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Gα₁₂ overexpression induced by miR16 dysregulation contributes to liver fibrosis by promoting autophagy in hepatic stellate cells

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Abstract

Background & aims—Hepatic stellate cells (HSCs) play a role in liver fibrosis. Guanine nucleotide-binding α-subunit12 (Gα₁₂) converges signals from G protein-coupled receptors whose ligand levels are elevated in the environment during liver fibrosis; however, information is lacking on the effect of Gα₁₂ on HSC trans-differentiation. This study investigated whether Gα₁₂ is overexpressed in HSCs and, if so, its effect on liver fibrosis and the molecular basis.

Methods—Gα₁₂ expression was assessed by immunostaining, and immunoblot analyses of mouse fibrotic liver tissues and primary HSCs. The role of Gα₁₂ for liver fibrosis was estimated using toxicant injury mouse model with Gα₁₂ gene knockout and/or HSC-specific Gα₁₂ delivery using lentiviral vectors, and primary HSCs and LX-2 cells with microRNA (miR) inhibitor, overexpression vectors, or adenoviruses. miR-16, Gα₁₂, and LC3 were examined in fibrosis patient samples.

Results—Gα₁₂ was overexpressed in activated HSCs and fibrotic liver, and was colocalized with desmin. In a CCl₄-induced fibrosis mouse model, Gα₁₂ ablation prevented increases in fibrosis and liver injury. This effect was attenuated by HSC-specific lentiviral delivery of Gα₁₂. Moreover, Gα₁₂ activation promoted autophagy accompanying JNK-dependent ATG12-5 conjugation. In

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Authors contributions:

KM Kim, CY Han, JY Kim, SS Cho, YS Kim, JH Koo, JM Lee acquired experimental data. SC Lim, KW Kang, JS Kim, SJ Hwang provided administrative, technical, or material support. KM Kim, CY Han, SH Ki, and SG Kim were involved in study concept and design. KM Kim and SG Kim drafted the manuscript. SG Kim contributed to writing of the manuscript, study supervision, and obtaining funding.

addition, we found that miR-16 was a direct inhibitor of *de novo* synthesis of $G\alpha_{12}$. Modulations of miR-16 altered autophagy in HSCs. In a fibrosis animal model or patients with severe fibrosis, miR-16 levels were lower than their corresponding controls. Consistently, cirrhotic patient liver tissues showed $G\alpha_{12}$ and LC3 up-regulation in desmin-positive areas.

Conclusions—MiR-16 dysregulation in HSCs causes $G\alpha_{12}$ overexpression, which activates HSCs by facilitating autophagy through ATG12-5 formation, implying that $G\alpha_{12}$ and the regulatory molecules may serve targets in the amelioration of liver fibrosis.

Graphical Abstract

Keywords

liver fibrosis; activated stellate cell; G protein; non-coding RNA; lysosomal degradation

Introduction

Liver fibrosis is a scarring response promoting deposition of extracellular matrix (ECM) components against hepatocyte injury [1]. ECM deposition disturbs normal liver function and architecture and can progress to liver cirrhosis when untreated. When exposed to various external stimuli during chronic injury, hepatic stellate cells (HSCs) are primarily responsible for disproportionate fiber accumulation, leading to liver fibrosis. Additionally, HSCs cause ECM deposition through trans-differentiation from a quiescent phenotype to a myofibroblastic phenotype [2]. However, there is currently no clinically effective way to inhibit HSC activation and fibrosis progression [3]; therefore, identification of the regulatory molecules and mechanisms involved in this process may assist in the implementation of new diagnostic and therapeutic targets related to liver fibrosis.

Because of their roles in complex biological processes, increased attention has been paid to G protein-coupled receptors (GPCRs) that recognize extracellular ligands, and their related downstream molecules and pathways [4]. GPCR activation and subsequent downstream activity is promoted by guanine nucleotide-binding α -subunits ($G\alpha$) proteins. Among these, $G\alpha_{12}$ members have attracted particular interest owing to their potent transforming capacity. Specifically, the $G\alpha_{12}$ transduces signals to control cell proliferation, migration, and

inflammation [5, 6]. Additionally, ligands that promote specific GPCR coupling to $G\alpha_{12}$ [i.e., sphingosine-1-phosphate (S1P), lysophosphatidic acid, angiotensin 2 (Ang II), thrombin, and endothelin-1] are implicated in liver fibrosis [7, 8]. Because $G\alpha_{12}$ converges signals from multiple GPCRs and coordinates GPCR-specific signaling cascades to common downstream molecules [9], $G\alpha_{12}$ levels may have a profound effect on the blunting or amplification of biochemical and physiological activities.

Autophagy is an essential catabolic process involved in lysosomal degradation and recycling of intracellular organelles and proteins to maintain cellular homeostasis. In HSCs, autophagy increases the fibrogenic response through the breakdown of lipid droplets [10]. Autophagy is orchestrated by events and proteins, including microtubule-associated protein 1 light chain 3 (LC3), autophagy-related gene (ATG)12-5, and ATG16, with LC3-II being necessary for membrane extension and closure to form vesicles [11]. In HSCs, the autophagic process is activated by many ligands of GPCRs that interact with $G\alpha_{12}$ [12, 13]; therefore, we hypothesized that $G\alpha_{12}$ may affect autophagy.

Given the lack of understanding on the role of $G\alpha_{12}$ in HSC trans-differentiation and the potential link between $G\alpha_{12}$ and autophagy, this study investigated whether $G\alpha_{12}$ is upregulated in HSCs and, if so, what the impact is on liver fibrosis and how it is regulated in conjunction with autophagy. Here, we showed that $G\alpha_{12}$ was overexpressed in activated HSCs due to dysregulation of a specific microRNA (miR) abundant in HSCs, which facilitated liver fibrosis. In addition, HSC-specific delivery of $G\alpha_{12}$ to whole-body $G\alpha_{12}$ knockout (KO) mice or WT mice using two different sets of constructs ascertained the role of $G\alpha_{12}$ overexpression in HSCs in the progression of fibrosis. Moreover, our findings indicated that this event was mediated by c-Jun N-terminal kinase (JNK)-dependent ATG12-5 formation and the induction of autophagy.

Materials and Methods

Materials

Information on the materials used in this study is provided in the Supporting Information.

Animal treatment

The protocol of animal experiments was approved by the Institutional Animal Use and Care Committee at Seoul National University, and followed by ARRIVE guidelines. Detailed information is provided in the Supporting Information.

Targeted gene delivery

Lentiviral SMA- $G\alpha_{12}$ and SMA- $G\alpha_{12}$ -enhanced green fluorescence protein (EGFP) were cloned, and the produced viruses were administered to C57BL/6 mice via tail vein for HSC-specific gene delivery. Specific details are provided in the Supporting Information.

Additional details regarding the materials and methods are described in the Supplementary materials and methods online (see supplementary material).

Results

Gα₁₂ overexpression in HSCs and fibrotic liver

We analyzed Gα-isoform levels in different types of liver-resident cells using the proteome data of whole liver and individual hepatic cell types [14]. Of the proteins detected in whole-liver lysates, Gα₁₂ showed the highest signal intensities specifically in HSCs (Fig. 1A, left). We compared Gα₁₂ levels in primary cells isolated from the liver of healthy mice, and found that Gα₁₂ was more abundant in HSCs than hepatocytes. In addition, hepatocytes expressed Gα₁₂ to a greater extent than Kupffer cells (Fig. 1A, right). Desmin⁺ and F4/80⁻ staining validated the purity of HSCs (Supplementary Fig. 1). To understand the link between Gα₁₂ and liver fibrosis, we examined Gα₁₂ levels in animal models. Gα₁₂ was overexpressed in liver homogenates prepared from mice treated with CCl₄ (Fig. 1B, left). Additionally, Gα₁₂ was up-regulated in HSCs isolated from the mice treated with CCl₄. This effect was not seen in the primary hepatocytes (Fig. 1B, right upper). Moreover, Gα₁₂ was enhanced and colocalized with desmin in fibrotic liver samples, confirmative of Gα₁₂ overexpression in activated HSCs (Fig. 1B, right lower). The same changes occurred in animals with bile duct ligation (Fig. 1C). Gα₁₂ levels were gradually increased in primary HSCs during culture activation along with induction of α-SMA and desmin (Fig. 1D). Our data showed that Gα₁₂ was overexpressed in fibrotic livers and activated HSCs. Analysis of the human Gene Expression Omnibus database (GSE25097) revealed that the transcript levels of GPCRs capable of interacting with Gα₁₂ members were higher in patients with liver cirrhosis as compared with those observed in healthy individuals (Supplementary Fig. 2A). Next, to examine the role of Gα₁₂ on HSC activation, we used S1P as a representative GPCR ligand to activate Gα₁₂; Gα₁₂ knockdown prevented S1P from inducing α-SMA (Supplementary Fig. 2B), supporting the idea that Gα₁₂ mediates GPCR activation of HSCs. Gα₁₂ knockdown effect was not seen in vehicle-treated cells presumably due to lack of GPCR activation (i.e., un-activated state). These results provide evidence that Gα₁₂ is overexpressed in activated HSCs, resulting in liver fibrosis.

Improvement of liver fibrosis by Gα₁₂ ablation

To assess the effect of Gα₁₂ overexpression on liver fibrosis and accompanying hepatocyte injury, we introduced a toxicant-induced fibrosis model using wild-type (WT) and Gα₁₂ KO mice. In Masson's trichrome and α-SMA-staining assays, ECM accumulation was notably attenuated in the livers of Gα₁₂ KO mice as compared with that observed in corresponding controls (Fig. 2A and 2B). This effect was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays for plasminogen activator inhibitor-1 (PAI-1) and α-SMA (Fig. 2C). Additionally, Gα₁₂ KO ameliorated histopathological changes, including hepatocyte vacuolar degeneration and inflammatory cell infiltration (Fig. 2D). Consistently, HAI-Knodell scores, ALT, AST, and LDH activities were all significantly lowered (Fig. 2E and 2F). These results provided evidence that Gα₁₂ ablation diminished toxicant-induced liver fibrosis and hepatocyte injury.

Exacerbation of liver fibrosis by lentiviral delivery of Gα₁₂ to HSCs

To ascertain the effect of Gα₁₂ overexpression in HSCs on liver fibrosis, we created a lentiviral SMA-Gα₁₂ (LV-SMA-Gα₁₂)-delivery system (i.e., the construct comprising the

gene encoding α_12 downstream from the α -SMA promoter) to accomplish HSC-specific α_12 overexpression (Fig. 3A, left). The system already had been used for HSC-specific gene delivery in our previous studies, and the specificity was previously validated [15]. As expected, HSC-specific gene delivery resulted in α_12 overexpression and its colocalization with desmin (Fig. 3A, right). Notably, α_12 overexpression in HSCs considerably attenuated the beneficial effect of α_12 KO on fibrosis, as shown by Masson's trichrome and α -SMA staining, sircol collagen assays, and qRT-PCR for collagen 1A1 in the liver (Fig. 3B), with immunoblot results for α -SMA and PAI-1 in liver homogenates verifying this outcome (Fig. 3C).

We found that WT mice showed fiber accumulation, ballooning degeneration and hepatocyte necrosis in the periportal area, piecemeal necrosis of hepatocytes, and infiltration of inflammatory cells in the liver (Fig. 3D). HSC-specific α_12 overexpression in α_12 KO mice diminished histopathological improvements, as verified by changes in HAI-Knodell score. Similar tendencies were obtained for serum ALT, AST, and LDH activities (Fig. 3E). In addition, HSC-specific α_12 overexpression in α_12 KO mice diminished the protective effect of α_12 KO on hepatic inflammation, as indicated by changes in a macrophage marker (i.e., F4/80) and portal inflammation scores (Supplementary Fig. 3). To firmly prove the effect of HSC-specific gene delivery, another set of lentiviral constructs (LV-SMA- α_12 -EGFP and LV-MOCK) were created and utilized in WT mice (Supplementary Fig. 4A). α_12 overexpression in HSCs augmented collagen accumulation in mice treated with CCl_4 for 2 weeks (Fig. 3F, upper). Histopathology was consistently changed (Fig. 3F, lower), as corroborated by HAI-Knodell scores and serum transaminase activities (Fig. 3G). Immunoblotting for α -SMA in liver homogenates displayed the same result (Supplementary Fig. 4B). Cell-specific gene delivery was validated using HSCs and hepatocytes isolated from healthy mice injected with the constructs (Fig. 3H, left). CCl_4 treatments *in vivo* unaffected the efficiency of lentivirus-mediated gene expression, as shown by a minimal change in GFP level in primary HSCs although a slight increase in α_12 intensity may reflect increase of the endogenous protein upon CCl_4 treatment (Fig. 3H, right). These results supported that HSC-specific α_12 overexpression was sufficient to promote liver fibrosis.

Induction of autophagy by α_12

Autophagy is associated with establishment of activated HSC phenotypes responsible for liver fibrosis [10, 16]. We hypothesized that α_12 overexpression might facilitate autophagy. As expected, culture activation of primary HSCs enhanced LC3B-II (Fig. 4A, left). We observed that p62 level increased along with LC3B-II after chloroquine treatment, which may reflect accumulation of the proteins under our experimental conditions. Nevertheless, the increase of p62 remains to be studied further (e.g., p62 regulation by the α_12 signaling network) [17]. In animals, α_12 ablation completely prevented CCl_4 *in vivo* treatment from increasing LC3B-II in the isolated primary HSCs (Fig. 4A, right). Consistently, α_12 overexpression *in vivo* promoted LC3B-II in the primary HSCs (Supplementary Fig. 5A). Moreover, a decrease in autophagic flux by α_12 KO was visualized by fluorescence analysis using adenoviral mCherry-green fluorescence protein (GFP)-LC3, a tool used to monitor LC3 flux (GFP fluorescence is quenched in the acidic lysosomal environment,

whereas the red fluorescence of mCherry remains evident under such conditions). We observed that the number of mCherry red puncta was diminished in $G\alpha_{12}$ KO primary HSCs as compared with that observed in controls (Fig. 4B), where yellow and red fluorescence represented autophagosomes and autolysosomes, respectively [18]. Consistently, ectopic overexpression of an active mutant of $G\alpha_{12}$ ($G\alpha_{12}QL$) increased LC3B-II in LX-2 cells (Fig. 4C, left). Additionally, exposure of cells to 3-methyladenine, a pharmacological inhibitor of autophagy, prohibited this effect (Fig. 4C, right). Therefore, these findings implied $G\alpha_{12}$ -mediated autophagy promotion. Immunoblots for α -SMA or PAI-1 showed appropriate changes in HSC activation in accordance with $G\alpha_{12}$ (Fig. 4A, right and 4C, left), confirming the link between autophagy and HSC activation.

We found that ATG12-5 conjugate levels were downregulated in $G\alpha_{12}$ KO primary HSCs (Fig. 4A, right). Consistently, either *in vitro* infection of LX-2 cells with $G\alpha_{12}QL$ or HSC-specific infection of mice with $G\alpha_{12}WT$ enhanced ATG12-5 conjugate (Fig. 4C and Supplementary Fig. 5A), whereas Beclin1, ATG4B, and ATG7 levels minimally changed (Supplementary Fig. 5B). ATG5 and ATG12 mRNA levels did not change significantly (Supplementary Fig. 5C). Immunoblot results showed slight increases in LC3B-II, ATG12-5 conjugate, or p62 in the liver homogenates from $G\alpha_{12}$ KO mice exhibiting HSC-specific $G\alpha_{12}$ overexpression as compared with levels observed in $G\alpha_{12}$ KO mice injected with LV-con (Fig. 4D). In a separate experiment, LC3B-II was also elevated by a different construct specifically overexpressing $G\alpha_{12}$ -GFP in HSCs, fortifying the link of $G\alpha_{12}$ and autophagy (Supplementary Fig. 4B). LC3B was colocalized with desmin in the liver sections (Supplementary Fig. 5D).

Previously, we proposed JNK-dependent regulatory roles for $G\alpha_{12}$ in the induction of inflammatory signaling and cancer-cell biology [5, 6, 19]. Therefore, we sought to determine the role of JNK in ATG12-5 conjugation leading to autophagy activation downstream of $G\alpha_{12}$. Overexpression of either JNK1 or JNK2 increased LC3B-II, p62, ATG12-5 and α -SMA levels in LX-2 cells, whereas JNK inhibition using a dominant-negative mutant or chemical inhibitor (SP-600125) exerted the opposite effect against $G\alpha_{12}QL$ (Fig. 4E and 4F, left), which supported the ability of JNK1/2 to regulate ATG12-5 formation required for autophagy activation. JNK inhibitor treatment repressed LC3B, p62, ATG12-5 and α -SMA in primary HSCs, corroborative of the connections among $G\alpha_{12}$, JNK, and autophagy (Fig. 4F, right). Our results demonstrated that $G\alpha_{12}$ overexpression in HSCs might facilitate JNK-dependent formation of ATG12-5 to promote autophagy induction.

HSC activation by miR-16 dysregulation

To understand the molecular basis governing $G\alpha_{12}$ overexpression in HSCs, we measured $G\alpha_{12}$ -transcript levels, and found that they were not increased in activated HSCs (Fig. 5A). Similar results were observed in animal liver samples or patient specimens (Supplementary Fig. 6A and 6B). Therefore, it is highly probable that $G\alpha_{12}$ is post-transcriptionally regulated. miRs are major regulators of post-transcriptional control of target cells [20]. To assess whether miRs mediate $G\alpha_{12}$ -transcript levels, we predicted miR candidates targeting $G\alpha_{12}$ using the TargetScan algorithm (Release 5.2) and examined probabilities with mirSVR scores (<http://mirRNA.org>). Of these candidates, miR-16 showed the one of the miRs with

highest affinity for $G\alpha_{12}$ (Fig. 5B) and was the most abundant miR observed in quiescent HSCs (Fig. 5C). Moreover, miR-16 levels were much higher in quiescent HSCs as compared with levels observed in hepatocytes (Fig. 5D). By contrast, miR-122 expression, known as an abundant miR in hepatocytes, exhibited opposite trends as those observed for miR-16 in the same samples. Intriguingly, miR-16 levels markedly diminished at days 0–3 and then gradually lowered up to days 12 (Fig. 5E). In an additional assay, miR-16 levels were comparable in hepatocytes and Kupffer cells (Supplementary Fig. 7A). We also found that the expression of other miRNAs predicted to potentially inhibit $G\alpha_{12}$ and having the same or similar seed sequence(s) was also lower in these samples (Supplementary Fig. 7B). These results implied a potential link between miR-16 repression and HSC activation.

MiR-16 as a novel inhibitor of $G\alpha_{12}$

To assess whether miR-16 directly inhibits *de novo* $G\alpha_{12}$ synthesis (i.e., protein translation), we assessed the pairing between miR-16 sequence and $G\alpha_{12}$ 3'-untranslated region (UTR), and found nearly complete complementarity. Moreover, the miR-16-recognition sites present in the 3'-UTR of $G\alpha_{12}$ mRNAs were highly conserved across different species (Fig. 6A). In LX-2 cells or primary HSCs, the ability of miR-16 to inhibit $G\alpha_{12}$ was supported by changes in $G\alpha_{12}$ levels following miR-16-level modulation using an inhibitor or mimic (Fig. 6B). miR-16 inhibitor or mimic transfection appropriately modulated luciferase expression from Luciferase- $G\alpha_{12}$ -3'-UTR construct in LX-2 or HEK293A cells (Fig. 6C and Supplementary Fig. 7C). This effect was not seen when we used a mutant construct (Fig. 6D). These results indicated that miR-16 dysregulation resulted in $G\alpha_{12}$ overexpression in activated HSCs.

MiR-16 regulation of autophagy and fibrosis

We then assessed the functional role of miR-16 in autophagy. Transfection with miR-16 inhibitor enhanced autophagy flux in LX-2 cells as shown by fluorescence assays (Fig. 7A). Consistently, miR-16 inhibitor transfection facilitated LC3B-II accumulation in LX-2 or primary HSCs (Fig. 7B). miR-16 modulation also changed α -SMA and/or PAI-1 levels. Additionally, miR-16 levels were significantly lower in liver samples from mice or patients with severe fibrosis as compared with those from control mice or patients with mild fibrosis (Ishak scores: 0–3 vs. 4–6) (Fig. 7C). Therefore, our data corroborated the regulatory effect of miR-16 on autophagy in HSCs and consequent liver fibrosis.

$G\alpha_{12}$ and LC3 up-regulation in cirrhosis patients

In an effort to reinforce our findings in a clinical situation, we examined $G\alpha_{12}$, and the markers of HSC activation and autophagy in the cirrhotic and the adjacent normal specimens from patients with cancer. $G\alpha_{12}$, desmin and LC3 were all stained in the similar regions of the liver sections, and their expression levels were higher in the cirrhotic regions (Fig. 8A), supporting the conclusion that $G\alpha_{12}$ up-regulated in HSCs in conjunction with their activation may facilitate the progression of liver fibrosis via promotion of autophagy. Taken together, our data support the conclusion that miR-16 dysregulation causes $G\alpha_{12}$ overexpression, which activates HSCs by facilitating autophagy, demonstrating the link between G protein signaling and liver fibrosis progression (Fig. 8B).

Discussion

HSC activation is mediated by extracellular ligands [21, 22]. In addition, GPCR-signaling pathways have a profound effect on cell behavior during fibrogenesis. Levels of GPCR ligands are elevated in fibrotic livers as compared with healthy controls, and most GPCRs activated by ligands are coupled with $G\alpha_{12}$ [7, 8]. Despite the known effects of $G\alpha_{12}$ on cell proliferation and migration [5], the basis for $G\alpha_{12}$ signaling in the process of liver fibrosis remained unidentified. Our results demonstrated that $G\alpha_{12}$ was overexpressed in HSCs under conditions of liver fibrosis. Proteomic data showing that $G\alpha_{12}$ was most strongly expressed in HSCs among different $G\alpha$ members support the regulatory role of $G\alpha_{12}$ in HSC trans-differentiation, as strengthened by immunostaining data from two different fibrosis animal models. Our findings implied that signals converging from different GPCRs activated by fibrogenic mediators in HSCs were amplified by increased $G\alpha_{12}$ levels. This idea was supported by observations that $G\alpha_{12}$ deficiency attenuated fibrotic severity and accompanying inflammatory responses against toxicant challenges and that these events were reversible by HSC-specific gene delivery. Furthermore, we observed that $G\alpha_{13}$, another $G\alpha_{12}$ family member, was not comparably induced in fibrosis samples and activated HSCs (Supplementary Fig. 8), suggesting the greater role for $G\alpha_{12}$ in HSC activation. We have done cDNA microarrays using the liver tissues from WT and $G\alpha_{12}$ -null mice, and found that $G\alpha_{12}$ plays a role in mitochondrial energy expenditure (TH Kim et al., manuscript under review). In another study, HSC activation was accompanied by mitochondrial biogenesis (AY Kim et al., unpublished data), supporting the concept that $G\alpha_{12}$ contributes to HSC activation in conjunction with fuel oxidation. However, $G\alpha_{12}$ KO mice were viable and fertile without apparent phenotype change [23].

Our finding that HSC-specific delivery of $G\alpha_{12}$ facilitated liver fibrosis in $G\alpha_{12}$ KO animals strengthened the predicted functional role of $G\alpha_{12}$ overexpression in HSCs on fibrosis progression. This outcome agreed with the finding that $G\alpha_{12}$ members regulate transforming growth factor beta (*Tgf- β*) gene through AP-1 [24]. Here, HSC-specific $G\alpha_{12}$ overexpression partially reversed the protective effects of $G\alpha_{12}$ KO on ALT, AST, and LDH activities. In addition, the lack of statistical significance associated with the first experiment might have been due to animal variations and/or the involvement of other types of cells deficient of $G\alpha_{12}$ (e.g., macrophages). However, our additional outcomes accomplished using HSC-specific $G\alpha_{12}$ WT-EGFP construct strengthened the effect of $G\alpha_{12}$ on HSCs and liver injury.

Damaged hepatocytes contribute to pathogenesis of liver fibrosis. In turn, activated HSCs produce TGF- β and other cytokines, facilitating hepatocyte injury, macrophage infiltration and activation, and HSC activation which accelerate the disease progression [25]. Indeed, $G\alpha_{12}$ overexpression in HSCs nullified the beneficial effect of $G\alpha_{12}$ KO on hepatic inflammation and injury. Since $G\alpha_{12}$ aggravates inflammatory signaling through COX-2 and iNOS [6, 19], $G\alpha_{12}$ in macrophages may also contribute to inflammation and thereby HSC activation, which warrants further investigation on other types of cells for comprehensive understanding of the disease. Nonetheless, the concept is supported by liver fibrosis being a steadily progressing symptom involving the complicated interplay between hepatocytes and other cell populations [26].

Autophagy provides energy required to support HSC trans-differentiation through the mobilization and breakdown of lipid droplets and mitochondrial β -oxidation [10]. Such events would allow HSCs to cope with cellular energy demands conferred by cell proliferation, repair, and the maintenance of energy homeostasis [10]. Therefore, autophagy may be a modulator of signaling pathways related to the differentiation of several cell types, including HSCs [10, 27]. Nevertheless, the underlying basis by which autophagy controls HSC activation have remained elusive. Here, we reported the ability of $G\alpha_{12}$ overexpressed in HSCs to control autophagy; this event depended on increases in ATG12-5 conjugate, being consistent with reports that some ligands known to activate GPCRs coupled to $G\alpha_{12}$ stimulate autophagy [12, 13]. The hypothesis that $G\alpha_{12}$ promotes autophagy in HSCs was reinforced by changes in LC3B-II and ATG12-5 levels in liver homogenates following HSC-specific $G\alpha_{12}$ delivery. It can be inferred that $G\alpha_{12}$ plays a role in autophagic signal amplification through increase of ATG12-5 conjugate, as supported by the observation that ATG12-5 conjugate, but not unconjugated ATG12 or ATG5, enhances LC3-II formation [28].

JNK activation upregulates α -SMA in response to TGF- β , platelet-derived growth factor, and Ang II [29]. JNK also participates in autophagy, including interaction with ATGs and induction/activation of Beclin1, ATG7, and ATG5 [30]. Previously, JNK was identified as a kinase regulated by the $G\alpha_{12}$ -signaling pathway involved in various biological events [5, 6, 19]. In our findings, the JNK pathway downstream of $G\alpha_{12}$ regulated autophagy by promoting ATG12-5 conjugation. Despite the reports on the conflicting roles of JNK isoforms in HSC proliferation and activation [29], our findings showed that JNK1 and JNK2 were both involved in ATG12-5 formation. Additionally, their activities were equally increased in the cells transfected with $G\alpha_{12}QL$ [31]. The detailed molecular basis associated with JNK regulation of ATG12-5 remains unknown. Because JNK1/2 are involved in various mechanisms related to the pathogenesis of liver injury with shared and different functions [32, 33], additional research is needed to determine the discrete roles of JNK isoforms during the initiation and progression of HSC trans-differentiation.

Another important finding of our study involved an identification of miR-16 as an upstream $G\alpha_{12}$ regulator. Our findings that miR-16 was markedly repressed during HSC activation, and that miR-16 inhibited $G\alpha_{12}$ by binding to $G\alpha_{12}$ mRNA with high affinity supported our conclusion that miR-16 dysregulation contributed to HSC activation through $G\alpha_{12}$ overexpression. This was supported by changes in autophagic biomarker and flux. The time-course data for $G\alpha_{12}$ and miR-16 in HSCs corresponded to each other, being consistent with the idea that $G\alpha_{12}$ overexpression results from miR-16 dysregulation. Moreover, we validated the relationship between miR-16 dysregulation and liver fibrosis using human specimens. miR-16, as a liver-enriched miR, regulates HSC proliferation [34], further supporting the importance of miR-16 in HSC biology.

Of note, miR-16 was much higher in quiescent HSCs than hepatocytes. Because HSC number increases with activation, whereas hepatocyte number declines [1], modulation of miR-16 in HSCs would contribute more to liver fibrosis. We also found that miR-16 levels were comparable in hepatocytes and Kupffer cells. Because $G\alpha_{12}$ was much lower in Kupffer cells than hepatocytes, we speculate that miR-16 in Kupffer cells would have a

lesser effect on fibrosis. Nonetheless, our data does not exclude the possibility that miR-16 plays a role in $G\alpha_{12}$ in other types of cells in the liver.

Targeting multiple sites of miRNA enhances the degree of target gene inhibition [35]. We observed downregulation of miR-15a, miR-15b, miR-195, miR-424, and miR-497 in activated HSCs. Because these miRs have different genomic locations and possess the same seed sequence as miR-16, they might also affect *de novo* $G\alpha_{12}$ synthesis [35]. Two sites of the same or different miRNAs located closely to each other act synergistically [36]. However, the above mentioned miRs have the same seed sequence, therefore binding the same location in the 3'UTR of $G\alpha_{12}$ mRNA. HSC trans-differentiation and the consequent changes in expression of genes encoding ECM proteins are governed by various miRNAs, including miR-29b, miR-150, and miR-194 [37, 38]. Therefore, it is highly likely that HSC activation is regulated by the pleiotropic action of multiple miRs.

We found that α -SMA level in LX-2 was comparable to that in quiescent HSCs, but was lower than activated HSCs (data not shown). LX-2 cells seem to contain less lipids than activated HSCs, but much more than quiescent HSCs [39]. $G\alpha_{12}QL$ repeatedly increased autophagy in LX-2, supporting the notion that the cells are not fully activated. In our study, the results obtained using LX-2 were almost identical to those in primary HSCs, demonstrating that $G\alpha_{12}$ overexpression by miR-16 dysregulation enhances autophagy in HSCs. This idea was reinforced by the association among $G\alpha_{12}$, desmin, and LC3 in human samples. In another study, $G\alpha_{12}$ cross-talked with integrin-linked kinase [40]. Since $G\alpha_{12}$ promotes reactive oxygen species production [41] and inflammation [6, 19], HSC activation by $G\alpha_{12}$ may rely on these factors. In our experiment, however, treatment with TGF- β , PDGF, tert-butylhydroperoxide, or tumor necrosis factor- α failed to change $G\alpha_{12}$ or miR-16 levels (data not shown). Since liver fibrosis induced drastic changes in the microenvironment of the tissue [26], we suppose that multiple fibrogenic stimuli would have direct and/or indirect effects on $G\alpha_{12}$ -dependent activation of HSCs. Collectively, miR-16 dysregulation elicits $G\alpha_{12}$ overexpression, which activates HSCs by facilitating autophagy via ATG12-5 conjugation. Thus, $G\alpha_{12}$ and the regulatory molecules may serve new targets for the prevention and treatment of liver fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

α-SMA	α -smooth muscle actin
Ang II	angiotensin 2

ATG	autophagy-related gene
CCl₄	carbon tetrachloride
ECM	extracellular matrix
EGFP	enhanced green fluorescence protein
Gα	guanine nucleotide-binding α-subunits
Gα₁₂QL	an active mutant of Gα ₁₂
GPCRs	G protein-coupled receptors
GFP	green fluorescence protein
HSCs	hepatic stellate cells
JNK	c-Jun N-terminal kinase
KO	knockout
LC3	microtubule-associated protein 1 light chain 3
miR	microRNA
PAI-1	plasminogen activator inhibitor 1
PDGF	platelet-derived growth factor
qRT-PCR	quantitative reverse transcription polymerase chain reaction
S1P	sphingosine-1-phosphate
TGF-β	transforming growth factor β
3'-UTR	3'-untranslated region
and WT	wild-type

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Lay Summary

G α_{12} is up-regulated in activated HSCs as a consequence of dysregulation of a specific microRNA abundant in HSCs, facilitating the progression of liver fibrosis. This event is mediated by JNK-dependent ATG12-5 formation and promotion of autophagy. We suggest that G α_{12} and associated regulators may serve as new targets in HSCs for treatment of liver fibrosis.

Highlights

- $G\alpha_{12}$ expression was enhanced during HSC activation due to miR-16 dysregulation.
- $G\alpha_{12}$ signaling facilitates autophagy via JNK-dependent ATG12-5 formation.
- $G\alpha_{12}$ associated molecules in HSCs may serve as targets for fibrosis treatment.

Fig. 1. Gα₁₂ is overexpressed in activated HSCs and in fibrotic liver tissue

(A) Left: heat map of Gα-subunits in the whole liver or individual hepatic cells. Data were extracted from a cell-type-resolved proteomics atlas of the mouse liver. Protein levels were normalized by their expression in whole-liver homogenates. Right: immunoblottings on the lysates of freshly isolated mouse hepatocytes, HSCs, and Kupffer cells. The difference in band intensity in hepatocytes was due to exposure time. (B) Left: immunoblotting for Gα₁₂. Mice were injected with vehicle (corn oil) or CCl₄ for 5 weeks ($n=4-6$ each). Right upper: immunoblotting on quiescent HSCs and hepatocytes freshly isolated from mice 24 h after a single dose of CCl₄. Right lower: Immunostaining (scale bar: 50 μm). White arrows indicate Gα₁₂ and desmin colocalization. (C) Left: immunoblotting for Gα₁₂ ($n=3$ each). Right: Immunostaining. (D) Immunoblottings on quiescent (freshly isolated), or culture-activated (5 or 10 days) HSCs. For B and C, values represent the mean±SEM. Statistical significance of the differences between each treatment and vehicle group ($*p<0.05$ and $**p<0.01$, significant versus respective controls) was determined by two-tailed *t*-test.

Fig. 2. Liver fibrosis is inhibited by Ga₁₂KO

(A) Upper: Masson's trichrome staining. Mice were treated as in the legend of Fig. 1B ($n = 4-6$ each). White arrows indicate periportal fibrosis (scale bar: 200 μm). Lower: immunostaining (scale bar: 200 μm) ($n=4-6$ each). (B) Relative staining area fractions using image analysis software. (C) qRT-PCR assays ($n=4-6$ each). (D) H&E staining. White arrows indicate vacuolar degeneration of hepatocytes, whereas red arrows show inflammatory cell infiltration in the periportal area (scale bar: 200 μm) ($n=4-6$ each). (E) HAI-Knodell scores ($n=4-6$ each). (F) Serum enzyme activities ($n=4-6$ each). For B, C, E, and F, values represent the mean±SEM. Statistical significance of the differences was determined by two-tailed t -test (* $p < 0.05$ and ** $p < 0.01$, significant versus vehicle-treated WT mice; and # $p < 0.05$ and ## $p < 0.01$, significant versus CCl₄-treated WT mice).

Fig. 3. HSC-specific lentiviral delivery of α_1 exacerbates liver fibrosis

(A) Schematic showing a construct encoding WT- α_1 (LV-SMA- α_1) downstream from the mouse α -sma promoter (SMP8). Mice were injected with each construct (or saline) through tail vein. After 1 week, they were subjected to CCl₄ treatment for 5 weeks ($n=3-9$ each). Immunostaining for α_1 and desmin confirmed colocalization (magnification: 40 $\times$). (B) Upper: Masson's trichrome staining. Symbols represent changes as in Fig. 2A, upper. Liver tissues of saline-alone group were stained separately (scale bar: 200 μ m) ($n=3-9$ each). Lower: immunostaining (scale bar: 200 μ m). Sircol assay and qRT-PCR were done on the liver samples ($n=3-9$ each). (C) Immunoblots and scanning densitometry. (D) H&E staining. Black arrows indicate ballooning degeneration of hepatocytes, whereas white ones show

infiltration of inflammatory cells and piecemeal necrosis of hepatocytes (scale bar: 200 μ m) ($n=3-9$ each). (E) HAI-Knodell scores and serum enzyme activities ($n=3-9$ each). (F) Upper: Masson's trichrome staining. Mice were injected with LV-SMA- $G\alpha_{12}$ -GFP or MOCK through tail vein. After 1 week, they were subjected to CCl_4 treatments for 2 weeks. Symbols represent changes as in Fig. 2A, upper (scale bar: 100 μ m) ($n=5$ each). Lower: H&E staining. Red arrows indicate eosinophilic degeneration, white ones show infiltration of inflammatory cells, and yellow ones present vacuolar degeneration (scale bar: 100 μ m) ($n=5$ each). (G) Relative staining area fractions, HAI-Knodell scores and serum enzyme activities ($n=5$ each). (H) Immunoblots on primary hepatocytes and HSCs. At 1 week after an injection of construct, hepatocytes and HSCs were freshly isolated from WT (left) or CCl_4 -treated mice as in Fig. 1B right upper (right). For B, C and E, values represent the mean $\pm$ SEM. Statistical significance of the differences was determined by two-tailed t -test (* $p<0.05$, ** $p<0.01$, significant versus saline-treated WT mice; # $p<0.05$ and ## $p<0.01$, significant versus CCl_4 - treated WT mice injected with LV-con; and \$ $p<0.05$, \$\$ $p<0.01$, significant versus CCl_4 - treated $G\alpha_{12}$ KO mice injected with LV-con, N.S., not significant). For G, values represent the mean $\pm$ SEM (** $p<0.01$, significant versus vehicle-treated mice injected with LV-MOCK; and ## $p<0.01$, significant versus CCl_4 -treated WT mice injected with LV-MOCK).

Fig. 4. $G\alpha_{12}$ activates autophagy in HSCs via ATG12-5 formation

(A) Left: immunoblots. Right: immunoblots on primary HSCs isolated from WT or $G\alpha_{12}$ KO mice 48 h after a single dose of CCl₄ treatment. HSCs were cultured in a growth medium for 5 days. (B) Fluorescent images for mCherry/GFP-LC3B puncta. Primary HSCs isolated from WT or $G\alpha_{12}$ KO mice treated with CCl₄, as in panel A, were cultured for 3 days, and then infected with ad-mCherry- GFP-LC3 for 24 h (magnification: 60 $\times$). (C) Left: immunoblots. LC3B-II levels were assessed by scanning densitometry ($n=3$ each). Right upper: immunoblots. Right lower: immunoblots on LX-2 cells treated with 5 μ M 3-MA (or vehicle) and CQ for 12 h following infection with ad- $G\alpha_{12}$ QL (24 h). (D) Immunoblots on liver homogenates prepared as in Fig. 3A (n.s., non-specific band). (E) Immunoblots. LC3B-II and ATG12-5 levels were assessed by scanning densitometry ($n=3$ each). (F) Left: immunoblots on LX-2 cells transfected with $G\alpha_{12}$ QL in combination with DNJNK (or MOCK) for 48 h or those treated with vehicle or JNKi (10 μ M) for 12 h following $G\alpha_{12}$ QL infection for 24 h. Right: immunoblots on primary HSCs treated with vehicle or JNKi. Mouse HSCs were cultured in a growth medium for 7 days. For immunoblottings for LC3B and p62, chloroquine (CQ, 10 μ M) was additionally treated for 12 h after transfection, or after infection plus 4 h serum starvation (For primary HSCs, CQ was additionally treated for 12 h at the indicated days, and cells were also exposed to CQ 4 h prior to JNKi treatment). For C, E, and F, values represent the mean $\pm$ SEM. Statistical significance of the differences was determined by two-tailed t -test (* $p<0.05$, ** $p<0.01$, significant compared with respective controls).

Fig. 5. miR-16 is dysregulated during HSC activation

(A) qRT-PCR assay ($n=3$ each). (B) miRs putatively targeting $G\alpha_{12}$. The candidate miRs were ranked by the sum of mirSVR scores obtained using microRNA algorithm. (C–E) qRT-PCR assays ($n=3$ –4 each). For A and E, values represent the mean $\pm$ SEM. Statistical significance of the differences was determined by two-tailed t -test (* $p<0.05$, ** $p<0.01$, significant compared with quiescent primary HSCs, N.S., not significant).

Fig. 6. miR-16 directly inhibits $G\alpha_{12}$.

(A) Alignments of miR-16 binding to the 3'-UTRs of $G\alpha_{12}$ mRNAs. (B) Upper & Lower: immunoblots for $G\alpha_{12}$. Middle: qRT-PCR for miR-16 ($n=3$ each). (C) 3'-UTR reporter assays ($n=3$ each). (D) hsa-miR-16 sequence and its target sequences in the $G\alpha_{12}$ 3'-UTR, or $G\alpha_{12}$ 3'-UTR mutant (changed bases are underlined), and reporter assays ($n=3$ each). For B–D, values represent the mean $\pm$ SEM. Statistical significance of the differences was determined by two-tailed t -test (* $p<0.05$ and ** $p<0.01$, significant as compared with respective controls, N.S., not significant).

Fig. 7. Dysregulation of miR-16 promotes autophagy in HSCs

(A) Fluorescent images for mCherry/GFP-LC3B puncta (magnification: 60×). (B) Left, Left middle: immunoblots and scanning densitometry ($n=3$ each). Right middle, Right: immunoblots on transfected quiescent HSCs. For immunoblotting for LC3B and p62, the cells were additionally treated with 10 μ M chloroquine (CQ) for 12 h after transfection. (C) qRT-PCR assays in liver samples prepared as in Fig. 1B (left) ($n=3-4$ each) or in the liver sections of patients with mild fibrosis or severe fibrosis (Ishak scores: 0-3 vs. 4-6; right) ($n=4-5$ each). For B left and C left, values represent the mean $\pm$ SEM. Statistical significance of the differences was determined by two-tailed t -test (* $p<0.05$, ** $p<0.01$, significant compared with respective controls). For C right, means are indicated.

(A)

(B)

Fig. 8. Immunostaining for Gα₁₂, desmin and LC3 in cirrhotic and the adjacent normal specimens from cancer patients

(A) Immunostaining (scale bar: 50 μm). White arrows indicate colocalization of Gα₁₂, desmin, and LC3. The result was confirmed using another set of human samples. (B) A schematic diagram.