 18S (Forward 5'- GGA ATTGACGGAAGGGCACCACC-3'; Reverse 5'-GTG CAGCCCCGGACATCTAAGG-3').

Western blot analysis

Western blot analysis of protein expression in LX-2 cells was performed as previously described (Seo et al. 2020). β-actin was used as an internal control. The Image J software was used to quantify protein expression. The primary antibodies included anti- SLIT3, anti-α-SMA, anti-COL1A1 and anti-COL1A2 (1:1000; Abcam, Cambridge, UK), anti-YAP, anti-p-YAP, anti-Smad2/3, anti-p-Smad2/3, anti-CTGF, and anti-β-actin (1:1000; Cell Signaling Technology, Danvers, MA).

CCK-8 assay

Lx-2 cells transfected with siSLIT3, siNC, vector and pcDNA3.1-SLIT3 were seeded into 96-well plates. 24 h after transfection, 5 ng/ml TGF-β was added. Subsequently, CCK-8 solution was added into the culture medium (100 μl) for 3 h. Absorbance was measured at 450 nm.

Wound-healing assay

Transfected or untransfected L × 2 cells were inoculated into 12-well plates at a density of $3–7 × 10^5$ cells/ml. Straight wounds were made on the fused monolayer and the cells were incubated with TGF-β for 24 h. Wounded monolayers are washed with PBS and photographed using an inverted microscope.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software®. Results are expressed as mean ± standard deviation (SD), and statistical differences between groups were compared by Student's t-test or ANOVA. All

experiments were repeated done in triplicate. $p < 0.05$ was considered statistically significant.

Results

SLIT3 is up-regulated in TGF-β-induced HSCs

We first used the previously published RNA sequencing (RNAseq) dataset GSE163211 to assess the gene regulation associated with NASH-related liver fibrosis progression. The differential gene expression was compared between normal liver tissue and NASH F1-F4 (Fig. 1A). 45 differential genes were detected, of which 30 were up-regulated and 15 were down-regulated. Among them, SLIT3 was up-regulated in NASH F1–F4 with logFC = 0.930, p value = 0.027 and expression data were obtained from GSE163211 (Fig. 1B). In addition, it was found that SLIT3 was significantly positively correlated with the expression levels of fibrosis-related genes COL1A1 and COL1A2 (Fig. 1C). Previous reports have shown that TGF-β is capable of inducing HSCs activation. Next, LX-2 cells were incubated with 5 ng/mL TGF-β in vitro. The results showed that TGF-β induced upregulation of SLIT3 protein and mRNA levels (Fig. 1C, D). The expression level first increased with the treatment time and then decreased, and the expression level was the highest at 1 h. These results suggested that the differential expression of SLIT3 may be related to liver fibrosis.

Knockdown of SLIT3 inhibits TGF-β-induced HSCs fibrosis

To investigate the potential role of SLIT3 in HSCs fibrosis, SLIT3 was knocked down or overexpressed in LX-2 cells. Western blot analysis verified the effect of SLIT3 knockdown and overexpression (Fig. 2). TGF-β increased
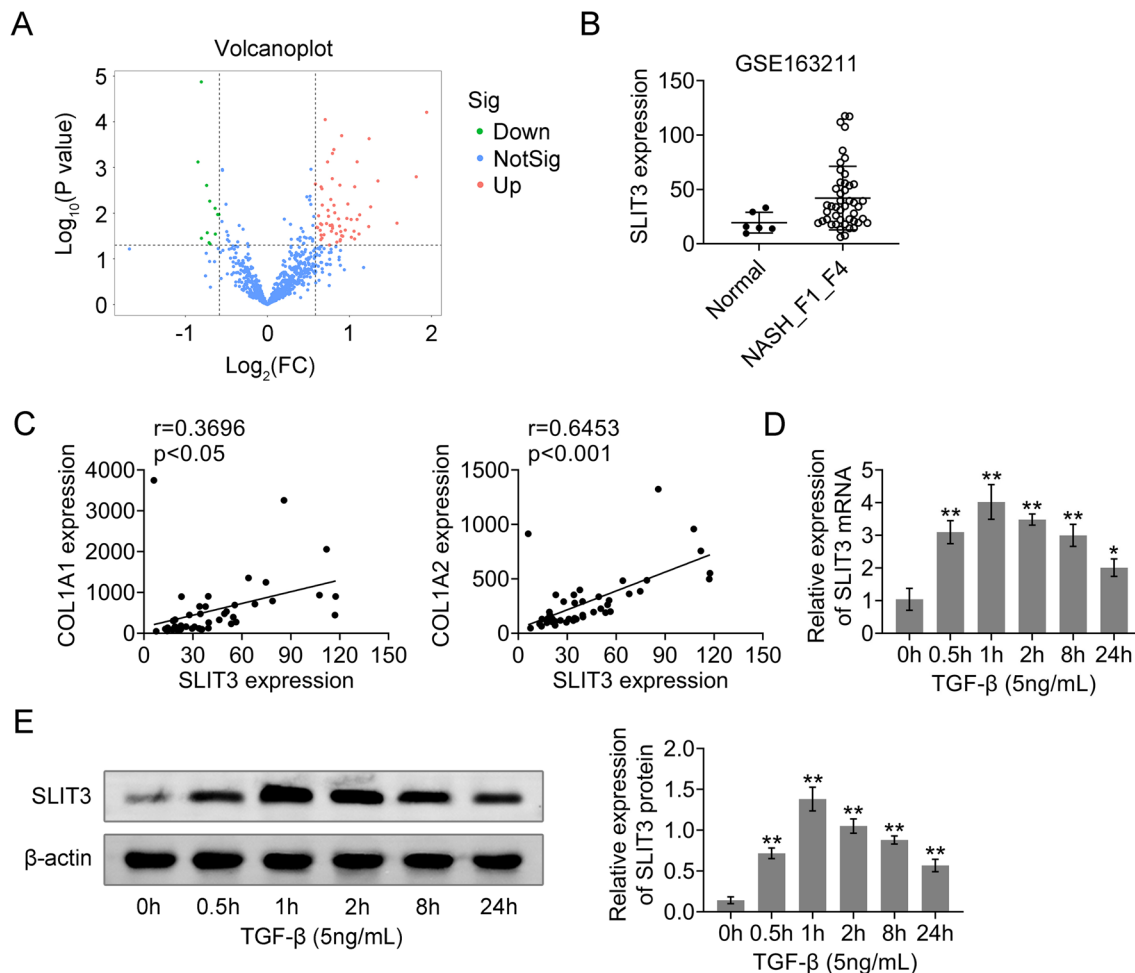

Fig. 1 SLIT3 is up-regulated in NASH F1-F4 patient's liver tissue and TGF-β-induced HSCs. **A** Volcano map of Normal vs NASH F1-F4 differentially expressed genes. **B** Expression level of SLIT3 in GSE163211 dataset. **C** In the GSE163211 dataset, SLIT3 was positively correlated with the expression levels of COL1A1 and COL1A2. D Western blot analysis showed the SLIT3 protein expression level of LX-2 cell after 5 ng/mL TGF-β treatment for 24 h. **$P < 0.01$ compared with 0 h group
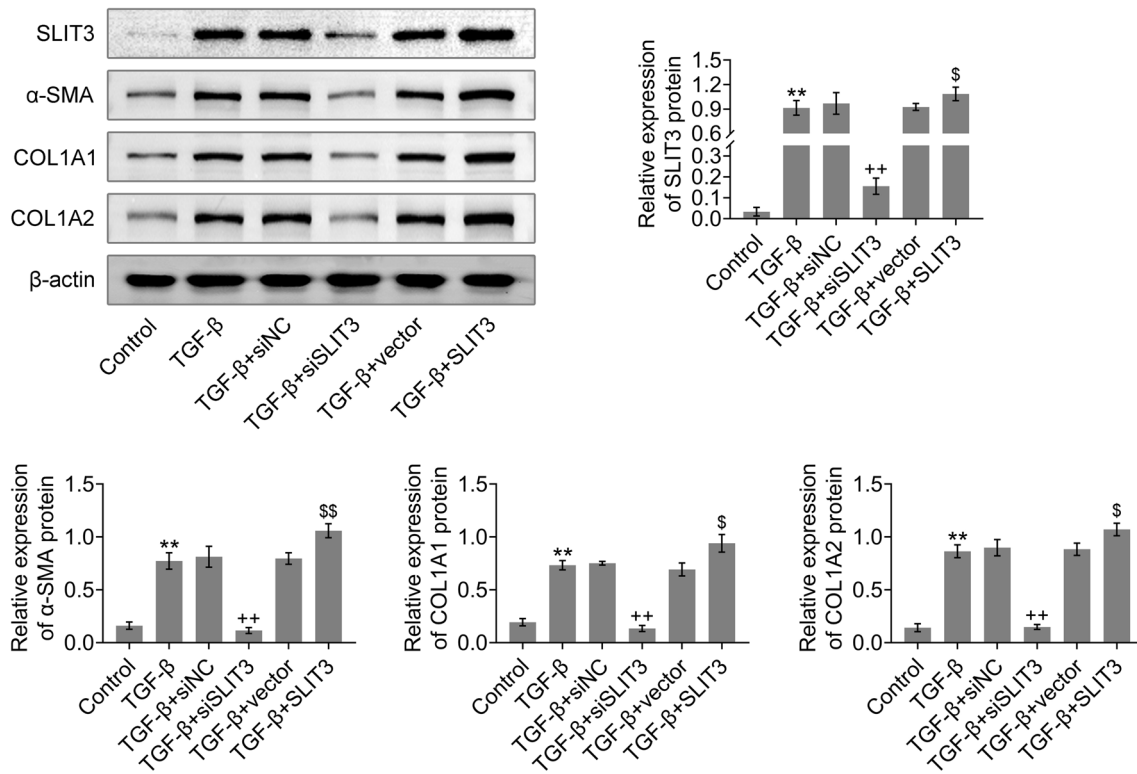
Fig. 2 Knockdown of SLIT3 inhibits TGF-β-induced HSCs fibrosisWestern blot analysis evaluated the expression of levels of SLIT3, α-SMA, COL1A2 and COL1A1. **$P < 0.01$ compared with control group; $+ + p < 0.01$ compared with TGF-β + siNC group; \$$p < 0.05$, \$\$$p < 0.01$ compared with TGF-β + vector group

the accumulation of α-SMA, COL1A2 and COL1A1 compared to control, but these increases were significantly suppressed by knockdown of SLIT3 (Fig. 2). In contrast, overexpression of SLIT3 further promoted α-SMA, COL1A2, and COL1A1 expression (Fig. 2). These results suggest that SLIT3 is involved in the induction of LX-2 cell fibrosis in response to TGF-β.

Knockdown of SLIT3 inhibits TGF-β-induced HSCs viability and migration

Another feature of HSCs activation is high proliferation and migration rates. To evaluate the effect of SLIT3 on proliferation and migration of HSCs, CCK-8 assay and wound-healing experiment were performed. As expected, TGF-β stimulation significantly enhanced the proliferation rate of LX-2 cells (Fig. 3A). Cells transfected with siNC or empty plasmid had no effect on TGF-β-induced cell proliferation. After down-regulation of SLIT3, the proliferation level of the cells was decreased and recovered to a level comparable to that of the control. Similar results were shown in wound-healing experiments. LX-2 cells transfected with SLIT3 siRNA or SLIT3 overexpression

plasmids showed significant differences in their migratory responses to TGF-β (Fig. 3B, C).

Knockdown of SLIT3 inhibits the YAP signaling pathway

Next, we investigated Fig. 4 whether SLIT3 mediates YAP signaling activation. The data showed that TGF-β significantly induced YAP expression and reduced YAP phosphorylation compared with the control. SLIT3 deficiency inhibited YAP expression and increased its phosphorylation. In addition, we also examined the protein expression of genes downstream of YAP. The results showed that SLIT3 deficiency inhibited TGF-β-induced CTGF expression and Smad2/3 phosphorylation.

Discussion

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease that affects one quarter of the world's population and imposes a huge global burden on public health systems (Koyama et al. 2017, Powell et al. 2021). NAFLD initially presents as hepatic steatosis and subsequently evolves
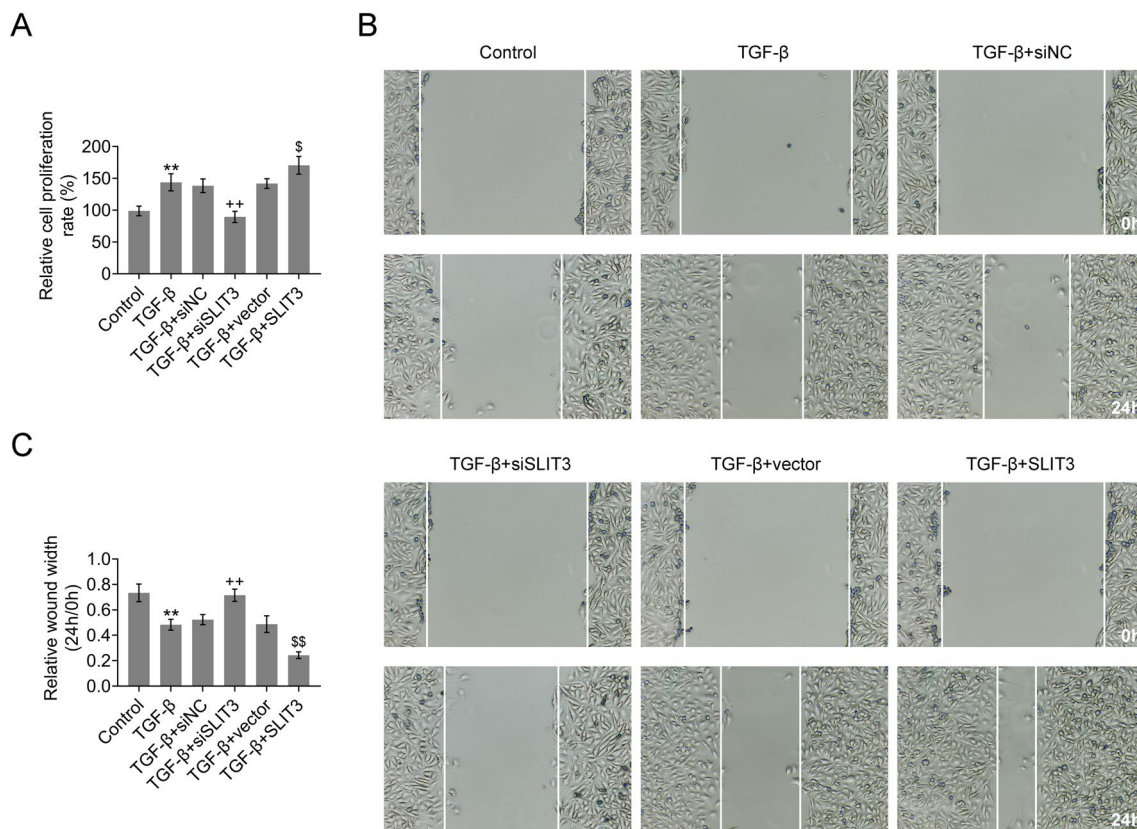
Fig. 3 Knockdown of SLIT3 inhibits TGF-β-induced HSCs viability and migration. A CCK-8 was used to examine the viability of LX-2 cell (B, C) Wound healing assay LX-2 cell migration. The statistical analysis was shown in the bar graphs. $**P < 0.01$ compared with control group; $++p < 0.01$ compared with TGF-β + siNC group; $p < 0.05$, $p < 0.01$ compared with TGF-β + vector group

into NASH with the potential for progressive fibrosis and increased risk of cirrhosis and hepatocellular carcinoma (Bruno et al. 2020; Challa et al. 2019). The progression of NASH to liver fibrosis is a reversible stage in this process (Liu et al. 2019), but the initial triggers for the development of liver fibrosis remain largely unknown.

With the development of bioinformatics, we can obtain RNA sequencing data from the GEO public database to identify differentially expressed genes related to the progression of liver fibrosis, providing new ideas for the study of the molecular mechanism of liver fibrosis. To this end, we explored differentially expressed genes between normal liver tissue and NASH-associated fibrotic tissue in the GSE163211 dataset and found 45 genes with abnormal expression. Among them, SLIT3 caught our attention. SLIT3 was up-regulated in NASH F1-F4 tissues, and its expression level was significantly positively correlated with the levels of liver fibrosis-related factors including COL1A1. SLIT3, one of the three SLIT ligands originally discovered in the central nervous system, mediates axon guidance through a roundabout family of receptors (Long et al. 2004). Several subsequent studies revealed that SLIT3 is expressed in tissues other than the

nervous system and is involved in physiological functions. For example, SLIT3 induces directional migration and proliferation of Mc3t3-e1 cells (Kim et al. 2018), and can promote the formation of H-type blood vessels and bone formation (Peng et al. 2020). In addition, SLIT3 is also involved in cardiac fibrosis and remodeling induced by pressure overload. Experiments have shown that SLIT3 is mainly present in fibrillar collagen-producing cells, and SLIT3 deficiency attenuates COL1A1 transcription in cardiac, aortic adventitia, lung, spleen and kidney tissues (Gong et al. 2020). Therefore, SLIT3 may be a potential target of fibrosis.

HSCs are a population of non-parenchymal hepatocytes (Trivedi et al. 2021). Under normal conditions, HSCs store lipids in the liver (Weiskirchen et al. 2019). During liver injury, HSCs are activated and transformed into proliferative and contractile myofibroblasts that simultaneously produce ECM, and numerous chemokines and cytokines such as COL1A1,α-SMA, fibronectin, laminin and proteoglycans, and cause tissue scaring (Mehta et al. 2019; Toosi 2015). TGF-β plays an important role in the pathogenesis of liver fibrosis and is one of the most potent fibrosis activators, which can induce quiescent HSCs differentiate into
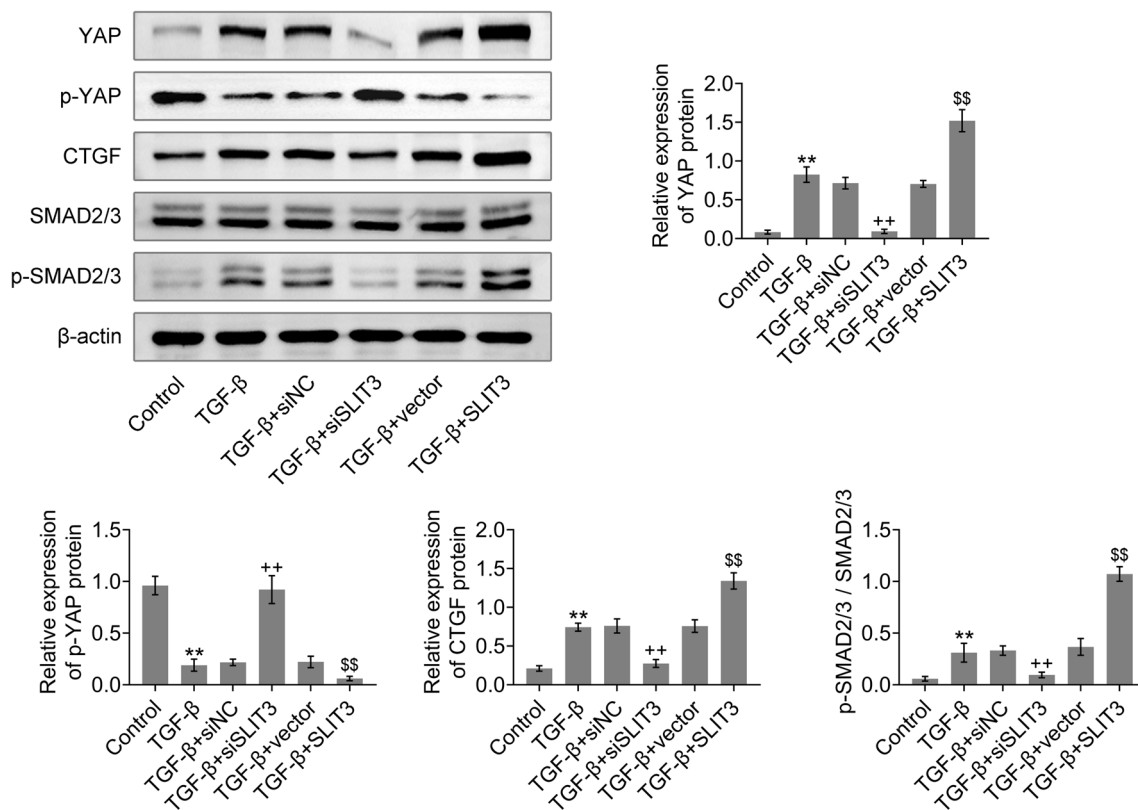
Fig. 4 Knockdown of SLIT3 inhibits the YAP signaling pathway. Western blot analysis evaluated the expression of levels of YAP, p-YAP, CTGF, SMAD2/3 and p-SMAD2/3. **$*P < 0.01$ compared with control group; $++p < 0.01$ compared with TGF-β + siNC group; $\$\$p < 0.01$ compared with TGF-β + vector group

myofibroblast-like cells and induce ECM production (Massagué 2012).

To explore the potential role of SLIT3 in liver fibrosis, we constructed a TGF-β-induced LX-2 cell model in vitro. Western blotting results showed that TGF-β induced the expression of SLIT3 in LX-2 cells, and at the same time induced the activation of LX-2 cells, as well as increased the expressions of α-SMA, COL1A2 and COL1A1. SLIT3 knockdown significantly alleviated ECM accumulation in LX-2 cells. Conversely, SLIT3-overexpressing further increased the protein levels of α-SMA, COL1A2, and COL1A1 in LX-2 cells.

HSCs have the characteristics of high proliferation and mobility after activation. Previous studies have shown that Slit3 knockdown can significantly inhibit the proliferation and migration of fibroblasts after TGF-β treatment (Gong et al. 2020). The above results are consistent with our experimental results. Compared with LX-2 cells transfected with siNC, Slit3 knockdown cells showed lower cell viability and migration ability under TGF-β treatment, while Slit3 overexpression further enhanced cell viability and promoted cell migration. In conclusion, knockdown of Slit3 can attenuate the response of HSCs to TGF-β.

The transcription factor YAP has been identified as an important mediator of mechanical transduction signals in fibroblasts and extensively regulates the biological activities of fibroblasts(Gong et al. 2020; Haak et al. 2019). Previous studies have demonstrated that SLIT3 regulates YAP signaling pathway activation in fibroblasts (Gong et al. 2020). Therefore, the potential association between SLIT3 and YAP in HSCs was examined. The results were consistent with previous reports that YAP phosphorylation levels were significantly increased in SISlit3-transfected LX-2 cells, indicating inhibition of TGF-β-induced YAP activation. Activation of HSCs is associated with upregulation of several genes involved in matrix remodeling, actin cytoskeleton, cell proliferation, and immune processes. *Ctgf*, for example, is a direct target of YAP (Zhubanchaliyev et al. 2016). It has been reported that YAP regulates SMad2/3 and participates in tissue fibrosis (Zhang et al. 2022). Therefore, the protein expression level of CTGF and the phosphorylation level of Smad2/3 were detected by Western blotting. The results showed that SLIT3 knockdown inhibited CTGF expression and Smad2/3 phosphorylation. In conclusion, our results demonstrated that loss of SLIT3 attenuated the effects of

TGF-β stimulation on HSCs by down-regulating YAP and its downstream target genes.

Declarations

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