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Original Article

Protein inhibitor of activated STAT 4 (PIAS4) regulates liver fibrosis through modulating SMAD3 activity

Huihui Xu[Δ], Zhiwen Fan[Δ], Wenfang Tian, Yong Xu[✉]

Key Laboratory of Cardiovascular Disease and Molecular Intervention, Department of Pathophysiology, Nanjing Medical University, Nanjing, Jiangsu 211166, China.

Abstract

Excessive fibrogenesis disrupts normal liver structure, impairs liver function, and precipitates the development of cirrhosis, an irreversible end-stage liver disease. A host of factors including nutrition surplus contribute to liver fibrosis but the underlying mechanism is not fully understood. In the present study, we investigated the involvement of protein inhibitor for activated stat 4 (PIAS4) in liver fibrosis in a mouse model of non-alcoholic steatohepatitis (NASH). We report that *PIAS4* silencing using short hairpin RNA (shRNA) attenuated high-fat high-carbohydrate (HFHC) diet induced liver fibrosis in mice. Quantitative PCR and Western blotting analyses confirmed that *PIAS4* knockdown downregulated a panel of pro-fibrogenic genes including type I and type III collagens, smooth muscle actin, and tissue inhibitors of metalloproteinase. Mechanistically, *PIAS4* silencing blocked the recruitment of SMAD3, a potent pro-fibrogenic transcription factor, to the promoter regions of pro-fibrogenic genes and dampened SMAD3 acetylation likely by upregulating SIRT1 expression. In conclusion, PIAS4 may contribute to liver fibrosis by modulating SIRT1-dependent SMAD3 acetylation.

Keywords: liver fibrosis, PIAS4, SMAD3, acetylation, transcriptional regulation

Introduction

Non-alcoholic steatohepatitis or NASH has become a significant health threat in a growing number of nations accompanying the global pandemic of obesity and type 2 diabetes[1]. Liver fibrosis is one of the many complications associated with NASH[2]. In response to nutrition surplus, several different types of cells including hepatic stellate cells and portal fibroblast cells trans-differentiate into pro-fibrogenic myofibroblasts and accelerate the production and deposition of extracellular matrix (ECM) proteins[3-4]. Although considered a host defense mechanism, excessive fibrogenesis can disrupt normal liver structure and interfere with liver functionalities precipitating the development of end-stage liver diseases such as cirrhosis and hepatocellular carcinoma[5]. The mechanism accounting for liver fibrosis during NASH pathogenesis is not fully appreciated.

Inside the ECM-producing cells, fibrogenesis is dictated by a network of growth factors, cytokines, and transcription factors[6]. Transforming growth factor (TGF-β) is by far the most extensively studied pro-fibrogenic factor in the liver, signaling primarily through the SMAD family of transcription factors[7]. Upon binding to its receptor, TGF-β triggers the

[Δ]These authors contributed equally to this work.

[✉]Corresponding author: Yong Xu, PhD, Nanjing Medical University, 101 Longmian Avenue, Jiangning District, Nanjing, Jiangsu 211166, China. Email:yjxu@njmu.edu.cn, Tel/Fax: + 86-25-86862888/+ 86-25-86862888

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phosphorylation and nuclear translocation of SMAD3. SMAD3 in turn binds to the promoter regions of pro-fibrogenic genes (e.g., type I collagen) and activates transcription. The ability of SMAD3 to promote pro-fibrogenic transcription is in part impacted by its post-translational modification status. In addition to phosphorylation, SMAD3 can also be acetylated in its lysine residues; acetylation of SMAD3 by the histone acetyltransferase p300/CBP enhances its activity[8]. In contrast, deacetylation of SMAD3 by the lysine deacetylase SIRT1 dampens its activity[9].

Previously we have shown that protein inhibitor of activated STAT 4 (PIAS4) downregulates SIRT1 expression at the transcriptional level in response to hypoxia in cancer cells[10-11]. Therefore, we hypothesized that PIAS4 could potentially contribute to liver fibrosis by modulating SIRT1-dependent SMAD3 (de) acetylation. Our data as summarized in this report support this hypothesis and indicate that targeting PIAS4 may provide novel therapeutic solutions against NASH-induced liver fibrosis.

Materials and methods

Animals

All animal protocols were approved by the NJMU Intramural Ethics Committee on Animal Studies. To induce steatohepatitis, 8 week-old male C57/BL6 mice were fed a high fat high carbohydrate (HFHC) diet (D12492, Research Diets) for 16 consecutive weeks[12]. To knock down *PIAS4* , the mice were injected *via* the tail vein with purified lentiviral particles ($1X10^9$ MOI) that carry short hairpin RNA (shRNA) targeting *PIAS4* (5'-GTGCTGTACGGGAAGTACTT-3') or scrambled shRNA (SCR) every 10 days for the duration of the experiments.

Protein extraction and Western blotting assay

Tissue lysates were obtained as previously described[13]. Western blot analyses were performed with anti-SIRT1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-type III collagen (Santa Cruz Biotechnology), anti-PIAS4 (Sigma), anti-β-actin (Sigma), anti-acetyl lysine (Cell Signaling Tech), anti-type I collagen (Rockland), anti-α-SMA (Abcam), and anti-SMAD3 (Abcam) antibodies.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed essentially as described before[14] with anti-SMAD3 antibody (Abcam). Precipitated genomic DNA was amplified by real-time PCR with primers as previously described[3,11,15].

Histology

Histological analyses were performed essentially as described before[11,13]. Briefly, paraffin sections were stained with picrosirius red (Sigma) or Masson's trichrome (Sigma) according to standard procedures. Pictures were taken using an Olympus IX-70 microscope.

Statistical analysis

Data are presented as mean ± SD. For experiments concerning multiple groups, one-way ANOVA with post-hoc Scheffe analyses were performed to evaluate the differences. The differences between two (control and experimental) groups were determined by two-sided, unpaired Student's *t*-test. *P* values smaller than 0.05 are considered significant. For the in vivo experiments, specific *P* values are spelled out.

Results

PIAS4 knockdown alleviates liver fibrosis in mice

We first examined the effect of PIAS4 on liver fibrosis in vivo. To induce liver fibrosis, C57/BL6 mice were fed with a HFHC diet for 16 weeks[12]. Picrosirius red (***Fig. 1A***) and Masson's trichrome (***Fig. 1B***) staining revealed extensive interstitial fibrosis in the livers of mice fed on the HFHC diet compared to the mice on a control (chow) diet. *PIAS4* knockdown was achieved *via* lentivirus-mediated delivery of shRNA injected through the tail vein. Western blotting analysis showed that compared to mice injected with control shRNA (SCR), PIAS4-specific shRNA (shPias4) significantly down-regulated PIAS4 levels in the liver (***Fig. 2B***). Histological measurements showed that *PIAS4* silencing largely abrogated HFHC diet induced liver fibrosis (***Fig. 1A*** and ***Fig. 1B***).

PIAS4 depletion downregulates expression of pro-fibrogenic genes

Next, we examined the effects of PIAS4 depletion on the expression of pro-fibrogenic genes in the liver. Quantitative PCR analyses showed that the HFHC diet stimulated the synthesis of a panel of pro-fibrogenic genes, including type I collagen (*col1a1* and *co1a2*), type III collagen (*col3a1*), alpha smooth muscle actin (*acta2*), tissue inhibitors of matrix metalloproteinase (*timp1* and *timp3*), lysyl oxidase (*lox*), and integrin subunit alpha 1 (*itga1*). PIAS4 depletion, to varying extents, downregulated all the pro-fibrogenic genes examined here (***Fig. 2A***). Western blotting experiments confirmed the observation that PIAS4 depletion systemically downregulated the induction of pro-fibrogenic

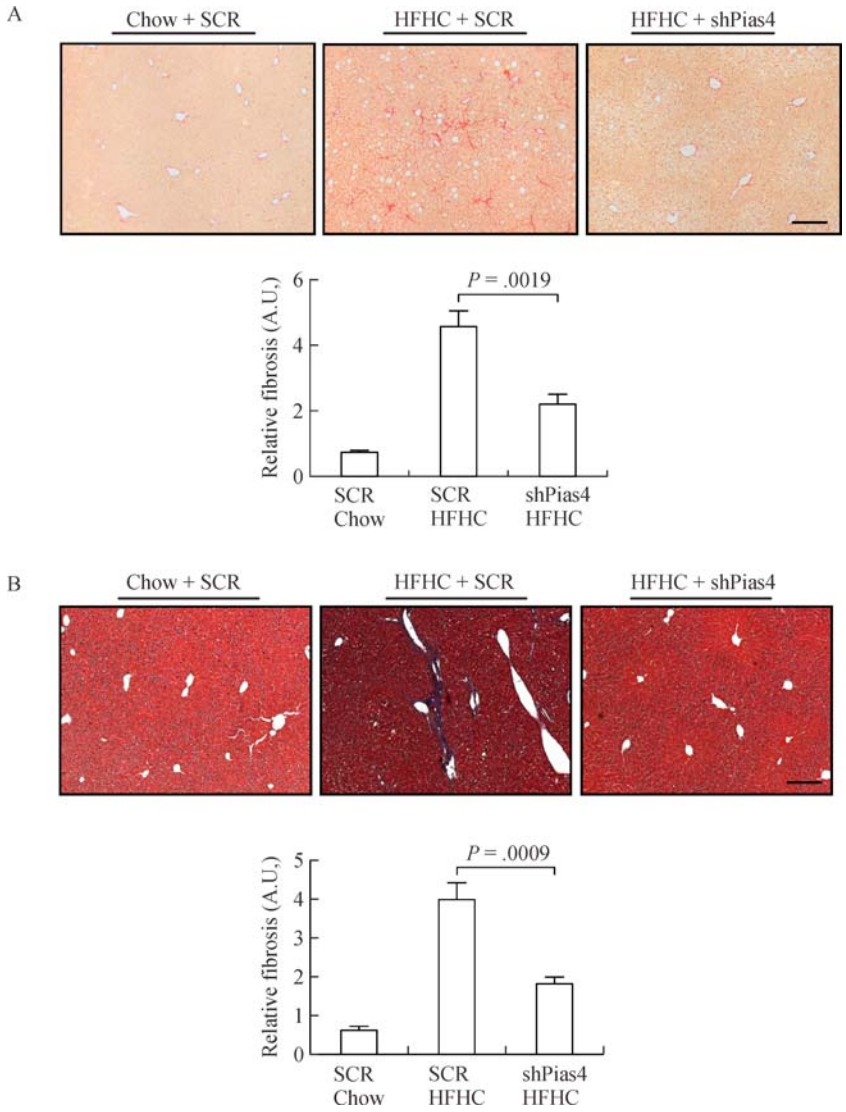

Fig. 1 **PIAS4 knockdown alleviates liver fibrosis in mice.** C57/BL6 mice were fed on a high fat high carbohydrate (HFHC)-diet or a chow diet for 16 weeks. Lentivirus carrying either PIAS4 targeting shRNA or a control shRNA was injected weekly *via* the tail vein. Picrosirius red (A) and Masson's trichrome (B) stainings were performed as described in Methods. Quantification was carried out using Image Pro. $N = 5$ mice for each group. Data are presented as mean±S.D. Scale bar, 50 μm.

gene expression by the HFHC diet (***Fig. 2B***). Taken together, we were able to conclude that PIAS4 might be essential for liver fibrosis in an HFHC diet-induced model of NASH.

PIAS4 modulates SMAD3 activity by influencing SIRT1-dependent deacetylation

SMAD3 is one of the most potent pro-fibrogenic transcription factors. We hypothesized that PIAS4 could modulate SMAD3 activity in the liver. ChIP assay showed that binding of SMAD3 to its target genes, including *col1a1*, *col1a2*, and *acta2*, was significantly upregulated in the livers of mice fed on the HFHC diet (***Fig. 3A***). On the contrary, PIAS4 silencing markedly dampened the occupancies of SMAD3 on its target promoters.

It has been documented that SIRT1 suppresses SMAD3 activity by promoting its deacetylation[9]. As shown in ***Fig. 2B***, HFHC diet feeding caused a decrease in SIRT1 expression, consistent with an increase in SMAD3 activity; PIAS4 knockdown, however, was able to normalize SIRT1 expression, again in keeping with suppressed SMAD3 activity. We then examined the acetylation status of SMAD3 in various settings. Immunoprecipitation combined with Western blotting showed that HFHC diet feeding resulted in a significant upregulation of SMAD3 acetylation, consistent with increased SMAD3 binding activity and decreased SIRT1 expression, in the liver (***Fig. 3B***). PIAS4 depletion, however, blocked the induction of SMAD3 acetylation, which was in agreement with restored SIRT1 expression and reduced SMAD3 binding on

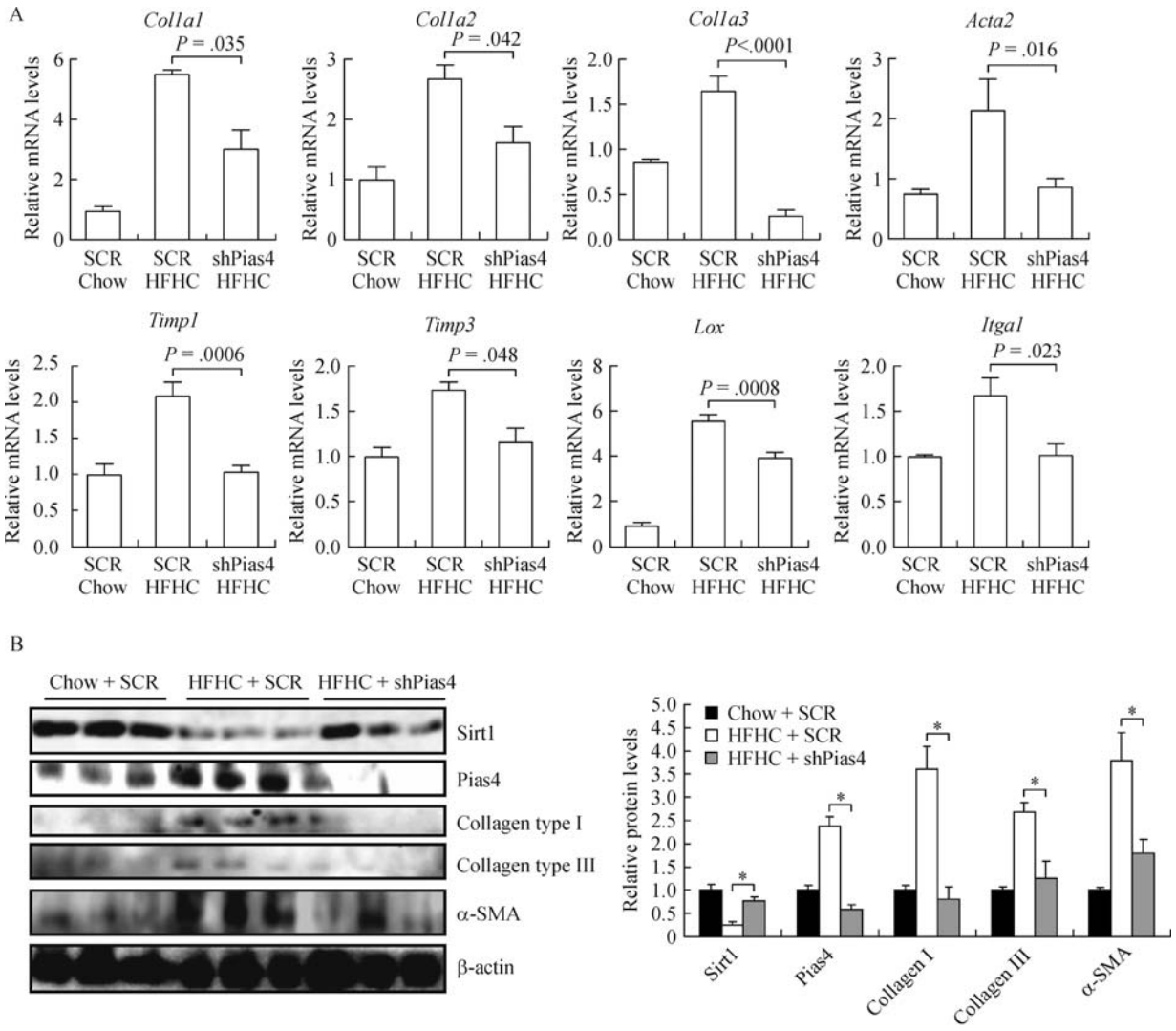

Fig. 2 **PIAS4 depletion downregulates expression of pro-fibrogenic genes.** C57/BL6 mice were fed on an HFHC-diet or a chow diet for 16 weeks. Lentivirus carrying either PIAS4 targeting shRNA or a control shRNA was injected weekly *via* the tail vein. Expression levels of pro-fibrogenic genes were examined by qPCR (A) and Western blotting assays (B). $N = 5$ mice for each group. Data are presented as mean±S.D. $^*P < 0.05$.

target promoters. Collectively, these data suggest that PIAS4 might contribute to liver fibrosis possibly by modulating SIRT1-dependent deacetylation of SMAD3.

Discussion

NASH serves as an intermediate disease state bridging the reversible and manageable steatosis and the irreversible and refractory cirrhosis[1]. Liver fibrosis is a hallmark event in the pathogenesis of NASH, the ineffective intervention of which may precipitate the development end-stage liver diseases and significantly dim the chance of patient survival. We report here that PIAS4 may play a critical role in liver fibrosis by modulating SMAD3 activity, likely through SIRT1-dependent deacetylation. Several previous investigations have implicated PIAS1 as a modulator of the TGF-β pathway although the conclusions seem to be contradictory. For instance, the Kurabayashi group has reported that PIAS1 is essential for TGF-β induced α-SMA trans-activation in smooth muscle cells by SUMOylating KLF4[16]. In contrast, Netherton and Bonni demonstrated that PIAS1 represses TGF-β initiated mesenchymal cell differentiation by SUMOylating SnoN[17]. Our data suggest that PIAS4 modulates TGF-β signaling in an indirect manner, namely, through SIRT1-dependent deacetylation of SMAD3. However, the possibility that PIAS4 could directly interact with and SUMOylate SMAD3 cannot be excluded especially in light of the finding that SMAD4, the common SMAD protein required for TGF-β signaling, have been found to be a direct substrate for SUMOylation[18]. In

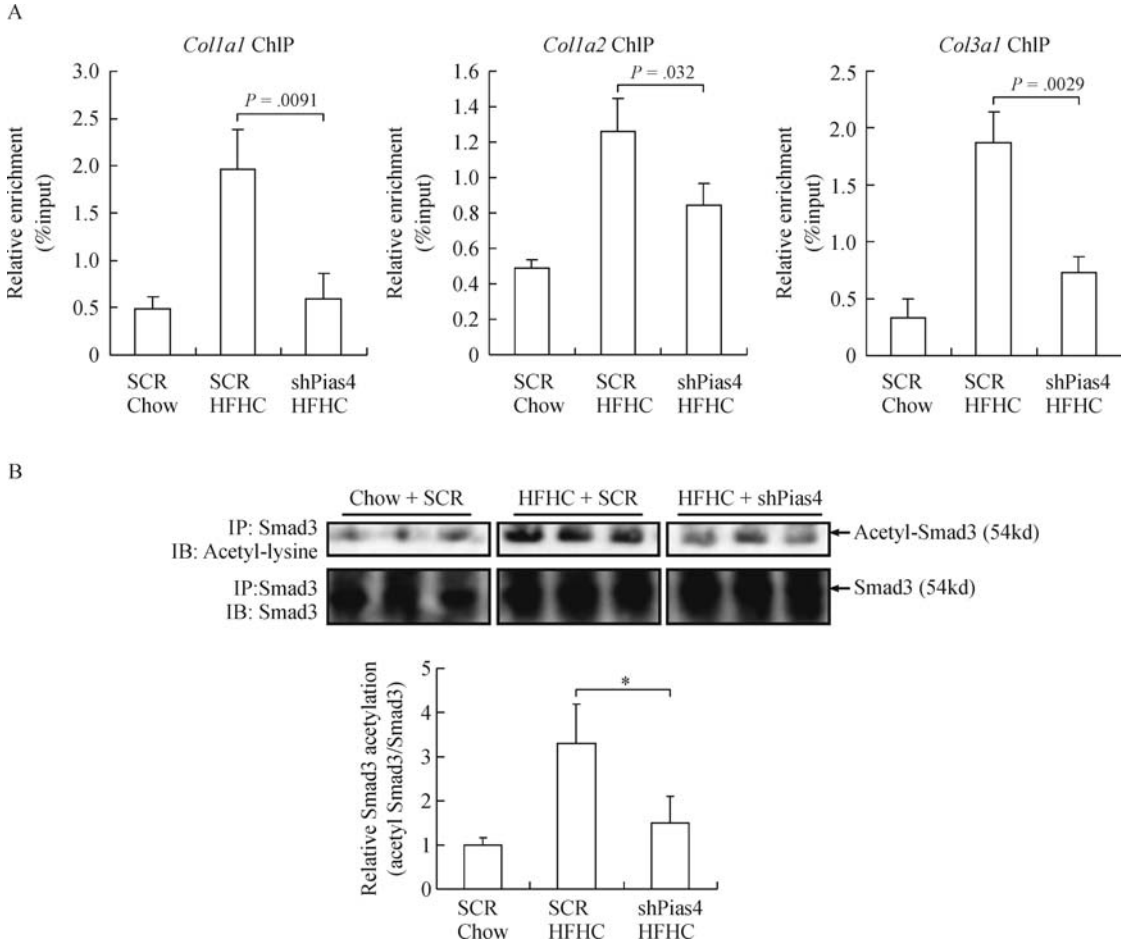

Fig. 3 **PIAS4 modulates SMAD3 activity by influencing SIRT1-dependent deacetylation.** C57/BL6 mice were fed on an HFHC-diet or a chow diet for 16 weeks. Lentivirus carrying either PIAS4 targeting shRNA or a control shRNA was injected weekly *via* the tail vein. (A) ChIP assay was performed using liver homogenates with anti-SMAD3 antibody. Precipitated DNA was amplified using primers surrounding the indicated gene promoters. (B) Immunoprecipitation was performed with anti-SMAD3 using liver homogenates. Western blotting was performed with anti-SMAD3 or anti-acetyl lysine. $n = 3$ mice for each group. Data are presented as mean±S.D. $^*P < 0.05$.

addition, it remains unclear whether other members of the PIAS family could play a non-redundant role in regulating liver fibrosis. Starkel et al. have shown that PIAS3 expression was progressively increased during the development of liver fibrosis/cirrhosis in patients infected with hepatitis C virus (HCV), suggesting that PIAS3 may also play a precipitating role in this process[19]. When further confirmed, our data add support to the argument of using a pan-PIAS inhibitor to stall or reverse fibrogenesis in the liver.

We show here that PIAS4 promotes liver fibrosis likely through SIRT1-dependent SMAD3 deacetylation. In fact, several alternative scenarios exist to interpret the data. First, PIAS4 has been known to modulate cellular response to hypoxia[10-11,20], which by itself is a risk factor for NASH and a promoter of liver fibrosis[21]. Second, liver fibrosis in the context of NASH often occurs as a result of excessive hepatic inflammation. PIAS4 can directly SUMOylate and thus activate NF-κB, the master regulator of cellular inflammation[22]. Therefore, our observation that *PIAS4* knockdown attenuated liver fibrosis could be secondary to reduced hepatic inflammation as a result of NF-κB deactivation. Finally, we used a lentivirus delivery system that did not differentiate the liver from other organs or cells in the circulation. It is possible that PIAS4 might influence liver fibrosis by regulating circulating myeloid cells (e.g., macrophages), which are considered a driving force of liver fibrosis[23]. These remaining issues will have to be sorted out by future investigations.

In summary, we provide evidence that *PIAS4* knockdown in a mouse model of NASH effectively attenuated liver fibrosis. Therefore, PIAS4 could become an attractive target for the development of novel therapeutic strategies to prevent excessive liver fibrogenesis.

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