Identification of GDF15 as A Pro-Fibrotic Factor in Mouse Liver Fibrosis Progression

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Research Article

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Abstract

Aim:

To elucidate the inhibitory role of growth differentiation factor 15 (GDF15) in liver fibrosis and its possible activation mechanism in hepatic stellate cells of mice.

Methods:

We generated a GDF15-neutralizing antibody that can inhibit TGF-β1-induced activation of the TGF-β/Smad2/3 pathway in LX-2 cells. All the mice in this study were induced by carbon tetrachloride and thioacetamide. In addition, primary hepatic stellate cells from mice were isolated from fresh livers using Nycodenz density gradient separation. The severity and extent of liver fibrosis in mice were evaluated by Sirius Red and Masson staining. The effect of GDF15 on the activation of the TGF-β pathway was detected using dual-luciferase reporter assays and Western blotting assays.

Results:

The expression of GDF15 in cirrhotic liver tissue was higher than that in normal liver tissue. Blocking GDF15 with a neutralizing antibody resulted in a delay in primary hepatic stellate cell activation and remission of liver fibrosis induced by carbon tetrachloride or thioacetamide. Meanwhile, TGF-β pathway activation was partly inhibited by a GDF15-neutralizing antibody in primary hepatic stellate cells. These results indicated that GDF15 plays an important role in regulating HSC activation and liver fibrosis progression.

Conclusions:

The inhibition of GDF15 attenuates chemical-inducible liver fibrosis and delays hepatic stellate cell activation, and this effect is probably mainly attributed to its regulatory role in TGF-β signalling.

Introduction

Liver fibrosis is a common chronic liver disease that is characterized by the accumulation of collagen and activation of hepatic stellate cells (HSCs) in the liver [1]. Liver fibrosis is a common precursor of liver cirrhosis and even hepatocellular carcinoma [1]. In addition, liver cirrhosis is irreversible and can only be effectively cured by liver implantation [2]. Therefore, preventing the progression from liver fibrosis to liver cirrhosis is highly beneficial to patients with chronic liver disease.

TGF-β signalling plays a key role in the progression of liver fibrosis and activation of HSCs [3], which may theoretically result in the blockage of liver fibrosis progression [3]. Nevertheless, the direct blockade of TGF-β signalling has been shown to cause many unacceptable adverse effects in animal trials [4]; thus, it is important to find new targets that can regulate TGF-β signalling as well as liver fibrosis progression.

Growth differentiation factor 15 (GDF15) is also known as macrophage inhibitory cytokine-1 (MIC-1), which plays an important role in regulating TGF-β signalling[5]. GDF15 is also known as a distant member of the TGF-β superfamily. It has been reported that GDF15 is closely related to lung fibrosis progression[6]. Moreover, GDF15 can ameliorate renal fibrosis by inhibiting fibroblast growth and activation[6]. The results above suggested that GDF15 could potentially regulate liver fibrosis due to similar mechanisms in the pathogenesis of pulmonary fibrosis and renal fibrosis.

We conducted this study and found that blocking GDF15 attenuated liver fibrosis in mice. In addition, we confirmed the effect of GDF15 on TGF-β signalling in mouse liver tissues and primary HSCs.

Materials And Methods

Clinical samples.

All human normal liver tissues and cirrhotic liver tissues were provided by the Department of Liver Surgery and Liver Transplantation at Shandong Provincial Hospital. The tissues were obtained with informed consent, and the study was approved by the Ethical Committee of Shandong Provincial Hospital.

Cell culture.

LX-2 cells were purchased from Millipore Corporation. These cells were cultured in Dulbecco's-modified Eagle medium (DMEM) supplemented with 10% foetal calf serum and 1% antibiotics at 37°C in a Thermo incubator containing 5% carbon dioxide.

Experimental animals.

All mice were purchased from Shandong University Laboratory Animal Centre and were housed and fed under specific pathogen-free (SPF) conditions. Among them, female BALB/c mice were prepared for neutralizing antibodies, and C57BL/6J mice were used for carbon tetrachloride and thioacetamide - induced liver fibrosis. We ensured that all animals in our study received humane care, and all study protocols complied with the institution's guidelines. The animal experiments were approved by the Ethical Committee of Shandong Provincial Hospital and the approve No. was NSFC:No.2019-033.

Preparation of the carbon tetrachloride- and TAA-induced liver fibrosis model.

Eight C57BL/6J mice were injected with carbon tetrachloride (CCl_4, 0.5 μL per gram body weight) in 25% olive oil twice per week at equal intervals for eight weeks. Another group of C57BL/6J mice (including eight mice) was injected with thioacetamide (TAA, 0.2 mg/g body weight) in double-distilled water three times per week at equal intervals for eight weeks. All chemical-inducible liver fibrosis mice were divided into two groups. The GDF15 mAb group mice (including eight mice) were treated with GDF15-neutralizing antibodies at a dose of 20 mg/kg twice a week by abdominal injection, and the control group mice (including eight mice) were injected with homologous IgG for six weeks.

Isolation of primary hepatic stellate cells from mice.

C57BL/6J mice were sacrificed using cervical dislocation method, and their livers were removed from the abdominal cavities. The fresh livers were perfused with a digestive solution consisting of 0.1% collagenase, 0.25% pronase E and 0.01% DNase and mechanically dissected. The digested livers were incubated in the same digestive solution at 37°C for approximately 25 to 30 minutes. Next, the suspension was removed and filtered through an iron mesh with 100-μm pores. Then, the filtered suspension was centrifuged through a Nycodenz gradient (Axis-Shield) at 8.2% concentration. The isolated primary HSCs were resuspended in DMEM supplemented with 20% foetal calf serum and 1% antibiotics (penicillin and streptomycin) and cultured at 37°C overnight in an environment containing 5% carbon dioxide. Twenty-four hours later, cell debris and non-adherent cells were removed.

Generation of GDF15-neutralizing antibodies.

GDF15-neutralizing antibody generation was performed using conventional hybridoma techniques. His-tag fusion constructs of GDF15 (which encoded Mouse GDF15 amino acids 10-303) were cloned into PET28 vectors and expressed according to previously established protocols. The purified His-tag fusion protein was used as an antigen to immunize female BALB/c mice for the generation of anti-GDF15 monoclonal antibodies. Afterwards, we identified one monoclonal antibody that effectively blocked TGF-β1-induced activation of the TGF-β/Smad2/3 signalling pathway and used this mAb as the primary GDF15-neutralizing antibody for all subsequent animal experiments.

Immunohistochemical and Sirius Red staining.

The paraffin-embedded liver tissues from mice and humans were sliced into 5-μm-thick sections followed by deparaffinization and stepwise rehydration in preparation for IHC and Sirius Red staining. For Sirius Red staining, the sections were stained with Sirius Red for approximately three minutes and dehydrated stepwise. For immunohistochemical staining, the sections were incubated with 0.3% hydrogen peroxide for thirty minutes and blocked with 10% bovine serum albumin. The sections were then incubated using an antibody targeting GDF15 (1:200, Abcam, ab189358) at 4ºC overnight and labelled with an HRP-conjugated secondary mouse antibody (Abcam) at room temperature for approximately one hour. Afterwards, the sections were incubated with a DAB substrate liquid (Thermo Fisher) and stained with haematoxylin for two minutes. All the sealed slides were imaged and recorded on a microscope manufactured by Carl Zeiss.

Masson staining.

The slides were deparaffinized and rehydrated stepwise before Masson staining. The slides were first stained with Weigert haematoxylin for 5 minutes and washed with water. The slides were then stained with acid ponceau for six minutes followed by quick immersion in 2% glacial acetic acid. Afterwards, the slides were immersed in 1% phosphomolybdic acid for approximately three minutes prior to immediate

staining with aniline blue for five minutes. The slides were subsequently immersed in 0.2% glacial acetic acid briefly and dehydrated stepwise until they were sealed with neutral balsam.

Immunofluorescence staining.

For cell staining, primary mouse HSCs were seeded onto rounded coverslips in 24-well plates and incubated at 37°C for approximately seven days in an environment containing 5%CO_2. For F-actin staining, cells were incubated with phalloidin-FITC (Sigma) for seventy minutes at room temperature. For α-SMA staining, cells were incubated with α-SMA antibody (Sigma) for seventy-five minutes at room temperature and subsequently treated with Alexa Fluor 594-conjugated secondary antibody, avoiding light. The nuclei were stained using DAPI (Sigma), and the immunofluorescence images were recorded using a fluorescence microscope (Carl Zeiss).

Western blotting.

Cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X 100, 1 mM $MgCl_2$, 1 mM PMSF) and boiled for five minutes. The proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane that was blocked using 1% bovine serum albumin in TBS. The NC membrane was incubated with antibodies targeting GDF15 (Abcam), Smad2 (Cell Signaling Technology, CST), phospho-Smad2 (CST), Smad3 (CST), phospho-Smad3 (CST), Smad4 (CST), and GAPDH (Abcam), followed by the addition of fluorescence-conjugated secondary antibodies. All the fluorescence signals were captured on an Odyssey imaging system (LI-COR).

Dual-luciferase reporter assay

We used the pGL4.19-TA-TGFBeta(V2) reporter vector (Promega) as a dual-luciferase reporter vector for TGF-beta signalling. pGL4.19-TA-TGFBeta was transfected into LX-2 cells and treated with GDF15, Ly364947, or TGF-β1. A Thermo Scientific Microplate Reader was used to detect the absorbance value of each group. Ly364947 is an inhibitor of the TGF-β signalling pathway.

Statistical analysis.

All the data are presented as the means ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 16.0 software. One-way ANOVA method was used for multigroup comparison. The comparisons of absorbance value between different groups were carried out by using ANOVA method. Two-tailed Student's t-test was used for comparisons between two groups. A P value less than 0.05 was considered statistically significant.

Results

GDF15 was upregulated in cirrhotic liver tissues and activated HSCs

We first detected the expression of GDF15 in human liver tissues and found that the expression of GDF15 in cirrhotic liver tissue was significantly higher than that in normal liver (Fig 1a and 1b, 1c). GDF15 expression was mainly localized in cirrhotic lesions in liver tissue, which indicated a correlation between GDF15 and liver cirrhosis (Fig 1d). The cell immunofluorescence staining assay showed that the expression of GDF15 in HSCs coincided with α-SMA (Fig 1e). Immunofluorescence staining revealed that the localization of GDF15 expression was nearly coincident with α-SMA, which reflected the activation of HSCs (Fig 1f). Moreover, Western blotting assays showed that GDF15 expression mainly originated from activated HSCs (Fig 1g).

GDF15 mAb attenuated liver fibrosis in mice.

Sirius Red and Masson staining are considered standard methods for evaluating liver fibrosis, and the two methods can accurately show collagen accumulation in fibrotic liver tissues. Sirius Red staining revealed that GDF15 mAb-treated fibrotic mice had attenuated liver fibrosis compared with IgG-treated fibrotic mice regardless of the chemical induction conditions (i.e., CCl_4 vs. TAA) [Fig 2a (1,3 CCl_4 induced; 2,4 TAA induced), Fig 2b, CCl_4 induced]. Masson staining showed that the collagen accumulation area in IgG-treated mice was significantly larger than that in the GDF15 mAb-treated group (Fig 2c,2d).

GDF15 mAb inhibited the activation of mouse HSCs

α-SMA expression is closely correlated with HSC activation and is considered an HSC activation marker. We successfully isolated primary HSCs from mouse livers and subjected the cells to aberrant treatment for five to seven days. Primary HSCs were fixed at five days and stained for immunofluorescence imaging. The results showed that the cell morphology of HSCs treated with GDF15 mAb was different from that of HSCs treated with control IgG. The GDF15 mAb-treated HSCs showed a lower activation state compared with cells in the IgG group (Fig 2e). The α-SMA expression levels in IgG-treated HSCs were significantly higher than those in the GDF15 mAb-treated group (Fig 2e).

GDF15 modulated HSC activation and liver fibrosis by regulating TGF-beta signalling

Compared with IgG-treated primary mouse hepatic stellate cells, the hepatic stellate cells treated with GDF15 mAb showed lower phospho-Smad2 and phospho-Smad3 levels, which reflected the activation of TGF-β signalling (Fig 3a). In addition, chemical-inducible mouse liver tissues from the GDF15 mAb group showed lower phospho-Smad2 and phospho-Smad3 levels than those from the IgG group (Fig 3b). Moreover, a luciferase reporter assay showed that TGF-β signalling in LX-2 cells can be activated by the GDF15 protein, and this activation effect can be blocked by the TGF-β pathway inhibitor Ly364947 (Fig 4).

Discussion

Liver fibrosis occurs during the early stages of liver cirrhosis and is mainly characterized by intrahepatic structural rearrangement, hepatic cell regeneration and excess accumulation of collagen [7-10]. Liver

fibrosis is a reversible process until liver cirrhosis develops. Progressive liver fibrosis causes increased morbidity and mortality in patients with chronic liver disease [11, 12]. The therapeutic methods for liver fibrosis are limited, with minimal effectiveness to date [13].

Hepatic stellate cells (HSCs), also known as fat-storing cells, vitamin A-storing cells [14] or Ito cells, play a central role in the progression of liver fibrosis [15, 16]. HSCs are a major source of extracellular matrix proteins during the liver fibrosis process [17, 18]. HSCs are intralobular connective tissue cells that present either myofibroblast-like[19] or lipocyte[20] phenotypes when converted from a quiescent state to an activated state[21]. All of the profibrotic factors that target HSCs affect the progression of liver fibrosis [22-24]. Activated HSCs can lead to excess secretion of extracellular matrix, which participates in intrahepatic structural rearrangement [25] and contributes to the increase in hepatic sinusoid pressure via self-contraction [26-28]. The activation of HSCs is regulated by many cytokines, such as tumour necrosis factor-α (TNF-α) [29], transforming growth factor-beta (TGF-beta) [30], insulin-like growth factor-1 (IGF-1) [31], hepatic growth factor (HGF) [32], platelet-derived growth factor (PDGF) [33], and endothelin-1 (ET-1) [34]. Among these cytokines, the TGF-beta[35] and related cell signalling pathways play central roles in regulating HSC activation[36]. TGF-beta and related cell signals can accelerate HSC activation[37] and can enhance HSC proliferation[38], migration[39], contraction[40] and the secretion of ECM proteins[41]. Therefore, TGF-beta and other related cell signals are ideal therapeutic targets in treating liver fibrosis [42]. However, directly blocking TGF-beta signalling causes severe adverse effects in animal models and cannot be instituted in clinical trials[42].

GDF15 was first isolated from macrophages after cytokine stimulation[43]. It has been reported that GDF15 expression can be regulated by several signalling pathways, including the p53/DEC1, GSK-3β, and EGR-1 pathways[44]. Moreover, it was reported that GDF15 plays important roles in regulating renal fibrosis and lung fibrosis[6]. However, the role of GDF15 in liver fibrosis progression remains unclear. Therefore, elucidation of the biological functions of increased GDF15 in liver disease pathogenesis may promote the potential application of GDF15 in diagnosis and targeted therapy. Similar to other TGF-β superfamily cytokines, GDF15 is synthesized as a precursor protein. N-terminal propeptides originating from mature GDF15 through proteolysis can be secreted as disulphide-linked homodimers[45]. The membrane receptors of GDF15 have not been identified. Moreover, it has been reported that GDF15 activates smad2 and smad3 through TGF-β I type receptors and promotes colorectal cancer metastasis[46].

In our study, we discovered evidence that illustrates the pro-fibrotic effect of GDF15 in liver fibrogenesis. The expression levels of GDF15 in cirrhotic liver tissue and activated HSCs were higher than those in normal liver tissue and quiescent HSCs. The differential expression of GDF15 in cirrhotic liver tissue and normal liver tissue suggested its potential role in liver fibrosis regulation.

Blocking GDF15 with a neutralizing antibody markedly delayed HSC activation and attenuated liver fibrosis in the chemical-inducible liver fibrosis mouse model. These results revealed the important role of GDF15 in promoting liver fibrosis progression. The activation of primary mouse HSCs treated with the

GDF15-neutralizing antibody was slower than that of the IgG-treated control group. Therefore, GDF15 mAb may inhibit liver fibrosis progression by regulating HSC activation. The activation of TGF-beta signalling in primary HSCs treated with GDF15-neutralizing antibody was inhibited compared with the control group. These results suggested that GDF15 may play an important role in regulating TGF-beta signalling. In addition, we also found that GDF15 regulated liver fibrosis in vivo via TGF-beta signalling. Activation of TGF-beta signalling has already been shown to play a central role in regulating the progression of tissue fibrosis. According to previous reports and our study results, the positive regulation of TGF-beta signalling by GDF15 can be recognized as the main mechanism explaining the progressive effect of GDF15 in liver fibrosis.

In summary, our research supports the role of GDF15 as a therapeutic target for attenuating liver fibrosis. In our study, no severe adverse effects caused by GDF15 inhibition were observed in either liver fibrosis mouse model. We confirmed the positive effects of GDF15 on HSC activation and liver fibrosis. According to our study, GDF15 can be further explored as an anti-fibrosis agent.

Declarations

Acknowledgements

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Disclosure of conflict of interest: None.

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References

1. Abu-Elsaad NM, Elkashef WF. Modified citrus pectin stops progression of liver fibrosis by inhibiting galectin-3 and inducing apoptosis of stellate cells. Can J Physiol Pharmacol. 2016;94:554–62.
2. Adams D, Larman B, Oxburgh L. Developmental expression of mouse Follistatin-like 1 (Fstl1): Dynamic regulation during organogenesis of the kidney and lung. Gene Expr Patterns. 2007;7:491–500.
3. Affo S, Rodrigo-Torres D, Blaya D, et al. Chemokine Receptor Ccr6 Deficiency Alters Hepatic Inflammatory Cell Recruitment and Promotes Liver Inflammation and Fibrosis. PLoS One. 2015;10:e0145147.
4. Ahn J, Son MK, Jung KH, et al. Aminoacyl-tRNA synthetase interacting multi-functional protein 1 attenuates liver fibrosis by inhibiting TGFbeta signaling. Int J Oncol. 2016;48:747–55.
5. Lerner L, Gyuris J, Nicoletti R, Gifford J, Krieger B, Jatoi A. Growth differentiating factor-15 (GDF-15): a potential biomarker and therapeutic target for cancer-associated weight loss. Oncol Lett. 2016;12:4219–23.

6. Kahli A, Guenancia C, Zeller M, et al. Growth differentiation factor-15 (GDF-15) levels are associated with cardiac and renal injury in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass. PLoS One. 2014;9:e105759.
7. Brener S. Transient Elastography for Assessment of Liver Fibrosis and Steatosis: An Evidence-Based Analysis. Ont Health Technol Assess Ser. 2015;15:1–45.
8. Chaly Y, Marinov AD, Oxburgh L, Bushnell DS, Hirsch R. FSTL1 promotes arthritis in mice by enhancing inflammatory cytokine/chemokine expression. Arthritis Rheum. 2012;64:1082–8.
9. Chen L, Li L, Chen J, et al. Oleoylethanolamide, an endogenous PPAR-alpha ligand, attenuates liver fibrosis targeting hepatic stellate cells. Oncotarget. 2015;6:42530–40.
10. Chen PJ, Cai SP, Yang Y, et al. PTP1B confers liver fibrosis by regulating. the activation of hepatic stellate cells. Toxicol Appl Pharmacol. 2016;292:8–18.
11. Cheng J, Hou J, Ding H, et al. Validation of Ten Noninvasive Diagnostic Models for Prediction of Liver Fibrosis in Patients with Chronic Hepatitis B. PLoS One. 2015;10:e0144425.
12. Coskun BD, Altinkaya E, Sevinc E, et al. The diagnostic value of a globulin/platelet model for evaluating liver fibrosis in chronic hepatitis B patients. Rev Esp Enferm Dig. 2015;107:740–4.
13. Ding K, Liu MR, Li J, et al. Establishment of a liver fibrosis model in cynomolgus monkeys. Exp Toxicol Pathol. 2014;66:257–61.
14. Ding N, Hah N, Yu RT, et al. BRD4 is a novel therapeutic target for liver fibrosis. Proc Natl Acad Sci U S A. 2015;112:15713–8.
15. Fiore EJ, Bayo JM, Garcia MG, et al. Mesenchymal stromal cells. engineered to produce IGF-I by recombinant adenovirus ameliorate liver. fibrosis in mice. Stem Cells Dev. 2015;24:791–801.
16. Ge L, Shi B, Song YE, Li Y, Wang S, Wang X. Clinical value of real-time elastography quantitative parameters in evaluating the stage of liver fibrosis and cirrhosis. Exp Ther Med. 2015;10:983–90.
17. Geng Y, Dong Y, Yu M, et al. Follistatin-like 1 (Fstl1) is a bone morphogenetic protein (BMP) 4 signaling antagonist in controlling mouse lung development. Proc Natl Acad Sci U S A. 2011;108:7058–63.
18. Geng 18, Li Y, Dong L, Liu Y, Li X, Ning XH. W. Impaired elastin deposition in Fstl1-/- lung allograft under the renal capsule. PLoS One. 2013;8:e81368.
19. Glinska-Suchocka K, Orlowska A, Spuzak J, Jankowski M, Kubiak K. Suitability of using serum hialuronic acid concentrations in the diagnosis of canine liver fibrosis. Pol J Vet Sci. 2015;18:873–8.
20. Hatori A, Yui J, Xie L, et al. Utility of Translocator Protein (18 kDa) as a Molecular Imaging Biomarker to Monitor the Progression of Liver Fibrosis. Sci Rep. 2015;5:17327.
21. Hatori A, Yui J, Xie L, et al. Utility of Translocator Protein (18 kDa) as a Molecular Imaging Biomarker to Monitor the Progression of Liver Fibrosis. Sci Rep. 2015;5:17327.
22. Kawabata D, Tanaka M, Fujii T, et al. Ameliorative effects of follistatin-related protein/TSC-36/FSTL1 on joint inflammation in a mouse model of arthritis. Arthritis Rheum. 2004;50:660–8.

23. Kudo-Saito C, Fuwa T, Murakami K, Kawakami Y. Targeting FSTL1 prevents tumor bone metastasis and consequent immune dysfunction. Cancer Res. 2013;73:6185–93.
24. Kumagai K, Tabu K, Sasaki F, et al. Glycoprotein Nonmetastatic Melanoma B (Gpnmb)-Positive Macrophages Contribute to the Balance between Fibrosis and Fibrolysis during the Repair of Acute Liver Injury in Mice. PLoS One. 2015;10:e0143413.
25. Kumar P, Smith T, Rahman K, Thorn NE, Anania FA. Adiponectin agonist ADP355 attenuates CCl4-induced liver fibrosis in mice. PLoS One. 2014;9:e110405.
26. Lee JI, Wright JH, Johnson MM, et al. Role of Smad3 in platelet-derived growth factor-C-induced liver fibrosis. Am J Physiol Cell Physiol. 2016;310:C436–45.
27. Leite SB, Roosens T, El Taghdouini A, et al. Novel human hepatic organoid model enables testing of drug-induced liver fibrosis in vitro. Biomaterials. 2016;78:1–10.
28. Li L, Chen N, He L, Wen X. [Significance of P53 and high mobility group box 1 protein in different levels of liver fibrosis in chronic hepatitis B]. Zhong Nan Da Xue Xue Bao Yi Xue Ban. 2015;40:1217–22.
29. Li Y, Lua I, French SW, Asahina K. Role of TGF-beta signaling in differentiation of mesothelial cells to vitamin A-poor hepatic stellate cells in liver fibrosis. Am J Physiol Gastrointest Liver Physiol. 2016;310:G262–72.
30. Liu J, Ji Y, Ai H, et al. Liver Shear-Wave Velocity and Serum Fibrosis Markers to Diagnose Hepatic Fibrosis in Patients with Chronic Viral Hepatitis B. Korean J Radiol. 2016;17:396–404.
31. Liu SB, Ikenaga N, Peng ZW, et al. Lysyl oxidase activity contributes to collagen stabilization during liver fibrosis progression and limits spontaneous fibrosis reversal in mice. FASEB J. 2016;30:1599–609.
32. Louka ML, Ramzy MM. Involvement of fibroblast-specific protein 1 (S100A4) and matrix metalloproteinase-13 (MMP-13) in CCl4-induced reversible liver fibrosis. Gene. 2016;579:29–33.
33. Marfa S, Crespo G, Reichenbach V, et al. Lack of a 5.9 kDa peptide C-terminal fragment of fibrinogen alpha chain precedes fibrosis progression in patients with liver disease. PLoS One. 2014;9:e109254.
34. Maruyama H, Shiha G, Yokosuka O, et al. Non-invasive assessment of portal hypertension and liver fibrosis using contrast-enhanced ultrasonography. Hepatol Int. 2016;10:267–76.
35. Miller M, Beppu A, Rosenthal P, et al. Fstl1 Promotes Asthmatic Airway Remodeling by Inducing Oncostatin M. J Immunol. 2015;195:3546–56.
36. Mitra A, Satelli A, Yan J, et al. IL-30 (IL27p28) attenuates liver fibrosis through inducing NKG2D-rae1 interaction between NKT and activated hepatic stellate cells in mice. Hepatology. 2014;60:2027–39.
37. Mouillet JF, Mishima T, Paffaro AM, et al. The expression and post-transcriptional regulation of FSTL1 transcripts in placental trophoblasts. Placenta. 2015;36:1231–8.
38. Osawa Y, Oboki K, Imamura J, et al. Inhibition of Cyclic Adenosine Monophosphate (cAMP)-response Element-binding Protein (CREB)-binding Protein (CBP)/beta-Catenin Reduces Liver Fibrosis in Mice. EBioMedicine. 2015;2:1751–8.

39. Ouchi N, Asaumi Y, Ohashi K, et al. DIP2A functions as a FSTL1 receptor. J Biol Chem. 2010;285:7127–34.
40. Rockstroh JK, Mohr R, Behrens G, Spengler U. Liver fibrosis in HIV: which role does HIV itself, long-term drug toxicities and metabolic changes play? Curr Opin HIV AIDS. 2014;9:365–70.
41. Rosenberg MI, Georges SA, Asawachaicharn A, Analau E, Tapscott SJ. MyoD inhibits Fstl1 and Utrn expression by inducing transcription of miR-206. J Cell Biol. 2006;175:77–85.
42. Rowland M, Gallagher C, Gallagher CG, et al. Outcome in patients with cystic fibrosis liver disease. J Cyst Fibros. 2015;14:120–6.
43. Lee J, Fricke F, Warnken U, Schnölzer M, Kopitz J, Gebert J. Reconstitution of TGFBR2-mediated signaling causes upregulation of GDF-15 in HCT116 colorectal cancer cells. PLoS One. 2015;10:e0131506.
44. Sasahara A, Tominaga K, Nishimura T, et al. An autocrine/paracrine circuit of growth differentiation factor (GDF) 15 has a role for maintenance of breast cancer stem-like cells. Oncotarget. 2017;8:24869–81.
45. Tanrikulu O, Sariyildiz MA, Batmaz I, et al. Serum GDF-15 level in rheumatoid arthritis: relationship with disease activity and subclinical atherosclerosis. Acta Reumatol Port. 2017;42:66–72.
46. Whitson RJ, Lucia MS, Lambert JR. Growth differentiation factor-15 (GDF-15) suppresses in vitro angiogenesis through a novel interaction with connective tissue growth factor (CCN2). J Cell Biochem. 2013;114:1424–33.

Figures

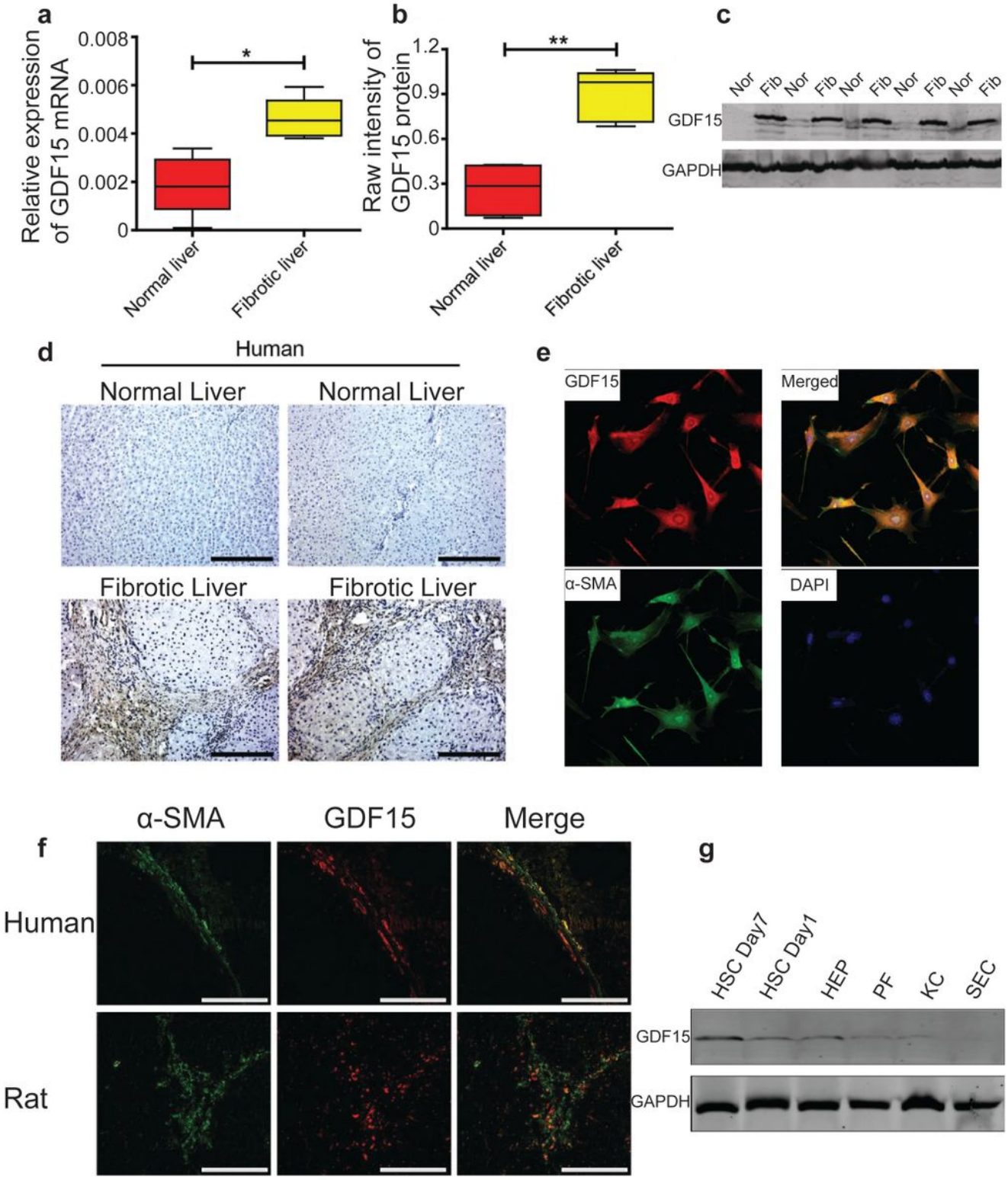

Figure 1

(a), qPCR assay showed that the expression of GDF15 in human fibrotic liver tissues was higher than that in normal liver. (b) Grey value analysis of Western blot assay showed the expression of GDF15 in human fibrotic liver tissues was higher than that in normal liver. (c) Western blot assay showed the GDF15 protein expression in fibrotic liver tissues (Fib) was higher than that in normal liver (Nor). (d) IHC images of GDF15 expression in human fibrotic liver tissues and normal liver tissues. (e) Cell immunofluorescence

staining showed that GDF15 was expressed at high levels in activated HSCs. (f) GDF15 expression was mainly localized to cirrhotic lesions of liver tissues. (g) Western blot assay showed that GDF15 protein expression mainly originated from activated HSCs compared with HEPs (hepatocytes), PFs (portal fibroblasts), KCs (Kupffer cells), and SECs (sinusoidal endothelial cells).

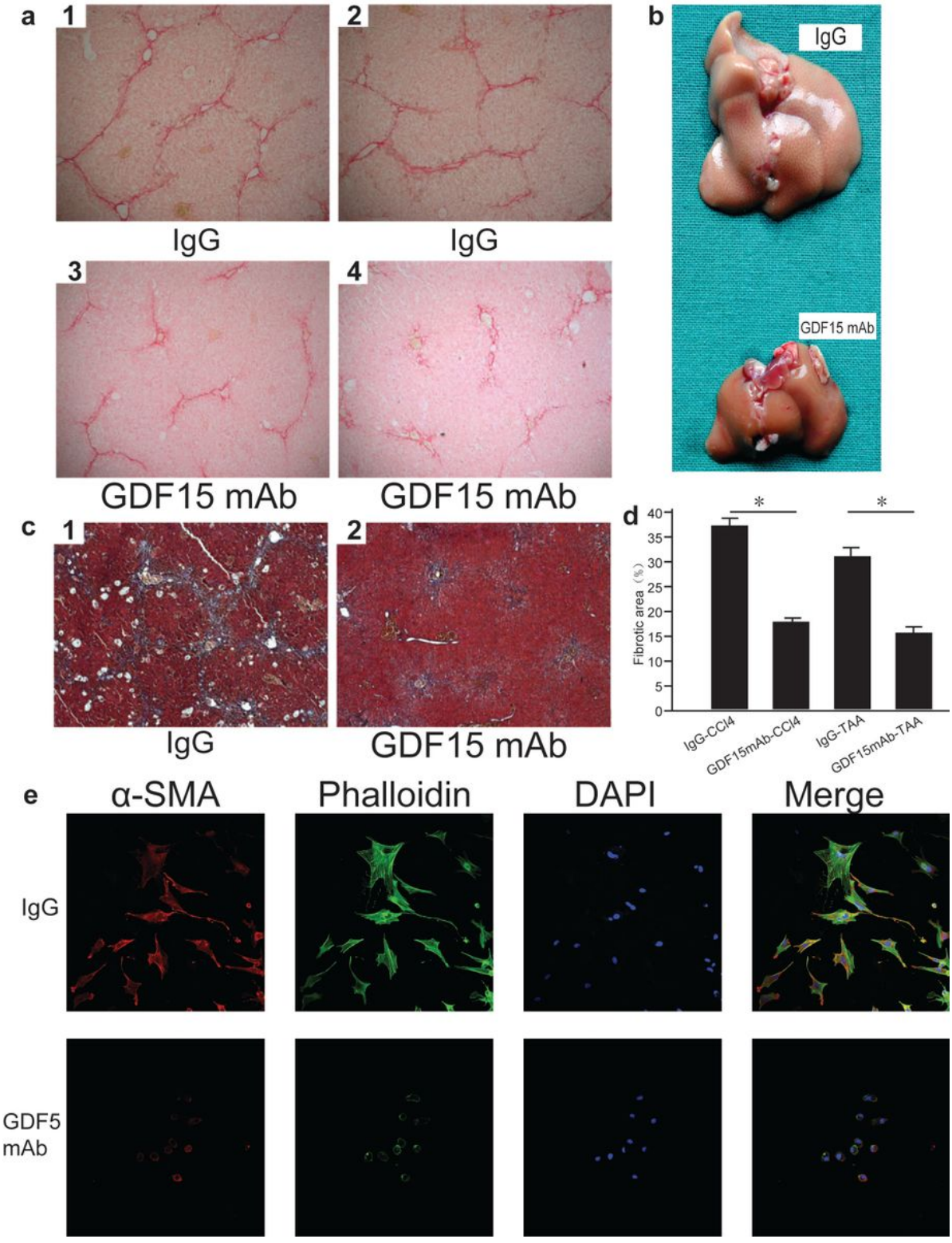

Figure 2

(a) 1 and 3 represent the CCl4-induced liver fibrosis animal model and show that the GDF15 mAb-treated group had attenuated liver fibrosis compared with the IgG group under Sirius Red stain; 2 and 4 represent the TAA-induced liver fibrosis animal model and show similar results to the CCl4-induced assay. (b) CCl4-induced liver fibrosis animal model showing that the GDF15 mAb-treated liver presented attenuated fibrosis. (c) CCl4-induced liver fibrosis animal model showing that the GDF15 mAb-treated group had attenuated liver fibrosis compared with the IgG group under Masson stain. (d) The fibrotic areas in representative histological images of Figure 2a (1, 2, 3, 4) were analysed. The fibrotic area ratios of the GDF15 mAb-treated group and the control group in CCl4- and TAA-induced conditions were analysed by ImageJ software. (e) Primary HSCs isolated from mice were fixed at five days. ICC images showed that the activation of HSCs treated with IgG was faster than that of HSCs treated with GDF15 mAb.

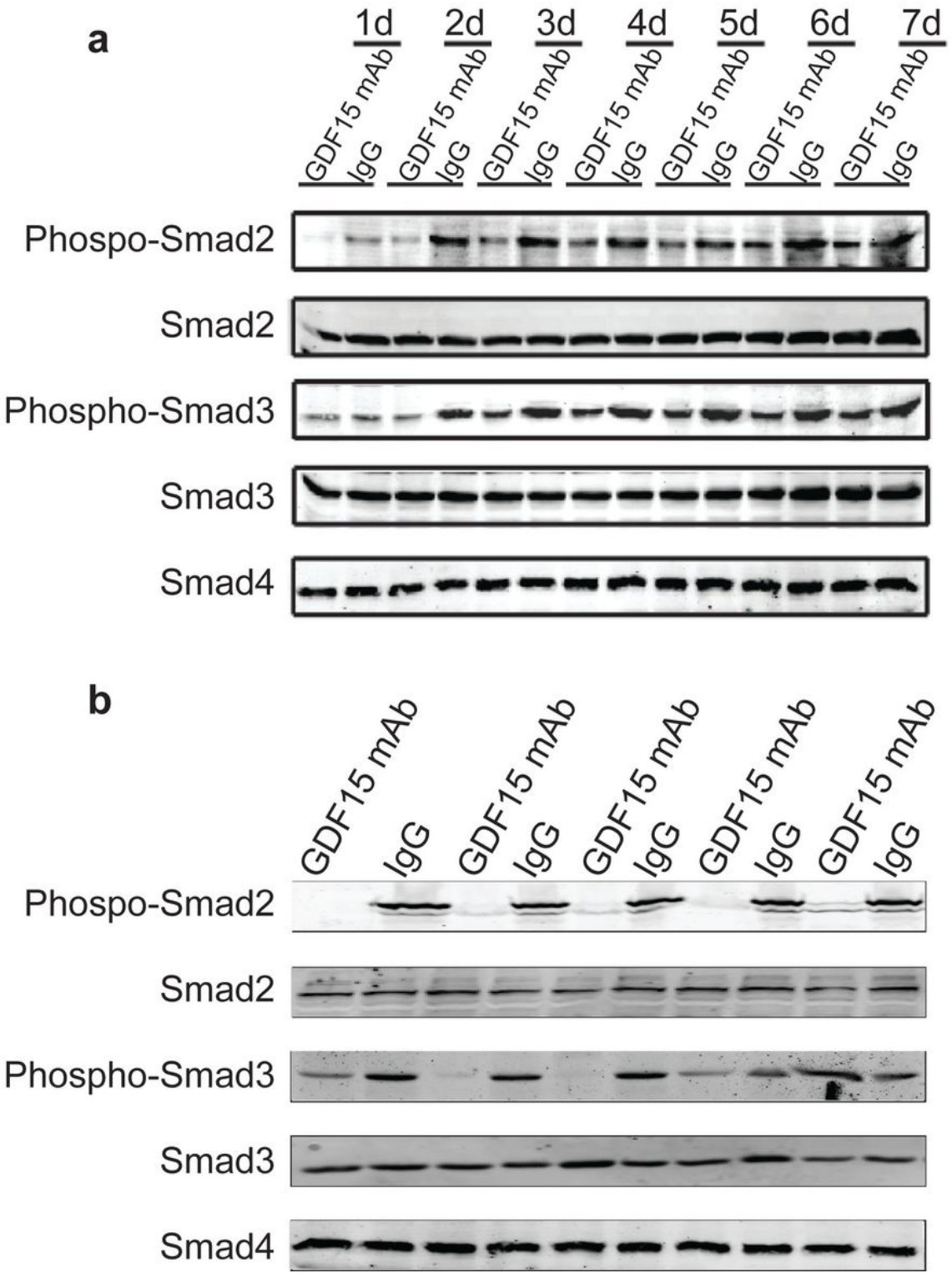

Figure 3

(a) Treatment of primary mouse HSCs with GDF15 mAb showed lower expression of phospho-Smad2 and phospho-Smad3 compared with the IgG-treated group. (b) Expression levels of phospho-Smad2 and phospho-Smad3 in GDF15 mAb group liver tissues were significantly lower than those in the IgG-treated group under CCl4-induced conditions.

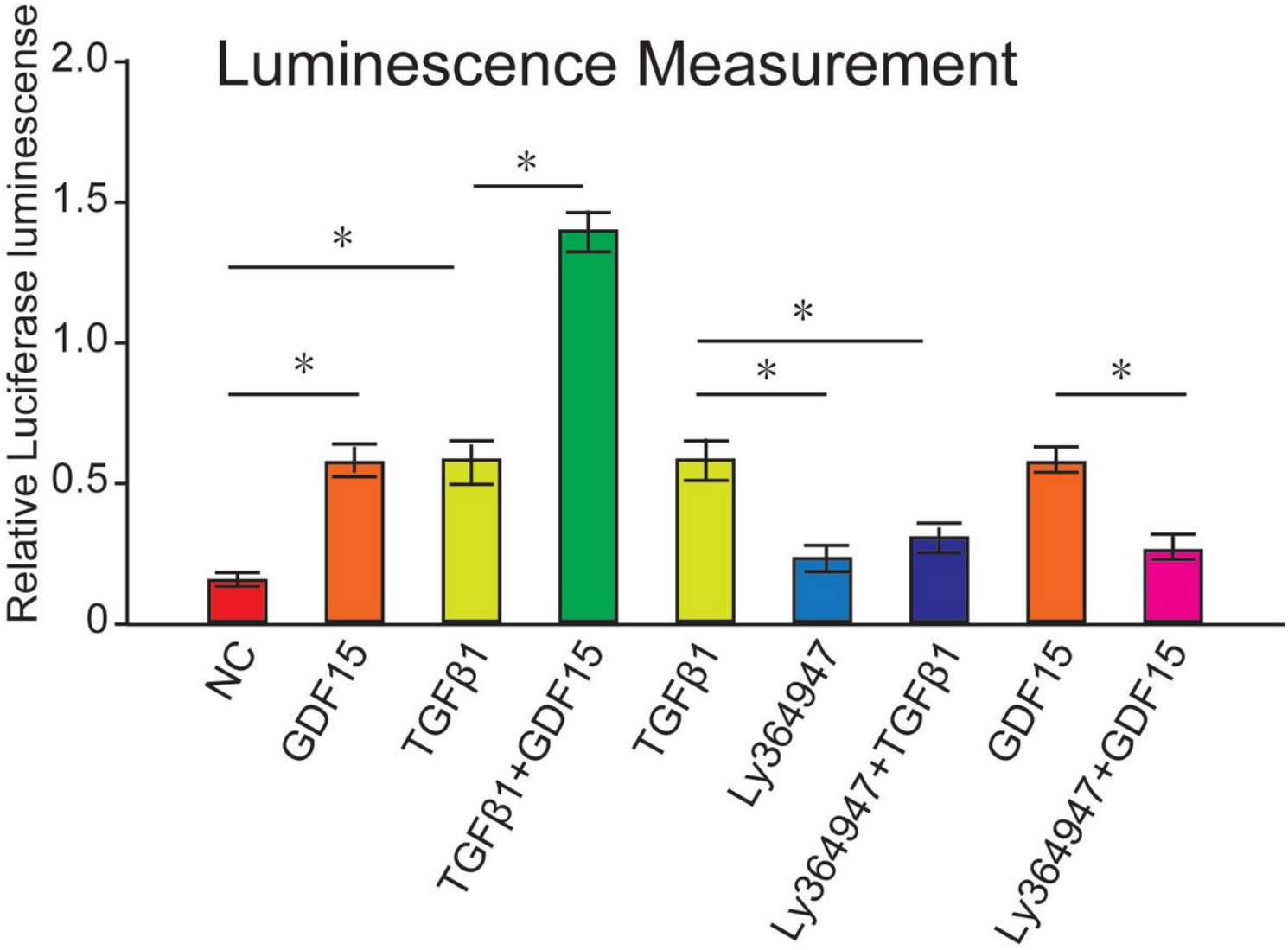

Figure 4

Luciferase reporter assay showing that GDF15 can activate TGFβ signalling in LX-2 cells, that TGFβ1 and GDF15 had cooperative effects in regulating TGFβ signalling, and that the effect of TGFβ1 and GDF15 on activating TGFβ signalling can be blocked by the inhibitor Ly364947.