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RACK1-mediated translation control promotes liver fibrogenesis

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ABSTRACT

Activation of quiescent hepatic stellate cells (HSCs) is the central event of liver fibrosis. The translational machinery is an optimized molecular network that affects cellular homoeostasis and diseases, whereas the role of protein translation in HSCs activation and liver fibrosis is little defined. Our previous report suggests that up-regulation of receptor for activated C-kinase 1(RACK1) in HSCs is critical for liver fibrogenesis. In this study, we found that RACK1 promoted macrophage conditioned medium (MCM)-induced assembly of eIF4F and phosphorylation of eIF4E in primary HSCs. RACK1 enhanced the translation and expression of pro-fibrogenic factors collagen 1α1, snail and cyclin E1 induced by MCM. Administration of PP242 or knock-down of eIF4E suppressed RACK1-stimulated collagen 1α1 production, proliferation and migration in primary HSCs. In addition, depletion of eIF4E attenuated thioacetamide (TAA)-induced liver fibrosis *in vivo*. Our data suggest that RACK1-mediated stimulation of cap-dependent translation plays crucial roles in HSCs activation and liver fibrogenesis, and targeting translation initiation could be a promising strategy for the treatment of liver fibrosis.

1. Introduction

Liver fibrosis is defined as excessive accumulation of extracellular matrix proteins including collagen that occurs in most types of chronic liver diseases [1]. While mild fibrosis remains largely asymptomatic, its progression toward cirrhosis is the major cause of liver-related morbidity and mortality worldwide [2]. Activation of quiescent HSCs into fibrogenic myofibroblast phenotype is considered as the central event in liver fibrosis. This process is associated with the increased expression of contractile filaments such as α-smooth muscle actin (α-SMA) and production of extracellular matrix (ECM), and a large amount of production of pro-fibrogenic factors such as cytokines and reactive oxygen species (ROS). Cytokines regulating the inflammatory response to injury contributes to hepatic fibrogenesis *in vivo* and *in vitro* [1]. Among the cytokines, transforming growth factor beta 1 (TGF-β1) functions as a key mediator in fibrogenesis by promoting the trans-differentiation of HSCs and deposition of ECM, while platelet-derived growth factor (PDGF) is recognized as the most potent mitogen for HSCs [1].

The translational machinery is an optimized molecular network that affects cellular homoeostasis and diseases [3]. Under most circumstances, the rate-limiting step in protein synthesis is translation initiation [4]. The mammalian target of rapamycin (mTOR) plays a key role in the regulation of translation initiation by phosphorylating eukaryotic initiation factor 4E binding protein 1 (4E-BP1), which inhibits 5'-cap-dependent mRNA translation by binding and inactivating eIF4E [5]. Phosphorylation of 4E-BP1 releases eIF4E, allowing the assembly of eIF4F and initiation of translation. In addition, the activity of eIF4E is tightly regulated via phosphorylation at Serine 209 by MAP kinase-interacting serine/threonine kinase 1 (MNK1) and PKCβII [6,7]. While most cellular mRNAs require only minimal eIF4E to be efficiently translated, phosphorylated eIF4E preferentially enhances translation of select mRNAs with lengthy G + C-rich 5′-UTRs, such as cyclin E1 [8], MYC [9], snail [10] and type I collagen [11]. Nevertheless, the role of translation control in liver fibrosis is little defined.

The receptor for activated C-kinase1 (RACK1) is a member of the family of WD40 repeat proteins and acts as a scaffold protein to interact with different molecules involving in many physiological processes, including mRNA translation, signal transduction as well as cell growth, migration, and differentiation [12]. Our

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previous research demonstrated that ribosomal RACK1 plays a key role in translation initiation and promotes chemoresistance and growth of hepatocellular carcinoma [7]. We also have reported that RACK1 is up-regulated in activated HSCs in TGF-β1-dependent manner and promotes liver fibrosis in mice [13]. However, whether RACK1 modulates protein translation in liver fibrosis is not understood. In this study, we found that translational regulation through mTOR and MNK1/eIF4E pathways was crucial for RACK1-induced HSCs activation, and our data suggest that aberrant activation of cap-dependent translation may play a critical role in liver fibrogenesis.

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences and published by the National Institutes of Health, and also approved by the ethics committee of Fudan University. The surgery of liver perfusion was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Mouse model of liver fibrosis

The wild-type BALB/C mice were obtained from SLAC Laboratory Animal Corp (Shanghai, China) and randomly grouped. All mice were maintained at 25 °C with a 12 h dark/light cycle. Liver fibrosis was induced by intraperitoneal injection of TAA at 0.2 mg/g body weight 3 times each week for 8 weeks. For the eIF4E-specific antisense oligonucleotides (ASOs) experiment *in vivo*, intravenous ASO administration was designed and performed according to a previous report [14]. Administration of ASO started on the second day when TAA was firstly given at the dose of 20 mg/kg (either the eIF4E ASO or the scramble ASO) thrice weekly. Mice were sacrificed 8 weeks later, and samples of liver tissues were fixed in formaldehyde or immediately frozen in liquid nitrogen.

2.3. Histological analysis

In brief, left, middle and right lobes of liver were harvested, fixed with 10% buffered formalin, dehydrated in ethanol, embedded with paraffin, For HE staining, the sections were stained with hematoxylin, rinsed with water, and stained with eosin, dehydrated and mounted. For Sirius red staining, sections were stained with Sirius red for 25 min, rinsed with 100% ethanol, dehydrated and mounted. For immunohistochemical staining, sections were soaked in 0.3% hydrogen peroxide, and incubated with α-SMA primary antibody overnight at 4 °C. All slides were processed using the peroxidase-antiperoxidase method.

2.4. Antibodies and reagents

Rabbit anti-phospho-4E-BP1, -4E-BP1, -phospho-eIF4E, -eIF4E, -phospho-S6K1, -S6K1, -phospho-MNK1, -MNK1, eIF4G, snail and cyclin E1 antibodies were purchased from Cell Signaling Technology. Mouse anti-GAPDH and goat anti-Col1A1 antibodies and eIF4E specific siRNA were obtained from Santa Cruz Biotechnology. M^7GDP sepharose was from Jena Bioscience. Mouse anti-α-SMA antibody and PP242 hydrate was from Sigma Aldrich. Recombinant human TGF-β1 and PDGF-BB were obtained from R&D system.

2.5. Primary hepatic stellate cells

Primary hepatic stellate cells were isolated from mice according to a previous report [15].

2.6. Preparation of macrophage conditioned medium (MCM)

Macrophages were obtained from fibrotic liver of mice induced by TAA for 8 weeks, and cultured in RPMI1640 medium plus 10% FCS. For the preparation of MCM, macrophages were incubated in serum-free medium for 48 h. Conditioned medium was collected by centrifugation, filtered with 0.45 μm membrane filter and stored at −80 °C until use.

2.7. Western blot

Western-blot analysis was carried out according to our previous report [7].

2.8. M^7GDP affinity chromatography

The protein extracts were prepared from HSCs and incubated with M^7GDP sepharose. The beads were pelleted and washed extensively with extraction buffer. The proteins retained were eluted with extraction buffer containing m^7GDP and applied to analysis.

2.9. [^{35}S]-methionine incorporation assay

HSCs were pre-incubated with DMEM depleted of methionine for 30 min and then labeled with [^{35}S]-methionine. For rate of synthesis of individual proteins, cell extracts with equal CPM were subjected to immunoprecipitation with relevant antibodies. The immunoprecipitates were eluted and applied to SDS-PAGE, and the gel were dried and visualized with phosphor-imaging.

2.10. Polysome profiling

Polysome profiling was carried out according to our previous report [7]. Briefly, HSCs were harvested and lysed in lysis buffer in the presence of 100 μg/ml cycloheximide. The lysates were clarified, loaded onto 10%–45% sucrose gradients, and ultracentrifuged. Individual fractions were digested with proteinase K, followed by phenol/chloroform extraction, and total RNA was recovered by ethanol precipitation. Equal volumes of RNA from each fraction were used to generate cDNA, followed by real-time PCR analysis.

2.11. Cell proliferation assay

Cell proliferation was determined using Cell Counting Kit-8 (CCK-8) according to our previous report [13].

2.12. Transwell assay

Transwell assay was performed according to our previous report [13]. Briefly, the membranes with 8 μm pores were coated with type IV collagen on the upper side and with type I collagen on the lower side. HSCs were added into the upper chamber. The number of infiltrating cells was counted from five visions and the experiments were repeated three times.

2.13. Evaluation of hydroxyproline content

Liver hydroxyproline content was measured as previously reported [16].

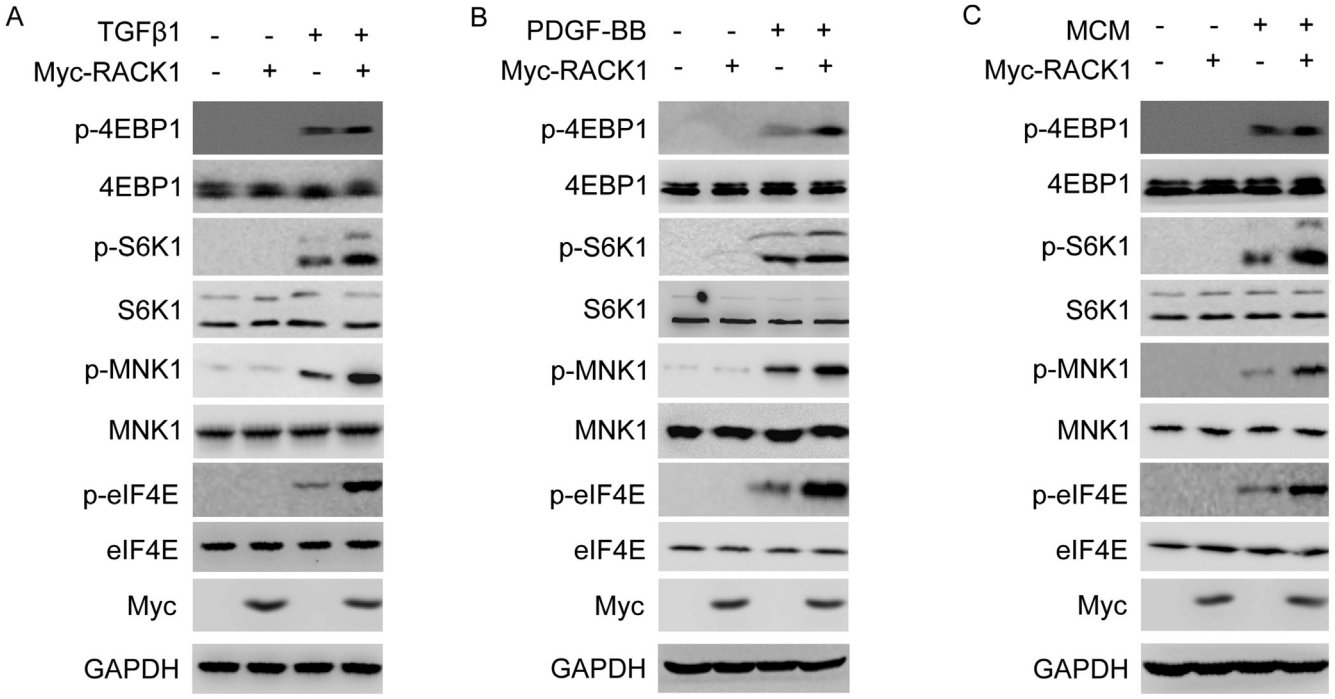
2.14. Statistical analysis

SPSS software package was used to analyze the statistics. Differences between two groups were tested using One-way ANOVA. Statistical significance was determined at the level of $P < 0.05$.

3. Results

3.1. RACK1 promotes the activation of mTOR and MNK1/eIF4E pathways in primary HSCs

RACK1 is a classical scaffold protein involved in the regulation of various signaling pathways, and our previous report demonstrated that up-regulation of RACK1 contributed to HSCs activation *in vitro* and *in vivo* [13]. To investigate the potential role of RACK1 in regulating protein translation during liver fibrogenesis, we



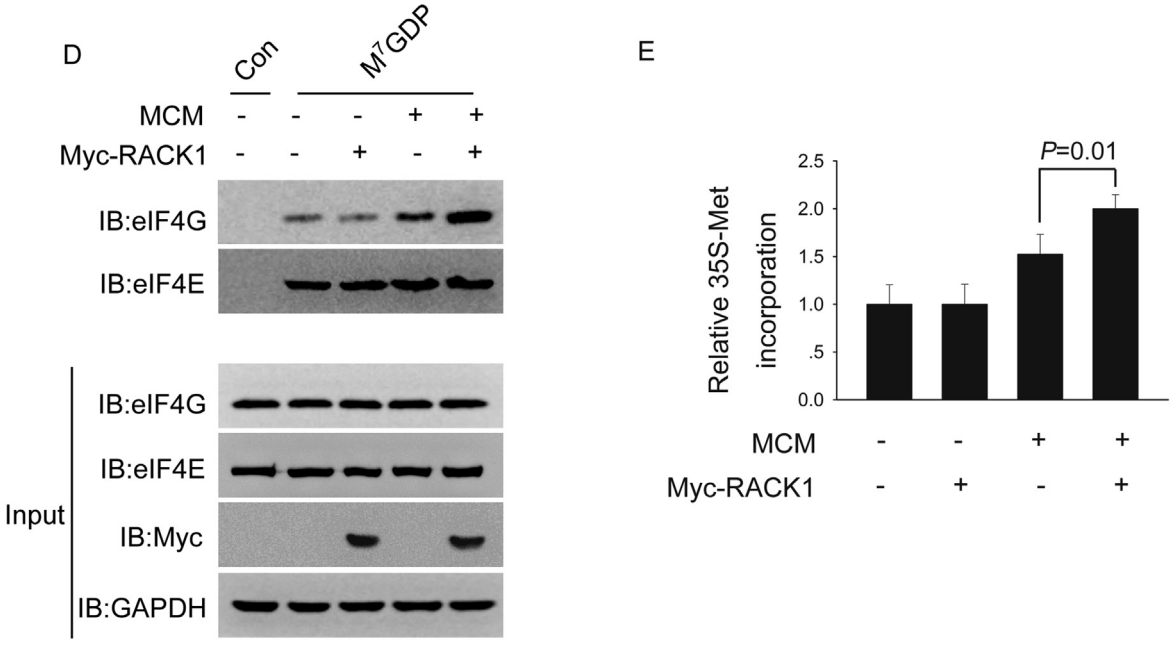


Fig. 1. RACK1 promoted the activation of mTOR and MNK1/eIF4E pathways in primary HSCs. (A, B) Primary mouse HSCs were transfected as indicated, serum-starved, and treated with TGFβ1 (10 ng/ml) or PDGF-BB (10 ng/ml) for 1 h. Total cell lysates were subjected to western blot analysis. (C) Primary mouse HSCs were transfected as indicated, serum-starved, and treated with MCM for 1 h. Total cell lysates were subjected to western blot analysis. (D, E) HSCs were transfected as indicated and treated with MCM for 24 h. Cells were subjected to M^7GDP affinity chromatography (D) or [^{35}S]-methionine incorporation assay (E).

examined the effect of RACK1 on the activation of signaling pathways that were related to translational control in primary HSCs. Treatment of TGF-β1, a potent fibrogenic factor, induced the phosphorylation of mTOR downstream target 4E-BP1 and p70S6K as well as the activation of MNK1/eIF4E signaling, and overexpression of RACK1 enhanced TGF-β1-mediated activation of mTOR and MNK1/eIF4E pathways (Fig. 1A). Similar regulatory effect of RACK1 on mTOR and MNK1/eIF4E pathways was also observed in PDGF-BB-induced signaling (Fig. 1B).

It has been known that hepatic macrophages are found in close proximity with collagen-producing myofibroblasts and indisputably play a key role in fibrosis [17]. Several studies have identified that hepatic macrophage is a critical source of TGF-β1 and PDGF in fibrosis and directly activates HSCs in a paracrine-dependent manner [17,18]. We next examined the effect of RACK1 on macrophage conditioned medium (MCM)-mediated signaling in primary HSCs [19,20]. As shown in Fig. 1C, treatment of MCM induced the activation of mTOR and MNK1/eIF4E signaling, and this effect was further enhanced by RACK1 overexpression (Fig. 1C). We also evaluated the effect of RACK1 on the formation of eIF4F complex, which is a rate-limiting step in cap-dependent protein synthesis and regulated by mTOR pathway. M^7GDP affinity chromatography assay demonstrated that administration of MCM enhanced the binding of eIF4G to eIF4E, and overexpression of RACK1 promoted MCM-induced formation of eIF4E/eIF4G complex (Fig. 1D). Metabolic labeling studies also revealed that MCM promoted [^{35}S]-methionine incorporation, and overexpression of RACK1 further enhanced *de novo* protein synthesis (Fig. 1E). These results indicate that RACK1 promotes the activation of mTOR and MNK1/eIF4E pathways in primary HSCs.

3.2. RACK1 promoted the translation and expression of pro-fibrotic factors in primary HSCs

While most cellular mRNAs require only minimal eIF4E to be efficiently translated, phosphorylated eIF4E preferentially enhances translation of select mRNAs with lengthy G + C-rich 5′-UTRs. We next evaluated the effect of RACK1 on the expression and translation of pro-fibrogenic factors in primary HSCs, including collagen 1α1, snail [21] and cyclin E1 [22]. Western blot analysis revealed that RACK1 promoted MCM-mediated up-regulation of collagen 1α1, snail and cyclin E1 in primary HSCs (Fig. 2A). Moreover, depletion of RACK1 using siRNA attenuated MCM-induced protein expression of these pro-fibrotic factors (Fig. 2B). To understand whether RACK1 modulated their protein expression at translational level, [^{35}S]-methionine incorporation assay was performed. In accordance with the alterations in protein expression, administration of MCM up-regulated the *de novo* protein level of collagen 1α1, snail and cyclin E1, and this effect was further increased by overexpression of RACK1 (Fig. 2C). Polysome profiling also showed that in MCM-treated primary HSCs, overexpression of RACK1 induced a significant right shift of collagen 1α1, snail and cyclin E1 mRNAs from light polysomes to heavier polysomes, while the distribution pattern of GAPDH mRNA in polysomes was marginally affected (Fig. 2D). Taken together, these results suggest that RACK1 regulates translation and expression of pro-fibrotic factors in primary HSCs.

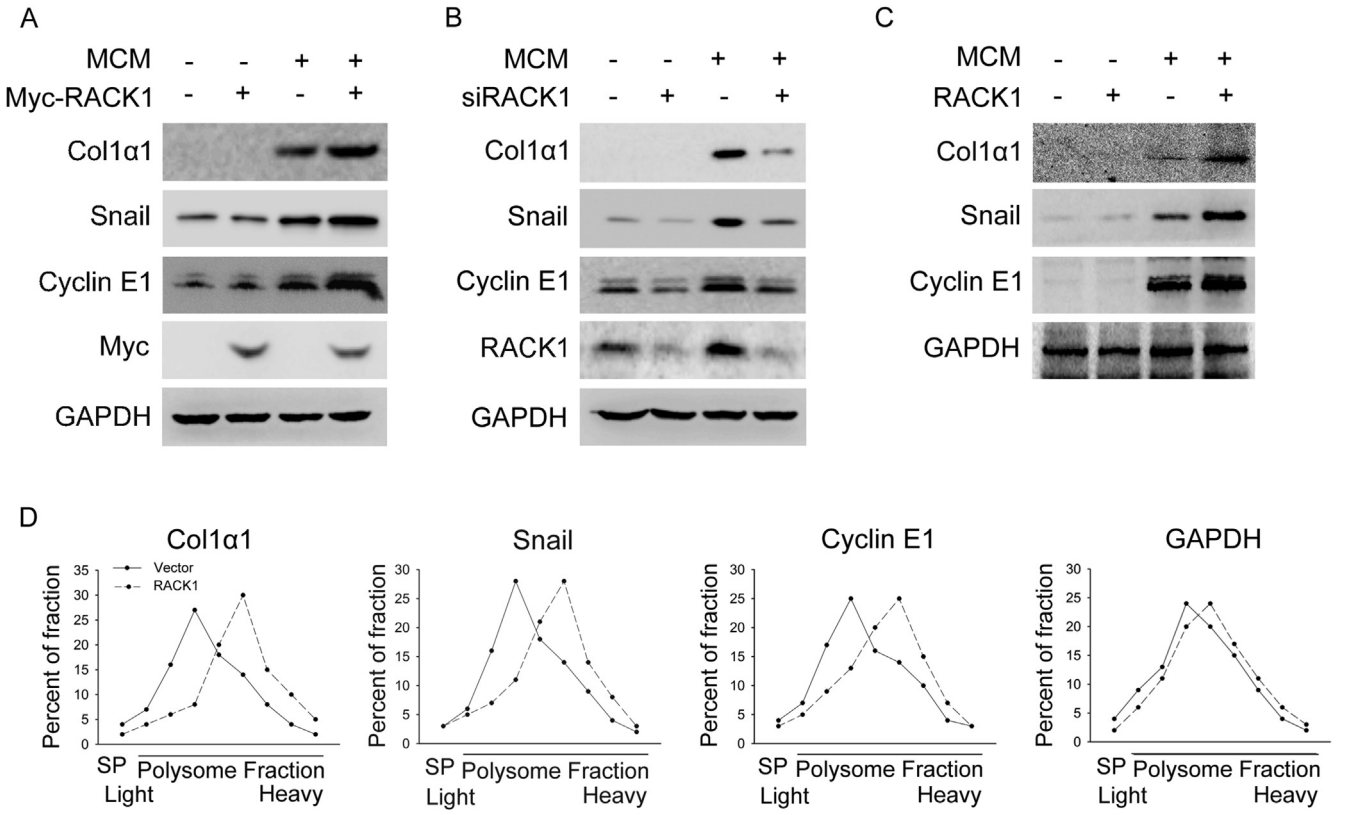


Fig. 2. RACK1 promoted the translation and expression of pro-fibrogenic factors in primary HSCs. (A) Primary mouse HSCs were transfected as indicated. 48 h later, cells were treated with MCM for another 24 h. Total cell lysates were subjected to western blot analysis. (B) Primary mouse HSCs were transfected with small interfering RNAs as indicated. 72 h later, cells were treated with MCM for another 24 h. Total cell lysates were subjected to western blot analysis. (C) Primary HSCs were treated as in (A) and labeled with [^{35}S]-methionine. Cell extracts of HSCs were subjected to immunoprecipitation. (D) Transfected HSCs were treated with MCM. Then cell lysates were applied to polysome profiling, followed by real-time PCR analysis. SP, a pool of sub-polysome fractions containing 40S, 60S and 80S ribosomes.

3.3. The fibrogenic effect of RACK1 in primary HSCs was blocked by PP242 or sieIF4E

We next examined whether RACK1 promoted the expression of pro-fibrotic factors through translational regulation. As shown in Fig. 3A, administration of PP242, a potent inhibitor of cap-dependent translation, suppressed RACK1-mediated up-regulation of collagen 1α1, snail and cyclin E1 in MCM-treated primary HSCs. Similar results were also observed in eIF4E specific siRNA-treated HSCs (Fig. 3B). These results suggest that cap-dependent translation is involved in RACK1-mediated up-regulation of pro-fibrotic factors.

Our previous report demonstrated that up-regulation of RACK1 promoted cytokine-induced proliferation and migration of primary HSCs [13]. We next assessed whether repression of cap-dependent translation attenuated the pro-fibrotic effect of RACK1. CCK8 assay revealed that overexpression of RACK1 promoted MCM-induced increase in HSCs viabilities, and treatment of PP242 or eIF4E siRNA inhibited RACK1-mediated up-regulation of cell viability in MCM-treated HSCs (Fig. 3C, D). Moreover, transwell analysis demonstrated that PP242 or sieIF4E also blocked RACK1-induced migration of MCM-treated HSCs (Fig. 3E–H). These results suggest that stimulation of cap-dependent translation is critical for the fibrogenic effect of RACK1 in primary HSCs *in vitro*.

3.4. Depletion of eIF4E attenuated liver fibrosis induced by TAA in mice

Since cap-dependent translation plays critical roles in the activation of primary HSCs *in vitro*, we next evaluated targeting cap-dependent translation by using specific eIF4E antisense oligonucleotides (ASO) possessed potential anti-fibrotic activity in TAA-induced mouse model of liver fibrosis. Real-time PCR analysis confirmed that eIF4E ASO efficiently suppressed eIF4E expression in HSCs (Fig. 4A). Western blot assay indicated that depletion of eIF4E inhibited TAA-induced expression of α-SMA and collagen 1α1 in fibrotic liver (Fig. 4B). Histological analysis revealed that eIF4E ASO treatment suppressed the formation of fibrotic septa (Fig. 4C, top panel), the accumulation of α-SMA positive cells (Fig. 4C, middle panel) and collagen deposition (Fig. 4C, bottom panel and Fig. 4D) in liver tissues of TAA-treated mice. Moreover, additional administration of eIF4E ASO significantly decreased hepatic hydroxyproline level in TAA-treated mice (Fig. 4E). These results demonstrate that depletion of eIF4E efficiently suppresses the development of hepatic fibrosis in mice.

4. Discussion

Liver fibrosis is a wound-healing response to a variety of chronic stimuli and represents a major medical problems worldwide. It is

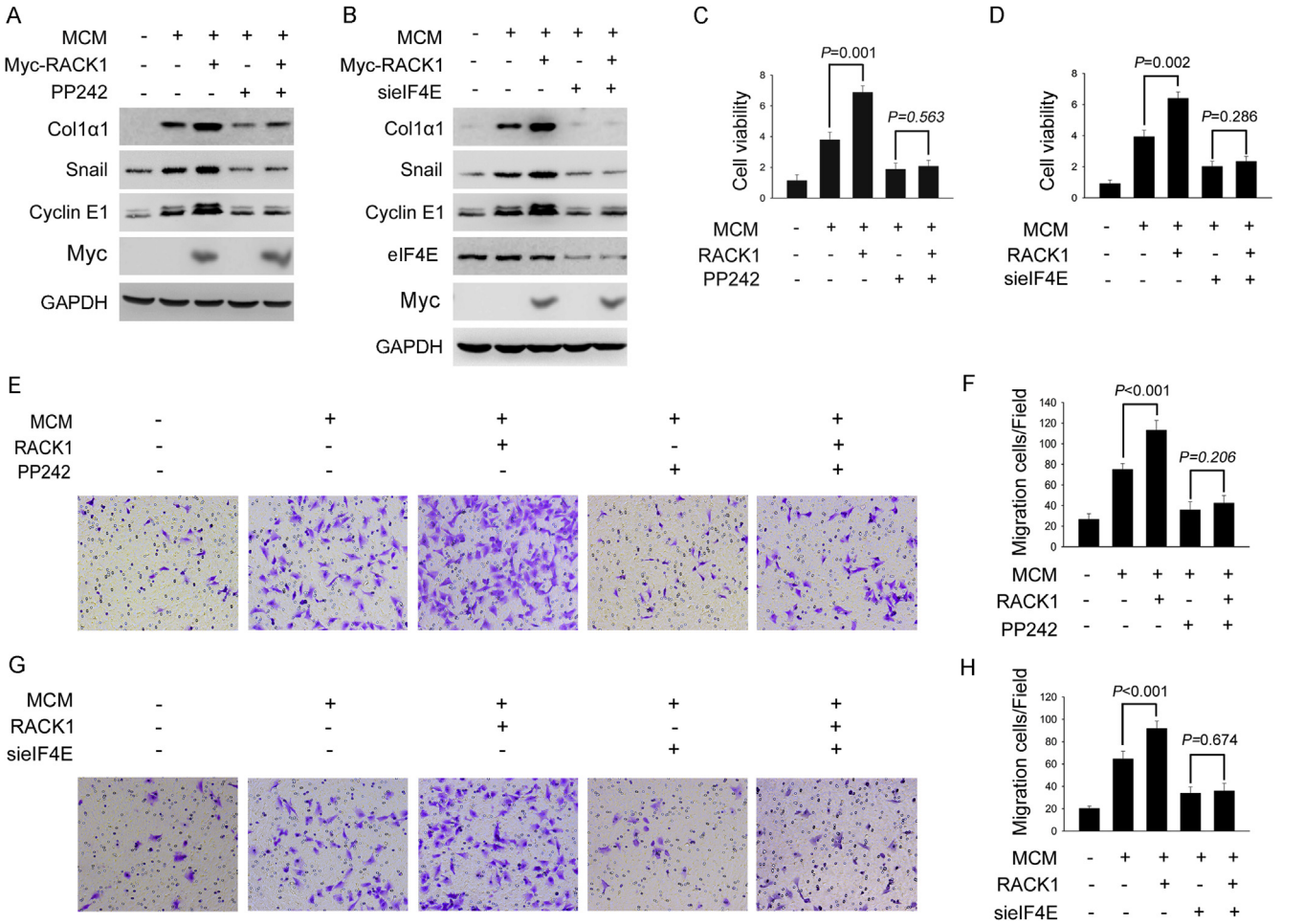


Fig. 3. The pro-fibrogenic effect of RACK1 was blocked by PP242 or small interfering eIF4E. (A) Transfected HSCs were treated with or without PP242 and/or MCM for 24 h, and total cell lysates were subjected to western blot analysis. (B) Primary HSCs were transfected as indicated. 48 h later, cells were treated with MCM for another 24 h, and total cell lysates were subjected to western blot analysis. (C, D) Primary HSCs were transfected and treated as indicated, followed by CCK-8 assay. (E–H) Primary HSCs were transfected and treated as indicated, followed by transwell assay. In (E) and (G), images are representative of three independent experiments.

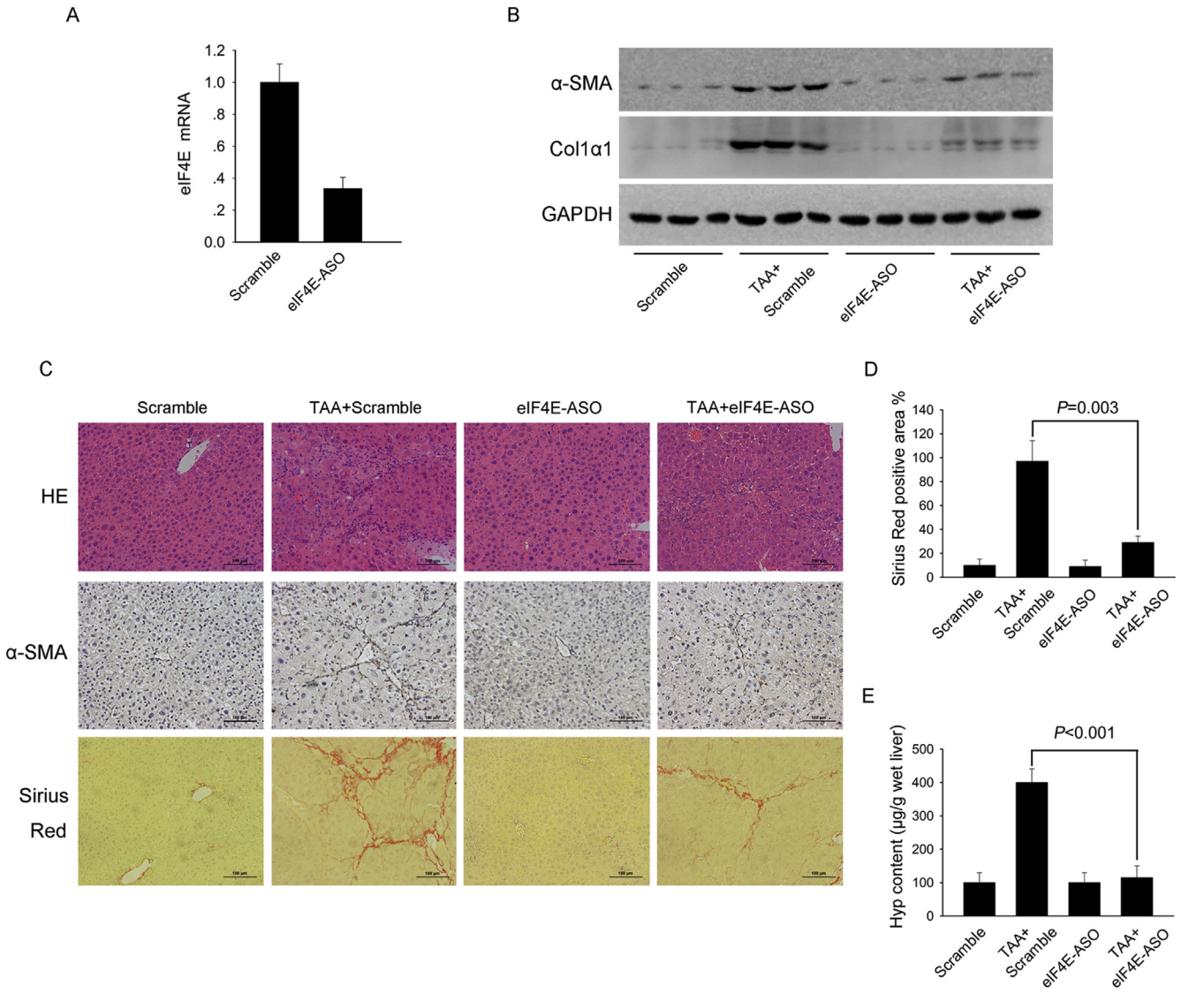


Fig. 4. Depletion of eIF4E attenuated liver fibrosis induced by TAA in mice. (A) Primary HSCs was isolated from the liver and applied to real-time PCR analysis to detect relative eIF4E expression. (B) Total protein lysates extracted from the livers were applied to western blot for α-SMA and col 1α1. (C) Liver sections were subjected to hematoxylin-eosin, α-SMA and Sirius Red staining. Scale bar, 100 μm. (D) Sirius Red staining areas were subjected to quantitative analysis using Image Pro plus software. (E) The hydroxyproline content in liver tissues of different groups were examined (n = 8). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

characterized by an excessive deposition of ECM proteins, which results in liver failure and portal hypertension and is associated with increased risk of liver cancer [23]. Though it has been recognized that derangements of translational control occurs in several kinds of diseases, the relationship between protein translation and liver fibrosis remains little defined. We herein demonstrated that RACK1-mediated translation control promoted liver fibrogenesis, and depletion of eIF4E attenuated TAA-induced liver fibrosis *in vivo*. Our data imply that aberrant activation of cap-dependent translation plays critical roles in the progression of liver fibrosis.

Activation of quiescent HSCs into a myofibroblast-like phenotype is considered as the central event of liver fibrosis. Among the key regulators of liver fibrogenesis, it is well established that hepatic macrophages play a crucial role in the development of hepatic inflammation and liver fibrosis [24]. Hepatic macrophages are found in close proximity with collagen-producing myofibroblasts and produce profibrotic mediators that directly activate fibroblasts, including TGF-β1 and PDGF [17]. In our study, we found that administration of MCM stimulated the activation of mTOR/4E-BP1 and MNK1/eIF4E pathways as well as the translation of profibrogenic factors including collagen 1α1, snail and cyclin E1 in HSCs (Figs. 1C and 2). Therefore, translational regulation may occur in HSCs in response to inflammation induced by macrophages, and up-regulation of RACK1 acts as a potent enhancer of protein synthesis in HSCs.

Emerging evidence indicates that translation regulation is organized in a highly optimized network, and perturbations to the optimal network that significantly affect translational activity therefore result in nonoptimal proteomes, fitness losses and diseases [3]. In general, translation initiation, which is the rate-limiting step in protein synthesis, is tightly regulated by eukaryotic initiation factors (eIFs), including eIF2, which controls loading of the ternary complex on the 40S subunit, and eIF4E, which regulates binding of capped mRNA to 40S subunit [4]. Elevated eIF4E activity preferentially enhances translation of select mRNAs [9], several of which encode potent fibrogenic factors, such as collagen 1α1, snail and cyclin E1. Our data demonstrated that RACK1-mediated regulation of cap-dependent translation plays a critical

role in HSCs activation, thus providing a better understanding in the molecular framework of translational regulation during liver fibrogenesis.

RACK1 serves as a binding partner for many kinases and receptors and plays a pivotal role in a wide range of biological responses [12]. It is also reported that RACK1 associates with the 40S subunit of ribosome, and is present in both ribosome- and nonribosome-bound form [25]. The role of RACK1 in modulating protein translation has been linked to its ribosomal localization. Ribosomal RACK1 is involved in the assembly of translation preinitiation complexes by interacting with eukaryotic translation initiation factor 3c (eIF3c) [26], and also modulates the phosphorylation of eIF6 and eIF4E via PKCβII [7,27]. A recent study demonstrated that ribosomal RACK1 functioned as a cellular factor required for infection by internal ribosome entry site (IRES)-containing viruses [28]. However, in our study, we found that RACK1 promoted protein translation by modulating the activation of translation-related pathways in HSCs, including mTOR and MNK1/eIF4E signaling. Our data suggest a novel role of RACK1 in modulating *de-novo* protein synthesis, and the underlying mechanism for RACK1-mediated regulation of protein translation may be multifaceted in different types of cells.

The identification of molecular mechanisms involved in the regulation of liver fibrosis is considered as a key issue for designing anti-fibrogenic strategies. Nowadays, research has delineated key mechanisms and cells that determine fibrosis progression, and mounting clinical evidences suggest that liver fibrosis can regress either by removing the cause of liver injury or treating the underlying diseases; however, anti-fibrotic treatment of fibrosis remains an unconquered area for drug development [29]. Therefore, it is of great potential in developing new agents and strategies to regress liver fibrosis. Our data suggests that administration of eIF4E-specific ASO in experimental liver fibrosis significantly suppressed fibrosis progression (Fig. 4). Since eIF4E-specific ASO has shown little side effects on body weight, organ weight or liver transaminase levels in a previous report [14], targeting cap-dependent translation through depleting eIF4E might be a safe and efficacious strategy for treating liver fibrosis in human beings.

Conflict of interest

We declare that authors have no conflict of interest.

Acknowledgments

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