Nintedanib inhibits the development and progression of peritoneal fibrosis

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Research

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Abstract

Background

Nintedanib, an FDA approved triple tyrosine kinase inhibitor, exhibits an antifibrotic effect in idiopathic pulmonary and renal fibrosis. Its effect on peritoneal fibrosis remains unexplored.

Methods

The present study investigated the effect of nintedanib on the development and progression of peritoneal fibrosis by administration fo nintedanib immediately after peritoneal injury or starting at day 21 of the injury in a in a mouse model of chlorhexidine gluconate-induced peritoneal fibrosis. Peritoneal fibrosis and associated mechanisms were examined by immunohistochemistry and immunoblot analysis.

Results

Administration of nintedanib immediately after peritoneal injury attenuated peritoneal fibrosis, whereas delayed administration of nintedanib not only halted the progression of peritoneal fibrosis, but also in part reversed the established fibrosis. Mechanistically studies showed that nintedanib inhibited injury-induced mesothelial-to-mesenchymal transition, expression of several cytokines/chemokines, vascularization and infiltration of macrophages to the injured peritoneum. Nintedanib also blocked phosphorylation of platelet derived growth factor receptor, fibroblast growth factor receptor, vascular endothelial growth factor receptor, and Src, downregulated expression of Snail and Twist, two transcription factors and inactivated several signaling pathways associated with peritoneal fibrosis, including Smad3, signal transducer and Activator of transcription 3, and nuclear factor-κB. Moreover, late treatment with nintedanib promoted expression of matrix metallopeptidase 2 and reduced expression of tissue inhibitor of metalloproteinases 2 in the injured peritoneum. Finally, nintedanib abrogated transforming growth factor β1–induced mesothelial-to-mesenchymal transition and phosphorylation of aforementioned signaling molecules in cultured human peritoneal mesothelial cells.

Conclusions

These results suggest that nintedanib may inhibit peritoneal fibrosis development and progression by blocking mesothelial-to-mesenchymal transition, inflammation, and angiogenesis, and partially reversed established peritoneal fibrosis through metalloproteinases-mediated extracellular matrix degradation. Therefore, nintedanib holds therapeutic potential for the prevention and treatment of peritoneal fibrosis.

Background

Peritoneal dialysis (PD) is one of the renal replacement therapies for the treatment of end stage of renal disease [1, 2].During this process, the peritoneal membrane (PM) is continuously exposed to hyperglycemic and acidic dialysis solutions. Long-term PD has been associated with progressive transformation of the peritoneum, submesothelial fibrosis, angiogenesis, vasculopathy, leading to ultrafiltration failure and discontinuation of PD [1, 2]. Currently, there is limited evidence for clinical interference of peritoneal fibrosis [2, 3]; attempts to ameliorate peritoneal fibrosis are hampered by the lack of targeted pharmacological therapies. As such, a strategy for developing an effective anti-fibrotic therapy is needed to target the key mechanisms that lead to peritoneal fibrogenesis.

Increasing evidence indicates that peritoneal mesothelial to mesenchymal transition (MMT) is an early mechanism for peritoneal fibrosis and functional deterioration of peritoneal membrane. MMT is characterized by the disruption of intercellular junctions and apical–basolateral polarity, and acquisition of a mesenchymal phenotype. This phenotype of mesothelial cells is able to secrete profibrotic and angiogenetic cytokines that contribute to producing a large amount of extracellular matrix components and inducing vascularization and mononuclear cell infiltration [4, 5].Like MMT in other tissues, peritoneal MMT also results from the activation of diverse signaling pathways and transcription factor triggered by multiple growth factors. Among them, transforming growth factor-β1 (TGF-β1) has been shown to play a predominant role in inducing MMT and peritoneal fibrosis. Other growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and vascular growth factor (VEGF) also contribute to these processes. Importantly, all of these growth factors and their receptors are expressed in the injured peritoneum and the receptor activation is necessary for TGF-β1 production. Since the interaction of individual growth factors with their receptors can induce distinct pathological changes of peritoneal fibrosis, pharmacological targeting of each receptor may not achieve a full spectrum of inhibition of peritoneal fibrosis. On this basis, the application of a multiple receptor inhibitor might be an ideal strategy for the treatment of peritoneal fibrosis.

Nintedanib is such an inhibitor that can simultaneously inhibit the phosphorylation of PDGFR, VEGFR, FGFR and the Src family kinases [6]. Initially, it was developed to treat various tumors [6]. In 2014, the FDA approved it as a treatment of idiopathic pulmonary fibrosis (IPF) due to its powerful anti-fibrotic effect [7]. Given the similar profibrotic mechanisms implicated in other organs, the antifibrotic ability of nintedanib may not be limited to the lung [8]. Recently, we assessed the therapeutic effect of nintedanib on renal fibrosis in a murine model of obstructive nephropathy, and found that administration of nintedanib also attenuated the development and progression of renal fibrosis [9]. In vitro cultured of renal interstitial fibroblasts, nintedanib could remarkably suppressed TGF-β1 induced renal fibroblast activation and production of extracellular matrix (ECM) proteins as well [9]. Since peritoneal fibrosis development is also a consequence of stimulation with multiple cytokines and growth factors, we hypothesized that nintedanib might also have an antifibrotic effect in peritoneal fibrosis.

To test the hypothesis, we investigated the effect of nintedanib on peritoneal fibrosis in an animal model of peritoneal injury induced by chlorhexidine gluconate (CG) and in cultured human peritoneal mesothelial cells (HPMCs), and the mechanisms involved. Our results demonstrated that nintedanib not

only ameliorated the development and progression of peritoneal fibrosis, and also partially reversed the established peritoneal fibrosis.

Methods

Chemicals and antibodies

Antibodies to p-PDGFR-β, p-VEGFR2, p-Src, Src, p-STAT3,STAT3,p-Smad3, Smad3, p-NF-κBp65, p-Akt,Akt and β-Actin were purchased from Cell Signaling Technology (Danvers, MA). TGF-β1 and antibodies to type I collagen, fibronectin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).p-FGFR1 antibody was purchased from Life Span Biosciences (Seattle, WA).NF-κBp65 antibody was purchased from Prosci Inc.(San Diego, CA). Antibodies to CD68, CD31, MMP-2, TIMP-2, E-cadherin, vimentin, Snail, Twist, and MCP-1, TNF-α, IL-1β and IL-6 ELISA assay kits were purchased from Abcam Inc. (Cambridge, UK). Nintedanib was purchased from Cayman (Arbor, MI). α-SMA antibody, chlorhexidin gluconate (CG) and all other chemicals were purchased from Sigma (St. Louis, MO).

Establishment of mouse peritoneal fibrosis models and nintedanib administration

The peritoneal fibrosis model was established in male C57/BL6 mice that weighed 24-28 g (Shanghai Super–B&K Laboratory Animal Corp. Ltd.), as described in our previous study. Briefly, peritoneal fibrosis in mice was generated by daily intraperitoneal injection of 0.1% CG. Control rats were injected with an equal volume of 0.9% saline. To examine the time course of peritoneal fibrosis in this model, mice (n=6) were sacrificed at days 0, 7, 14, 21, and 35, respectively, after CG injection and peritoneum were collected. To examine the effect of nintedanib on the development of peritoneal fibrosis, nintedanib at 50 mg/kg was given by gavage immediately after CG injection and then administered daily. DMSO alone treated animals were used as controls. On day 21 after CG injection, the peritoneum was harvested. To examine the effect of delayed administration of nintedanib on peritoneal fibrosis, nintedanib at 50 mg/kg was given by gavage on day 21 after CG injection and then given daily for 14 days. At 35 days, mice were euthanized and the parietal peritoneum apart from the injection points was harvested for further analysis.

Cell culture and treatments

Human peritoneal mesothelial cells (HPMCs) were cultured in DMEM (Sigma-Aldrich) containing 10% FBS, 1% penicillin, and streptomycin in an atmosphere of 5% CO2 and 95% air at 37°C. To determine the effect of nintedanib on HPMCs in response to TGF-β1, cells were incubation with DMEM containing 0.5% FBS for 24 h by followed by stimulation with TGF-β1 (5 ng/ml) for 24h and then incubated with 400 nM nintedanib for an additional 48 h.

Immunoblot analysis

Immunoblot analysis of peritoneum tissue samples and HPMCs were conducted as described previously. The densitometry analysis of immunoblot results was conducted using Image J software developed at

the national institute of health. The quantification data is given as the ratio between the target protein and loading control.

Histochemical and immunofluorescent staining

Formalin-fixed were peritoneum embedded in paraffin and prepared in 3-μm-thick sections. Immunohistochemical staining was conducted on the basis of the procedure described in our previous study. To evaluate peritoneal fibrosis, Masson trichrome staining was performed according to the protocol provided by the manufacture (Sigma-Aldrich). The collagen tissue area (blue color) was quantitatively measured using Image Pro-Plus software (Media-Cybernetics, Silver Spring, MD) by drawing a line around the perimeter of the positive staining area, and the average ratio to each microscopic field (× 200) was calculated and graphed. The thickness of the submesothelial tissue was evaluated (in micrometers), and the average of ten independent measurements was calculated for each section (original magnification, ×200).CD31 and CD68 expression in peritoneum tissue were assessed by immunohistochemical staining of vimentin. Snail and Twist expression in peritoneum tissue were assessed by immunofluorescent staining using a Zeiss 710 Duo microscope. (Zeiss, Germany).

ELISA analysis

To examine the renal expression of MCP-1, TNF-α, IL-1β and IL-6, mouse peritoneum were homogenized in an extraction buffer. The supernatant recovered after centrifugation was used for determination of these chemokine/cytokines by the commercial Quantikine ELISA kits in accordance with the protocol specified by the manufacturer (AbcamInc, Cambridge, UK). Total protein levels were determined using a bicinchoninic acid protein assay kit. The concentration of cytokines in the peritoneum was expressed as picograms per milligram of total proteins.

Statistical analysis

All the experiments were conducted at least three times. Data depicted in graphs represent the means ± S.E.M for each group. Inter-group comparisons were made using one-way analysis of variance (ANOVA). Multiple means were compared using Tukey's test. The differences between the two groups were determined by the Student *t-test*. Statistically significant differences between mean values were marked in each graph. $P<0.05$ was considered a statistically significant difference between mean values. All the statistical analyses were conducted by SPSS 20.0.

Results

CG injection induces peritoneal fibrosis in a time-dependent manner in mice

In order to assess the effect of nintedanib on the progression of peritoneal fibrosis, we first established a murine model of peritoneal fibrosis by daily I.P injection of chlorhexidine gluconate (CG) for 35 days. Following CG injection, expression of α-smooth muscle antigen (SMA), type I collagen and fibronectin, three hallmarks of fibrosis, was examined by immunoblot analysis. As shown in Figure 1, A-D, a minimal

amount of α-SMA, fibronectin and type I collagen were detected in the peritoneum of control mice; their expression levels gradually increased over time with CG injection. The maximal levels were seen at 35 days under this experiment setting. To directly observe the thickening of the submesothelial area with an accumulation of collagens in this model, we also stained peritoneal tissue with Masson trichrome stain collected at 21 and 35 days after CG injection. As shown in Figure 1, E and F, CG injection caused the thickening of the submesothelial compact zone, and peritoneal interstitial expansion with increased cellularity. These changes were clearly seen on day 21 and more dramatically on day 35, with a fibrosis score of 2.0 and 3.2, respectively, over the control of peritoneal tissues. These changes were similar to the pathological changes observed in a rat model of peritoneal fibrosis induced by CG[10]. Therefore, we have successfully established a murine model of peritoneal fibrosis.

Nintedanib attenuates the development of peritoneal fibrosis and inhibits phosphorylation of multiple RTKs and Src in mice following CG injection

Our previous studies indicated that 50 mg/kg nintedanib attenuated renal fibrosis [9]; this dose was thus used to assess the effect of nintedanib on the peritoneal fibrosis in this model by oral administration of it immediately after CG injection and then daily for 21 days. As shown in Figure 2, A and B, the thickness of the submesothelial zone and the area of collagen fibrils in CG-injured mouse with nintedanib administration was significantly less than that in mouse subjected to CG alone by Masson trichrome staining. To demonstrate the specificity of nintedanib, we examined its effect on the activation of PDGFRβ, FGFR1, VEGFR2, and Src. As shown in Figure 2, C-G, CG injection for 21 days induced phosphorylation of PDGFRb, FGFR1, VEGFR2, and Src whereas treatment with nintedanib largely reduced the phosphorylation of each of them. These results suggest that nintedanib has a potential effect in preventing peritoneal fibrosis development.

Nintedanib reduces the expression of collagen 1 and fibronectin in mice following CG injection

To confirm the antifibrotic effect of nintedanib, we further examined the impact of nintedanib on the expression of collagen 1 and fibronectin, two major ECM proteins deposited in the submesothelial compact zone of peritoneum by immunoblot analysis and immunostaining. Immunoblot analysis demonstrated an increase in the expression of collagen 1 and fibronectin in the peritoneum after CG injection (Figure 3, A-C). Nintedanib significantly suppressed their expression. Similar results were also observed by immunochemical analysis (Figure, D-F). As such, nintedanib may reduce interstitial expansion through suppression of ECM protein accumulation.

Nintedanib inhibits MMT in the peritoneum after CG injury

MMT, characterized by increased expression of α-SMA and vimentin and decreased expression of E-Cadherin, has been shown to play a primary role in inducing fibrosis and functional deterioration of the peritoneal membrane [11]. It is driven by the activation of some transcriptional factors such as Snail and twist [12, 13]. Therefore, we examined the effect of nintedanib on the expression of these proteins in the peritoneum after CG injury by immunoblot analysis. As shown in Figure 4, A-D, CG injection resulted in

decreased expression of E-Cadherin and increased expression of α-SMA and vimentin; treatment with nintedanib largely preserved E-Cadherin expression but inhibited a-SMA and vimentin expression. Similarly, nintedanib treatment suppressed expression of Snail and Twist (Figure 4, A, E and F). These results illustrated that nintedanib protects against MMT development in CG injured peritoneum.

Nintedanib inhibits TGF-b1-induced MMT and expression of ECM proteins in cultured HPMCs

TGF-b1 can induce MMT and transformed MCs are able to produce ECM matrix and cause fibrosis [14]. To verify the effect of nintedanib on MMT *in vitro*, cultured HPMCs were exposed to TGF-b1 and then collected for immunoblot analysis of expression of MMT markers and Snail and Twist. As shown in Figure 5, A-H, exposure of TGF-β1 to HPMCs resulted in increased expression of α-SMA, vimentin, collagen I and fibronectin as well as Snail and Twist, whereas nintedanib treatment abolished their expression. In contrast, TGF-β1 reduced the expression of E-Cadherin, which was restored by nintedanib treatment. These data support our *in vivo* observations that nintedanib is able to suppress the development of MMT and the production of ECM components.

Nintedanib suppresses production of multiple proinflammatory cytokines/chemokines and infiltration of macrophages in the peritoneum after CG injury

Overproduction of inflammatory cytokines/chemokines and influx of inflammatory cells into the submesothelial compact zone is a typical pathologic feature of peritoneal fibrosis [15]. We thus examined whether nintedanib would be effective in suppressing expression of proinflammatory cytokines/chemokines and macrophages in the peritoneum after CG injury. The ELISA indicated that the expression of monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were elevated in the peritoneum after CG injury; administration of nintedanib significantly reduced this response (Figure 6, A-D). Immunohistochemistry staining demonstrated that the number of CD68-positive macrophages was increased in the submesothelial layer of mouse peritoneum after CG injury; nintedanib treatment also reduced their infiltration (Figure 6, E and F). Since NF-κB is a major transcriptional factor that regulates expression of proinflammatory cytokine and chemoattractants in peritoneal fibrosis [16], we examined its phosphorylation and expression under the same experimental settings (Figure 6, F and H). CG injury to the peritoneum induced phosphorylation of NF-κB, which was slightly suppressed by nintedanib. The expression of total NF-κB was not affected by CG and nintedanib treatment. Collectively, we suggest that nintedanib is also effective in suppressing the inflammatory responses in the fibrotic peritoneum after CG injury.

Nintedanib reduces angiogenesis in the peritoneum after CG injury

Long-term PD is frequently accompanied by angiogenesis in the fibrotic submesothelial zone of peritoneum, which is induced by overproduction and release of VEGF from injured mesothelial cells [17]. Due to the property of nintedanib in inhibiting the interaction of VEGF with its receptors, we assumed that this inhibitor might be able to interfere with angiogenesis in the peritoneum. To test this hypothesis, we examined the expression of endothelial cell marker CD31 in the peritoneum by both

immunohistochemical staining and immunoblot analysis. As shown in Figure 7, A-B, CG injury to the peritoneum resulted in the increase of CD31 (+) vessels in the peritoneum. Nintedanib significantly reduced the number of CD31(+) vessels. The results from immunoblot analysis also demonstrated that the administration of nintedanib reduced the protein expression of CD31 in CG injured peritoneum (Figure7, C-D). Collectively, nintedanib has a potent inhibitor effect on angiogenesis in the peritoneum injured by CG injection.

Delayed administration of nintedanib attenuates progression of peritoneal fibrosis and metabolism of ECM induced by CG injury

To assess the therapeutic effect of nintedanib on peritoneal fibrosis, nintedanib at 50 mg/kg was given starting day 21 after CG injection, when peritoneal fibrosis had already progressed to an advanced stage. Following 14 additional days of treatment, peritoneum was collected for examining the deposition of ECM and the expression of relevant proteins (Figure 8, A). As shown in Figure 8, B and C, increased thickness of the submesothelial compact zone and Masson trichrome–positive areas were observed at 21 days and were further elevated at 35 days after CG injury. In contrast, these pathological changes of peritoneum were not further increased at 35 days after treatment with nintedanib. Immunoblot analysis shows that delayed administration of nintedanib also reduced CG-induced expression of fibronectin and type I collagen to the level below that observed on day 21 after CG injection (Figure 8, D-F).

The metabolism of ECM protein is regulated by matrix metalloproteinase (MMPs) and tissue inhibitor of metalloproteinase (TIMPs). As shown in Figure 8, D, G and H, MMP-2 and TIMP-2 expression levels were increased after CG injury; delayed administration of nintedanib inhibited TIMP-2 expression, along with the increased expression of MMP-2. Hence, these data demonstrated that nintedanib was able to not only prevent progression of peritoneal fibrosis but also to partially reverse the established peritoneal fibrosis by MMP-mediated ECM degradation in the injured peritoneum.

Delayed administration of nintedanib inhibits expression of Snail and Twist in the peritoneum after CG injury

We also examined the effect of late nintedanib treatment on the expression of Snail and Twist induced by CG injury in the peritoneum by immunoblot analysis. CG injection induced a higher expression of Snail and Twist on day 35 compared to day 21. Delayed treatment with nintedanib reduced expression of these two proteins which was lower on day 35 than that of day 21 (Figure 9, A-C). As shown in Figure 9, D, costaining of vimentin and Snail or Twist indicates that vimentin was most expressed in Snail- or Twist–positive cells at day 21 after CG injury, suggesting that Snail and Twist are involved in the MMT induced by CG injury. Since Snail and Twist are two major transcriptional factors that promote MMT, a lower level of Snail and Twist at day 35 relative to that at day 21 suggest that nintedanib treatment is able to partially reverse MMT.

Delayed administration of nintedanib blocks phosphorylation of STAT3, NF-κB, and Smad3 after CG injury

Phosphorylation of RTKs can lead to activation of several downstream signaling pathways, of which, Smad3, STAT3, and NF-kB have been reported to be associated with peritoneal fibrosis [11, 18]. As shown in Figures 10, A, B, D, and F, increased phosphorylation levels of STAT3, NF-κB and Smad3 were detected in CG injured peritoneum at day 21 compared with control. Administration of nintedanib beginning at day 21 after CG injection for two weeks resulted in reduced phosphorylation of all of these three signaling molecules compared to levels on day 21. Expression of total STAT3 was also increased at day 21 and further advanced at day 35, but treatment with nintedanib did not affect its expression (Figure 10, A and C). The expression levels of NF-κB and Smad3 remained the same during the whole time course and nintedanib did not alter their expression (Figure 10, A, E, and G). Therefore, delayed administration of nintedanib may inhibit CG injury-induced phosphorylation of STAT3, NF-κB, and Smad3 in the fibrotic peritoneum.

Nintedanib blocks TGF-β1-induced phosphorylation of STAT3, AKT, Smad3 and Src in cultured HPMCs

To illustrate the effect of nintedanib on the activation of STAT3, NF-κB Smad3 signaling pathways in HPMCs, we examined the phosphorylation levels of these three signaling molecules in vitro cultured HPMCs. HPMCs were exposed to TGF-β1 in the presence or absence of nintedanib and then harvested for analysis of phosphorylation of STAT3, NF-κB Smad3, and Src. Figure 11, A-E shows that TGF-β1 induced the phosphorylation of STAT3, NF-κB, and Smad3 as well as Src; nintedanib treatment reduced the phosphorylation of all of them. These data suggest that TGF-β1 mediated activation of these signaling pathways can also be inhibited by nintedanib.

Discussion

Peritoneal fibrosis is a major complication occurring in patients undergoing long-term peritoneal dialysis, and may eventually lead to peritoneal ultrafiltration failure which can lead to the termination of peritoneal dialysis. Recent studies have demonstrated that some receptor tyrosine kinases (RTKs) and Src superfamily kinases are involved in the initiation and progression of peritoneal fibrosis [19]. Here, we demonstrated that nintedanib, an FDA-approved multiple tyrosine kinase inhibitor for the treatment for IPF, was effective in inhibiting peritoneal fibrosis in a preclinical model of peritoneal fibrosis induced by CG. Nintedanib not only attenuated peritoneal fibrosis but also inhibited peritoneal fibrosis progression and partially reversed the established peritoneal fibrosis. Moreover, nintedanib treatment inhibited MMT in cultured human peritoneal mesothelial cells. These results indicate that nintedanib is a potent antagonist for preventing and treating peritoneal fibrosis, and suggest its potential as a novel treatment for peritoneal fibrosis.

The peritoneum is composed of a continuous monolayer of the mesothelial cells, which have an epithelial-like cobblestone shape and cover a submesothelial region, composed of bundles of collagen fibers with few fibroblasts, mast cells, macrophages, and vessels [1]. During PD, continual exposure to hyperosmotic, hyperglycemic, acidic dialysis solutions and episodes of catheter complications can cause acute and chronic injury of the peritoneal membrane, along with the pathological change, such as

progressive fibrosis, inflammation, angiogenesis, and vasculopathy [8]. These processes are coincident with the activation of diverse cellular RTKs, including PDGFR, FGFR, VEGFR, and Src. PDGFR, FGFR and Src family kinases which have been shown to contribute to MMT and fibroblast activation. While VEGFR is a key regulator of angiogenesis, all of them can induce an inflammatory response [1, 19]. In this study, we found that the administration of nintedanib inhibited deposition of ECM proteins, inflammation, angiogenesis, suggesting a potent inhibitory effect of nintedanib on peritoneal fibrosis. In line with this observation, nintedanib largely inhibited phosphorylation of PDGFR, FGFR, VEGFR and completely blocked Src phosphorylation. It should be mentioned that Src phosphorylation is initiated by not only the aforementioned three RTKs, but also other cellular membrane receptors, such as TGF-β receptors and EGFR [20, 21]. Therefore, in addition to PDGFR, FGFR and VEGFR-mediated peritoneal fibrogenesis, nintedanib may also inhibit the profibrotic responses initiated by many other growth factor receptors. Thus, compared with the application of individual RTK inhibitor, nintedanib seems to have a more potent antifibrotic effect due to its triple tyrosine kinase inhibitory properties.

The peritoneal mesothelial cells (PMCs) undergoing MMT play a central role in the alterations of the peritoneal membrane leading to fibrosis [11]. In response to diverse stimuli, such as high glucose, growth factors or inflammatory factors, the PMCs have downregulated E-cadherin, upregulated vimentin, and loss of epithelial features and eventually gain the feature of mesenchymal cells [19]. These profibrotic PMCs produce inflammatory cytokines and ECM proteins, including fibronectin and type I collagen, leading to peritoneal fibrosis [19]. During this process, Twist and Snail are the two major transcription factors to induce E-cadherin downregulation [22] and to drive PMCs undergoing MMT [23]. In the present study, we found that CG injury induces the MMT of PMCs, coincident with increased the expression of Snail and Twist, while the administration of nintedanib immediately following CG inhibited the expression of Snail, Twist, and preserved expression of E-cadherin. These data suggest that nintedanib may attenuate renal fibrosis through a mechanism involved in the inhibition of MMT.

Nintedanib may also attenuate peritoneal fibrosis via inhibition of inflammation. It is well known that infiltration of the monocytes/macrophages and overproduction of proinflammatory cytokines accelerate peritoneal fibrosis [24]. In this study, we observed that CG injury induced a significant elevation of multiple proinflammatory cytokines/chemokines, including MCP-1, TNF-α, IL-6, and IL-1β, as well as infiltration of CD68-positive macrophages to the peritoneum; administration of nintedanib inhibited all these responses. In addition, nintedanib was effective in reducing phosphorylation of NF-κB, a transcriptional factor associated with the production of many proinflammatory cytokines. We thus suggest that nintedanib has a powerful anti-inflammatory effect on peritoneal fibrosis. These results are consistent with its suppression of inflammation in animal models of IPF and renal fibrosis [9, 25]. The protective effect of nintedanib from inflammation may be through its inhibition of multiple RTKs and Src, and subsequently, inactivation of their downstream signaling pathways, such as STAT3 and NF-κB.

Angiogenesis has been demonstrated to be an important event in the pathological process of peritoneal fibrosis [17]. Long-term exposure to peritoneal dialysates with high-dose glucose induces angiogenesis in peritoneum, and inhibition of angiogenesis could alleviate peritoneal fibrosis and dysfunction [17]. VEGF

is involved in the fibrogenesis and angiogenesis of peritoneal membrane through the TGF-β-VEGF pathway in mesothelial cells and fibroblasts [26], which could stimulate the formation of new capillaries, lead to angiogenesis and peritoneal fibrosis [27]. In the present study, we found that nintedanib was effective in reducing the number of CD31-positive cells and increased thickened peritoneum area in CG-injured mice. In addition to VEGF, inhibition of Src also reduced CD31 expression and CD31-positive cells population induced by CG injury as shown in our previous study [28]. These suggest that nintedanib may inhibit peritoneal angiogenesis through at least targeting VEGF receptor and Src.

Nevertheless, we cannot exclude the possibility that nintedanib may also suppress peritoneal fibrosis through inhibition of lymphangiogenesis. In this regard, Lin *et al* have reported that nintedanib inhibited suture-induced corneal lymphangiogenesis, accompanied by reduced inflammatory cell recruitment *in vitro* and *in vitro* [29]. During peritoneal fibrosis, TGF-β induced VEGF-C expression is able to trigger lymphangiogenesis [30, 31]; increased peritoneal lymphatic vessels constantly absorb dialysate during PD treatment, which reduces effective ultrafiltration [32]. Given that lymphangiogenesis contribute to inflammation and high solute transport and ultrafiltration failure in the peritoneum [33], it will be interesting to examine whether nintedanib also reduces peritoneal fibrosis and improves ultrafiltration failure by targeting lymphangiogenesis.

Our data suggest that nintedanib treatment can reverse established peritoneal fibrosis. Evidence of this is suggested by reduced expression of fibronectin and collagen 1 on day 35 to levels below that of day 21 post CG injection with delayed administration of nintedanib. This result is similar to what we have observed in an animal model of renal fibrosis induced by UUO [9]. Currently, the mechanism by which nintedanib reverses peritoneal fibrosis is not clear, but it may be associated with suppression of MMT or/and promotion of ECM degradation. In support of this hypothesis, it has been documented that MMT can be reversed, in particular, the early phase of MMT [11]; the reversal of MMT was also observed in the current study, as suggested by the reduction of expression of MMT markers and Snail and Twist after late treatment with nintedanib at day 21 after CG injection to levels lower than that observed on day 21. Conversely, expression levels of MMP2, a key ECM degrading enzyme in fibrosis, were higher in the kidney treated with nintedanib relative to those without nintedanib treatment. Nevertheless, we cannot exclude the possibility that other TIMPs and TIMPs may be activated and involved in this process. Additional studies are needed for identification of those enzymes.

Although nintedanib has been used as a therapeutic drug for lung fibrosis, its clinical value has not been tested for other fibrotic diseases. Recent preclinical studies have demonstrated a powerful anti-fibrotic effect of nintedanib in other organs, such as liver, kidney, heart, and skin [8]. The current studies have also proved the efficacy of nintedanib in suppressing peritoneal fibrosis and reversing the established peritoneal fibrosis. Since nintedanib is mainly metabolized in the liver, and only a very small amount of it is excreted from the kidney, implicating that it may not be toxic to the kidney. This biological property of nintedanib may make it an ideal antifibrotic drug for patients with diseased kidneys. On this basis, clinical trials are needed to show evidence for its suitability in patients with chronic kidney disease (CKD) or peritoneal fibrosis.

Conclusion

Our present study provides the first evidence that nintedanib attenuates peritoneal fibrosis. The underlying mechanism for anti-peritoneal fibrosis is associated with inhibition of multiple RTKs and Src as well as their downstream signaling pathways leading to the MMT, ECM synthesis, inflammation, and angiogenesis. Therefore, our results suggest a potential therapeutic use of nintedanib and other RTK antagonists in preventing and treating peritoneal fibrosis.

Abbreviations

PD
Peritoneal dialysis
PM
peritoneal membrane
MMT
Mesothelial to mesenchymal transition
TGF-β1
transforming growth factor-β1
PDGF
Platelet-derived growth factor
EGF
Epidermal growth factor
FGF
Fibroblast growth factor
VEGF
Vascular growth factor
FDA
Food and Drug Administration
IPF
Idiopathic pulmonary fibrosis
ECM
Extracellular matrix
CG
Chlorhexidine gluconate
HPMCs
Human peritoneal mesothelial cells
α-SMA
α-smooth muscle antigen
MCP-1
Monocyte chemoattractant protein-1

TNF-α
Tumor necrosis factor-α
IL-1β
Interleukin-1β
IL-6
Interleukin-6
MMPs
Matrix metalloproteinase
TIMPs
Tissue inhibitor of metalloproteinase
RTKs
Receptor tyrosine kinases
CKD
chronic kidney disease

Declarations

Ethics approval and consent to participate

All the animal experiments were performed according to the guidelines of the National Institutes of Health Guidelines on the Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee at Tongji University, China.

Consent for publication

All authors have given consent for publication

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Liu F. and Zhuang S designed experiments; Liu F. Yu C, Qin F, Fang, Wang Y, Wang J, Cui, Liu N conducted experiments; Liu F. performed data analysis; and Liu F and Zhuang S contributed to the writing of the manuscript:

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Figures

Figure 1

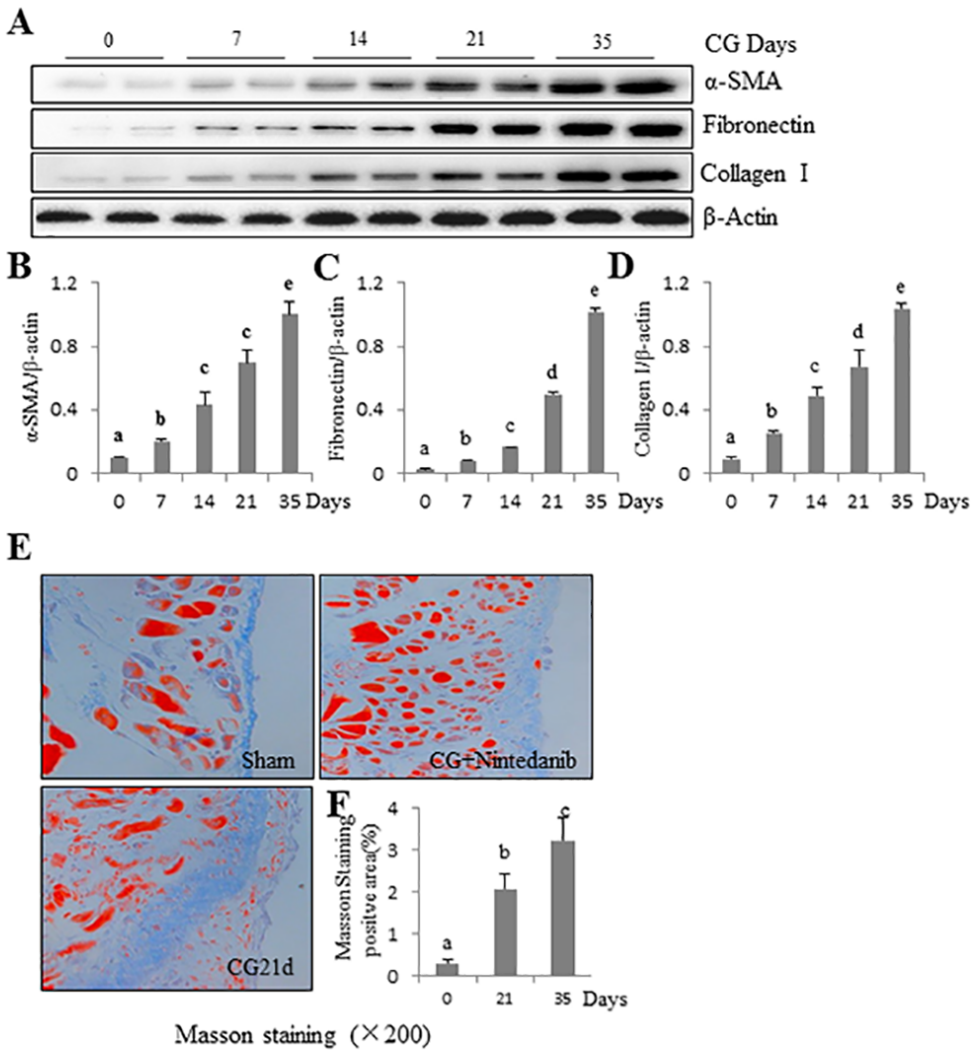

Figure 1

CG injection induces progression of peritoneal fibrosis in a time-dependent manner in mice. A. The peritoneum was taken for immunoblot analysis of α-SMA, fibronectin and collagen I and β-Actin as indicated. Representative immunoblots from 3 experiments are shown. Expression levels of α-SMA (B), fibronectin (C) and collagen I (D) were quantified by densitometry and normalized with β-Actin as indicated. Data are means±S.E.M. (n =6). E. Photomicrographs illustrate Masson trichrome staining of

the peritoneum at 0, 21 and 35 days after CG injection (×200). F. The graph shows the score of the Masson-positive submesothelial area (blue) from 10 random fields (200 ×) (means±S.E.M.)(n=6). Means with different superscript letters are significantly different from one another (P <0.05).

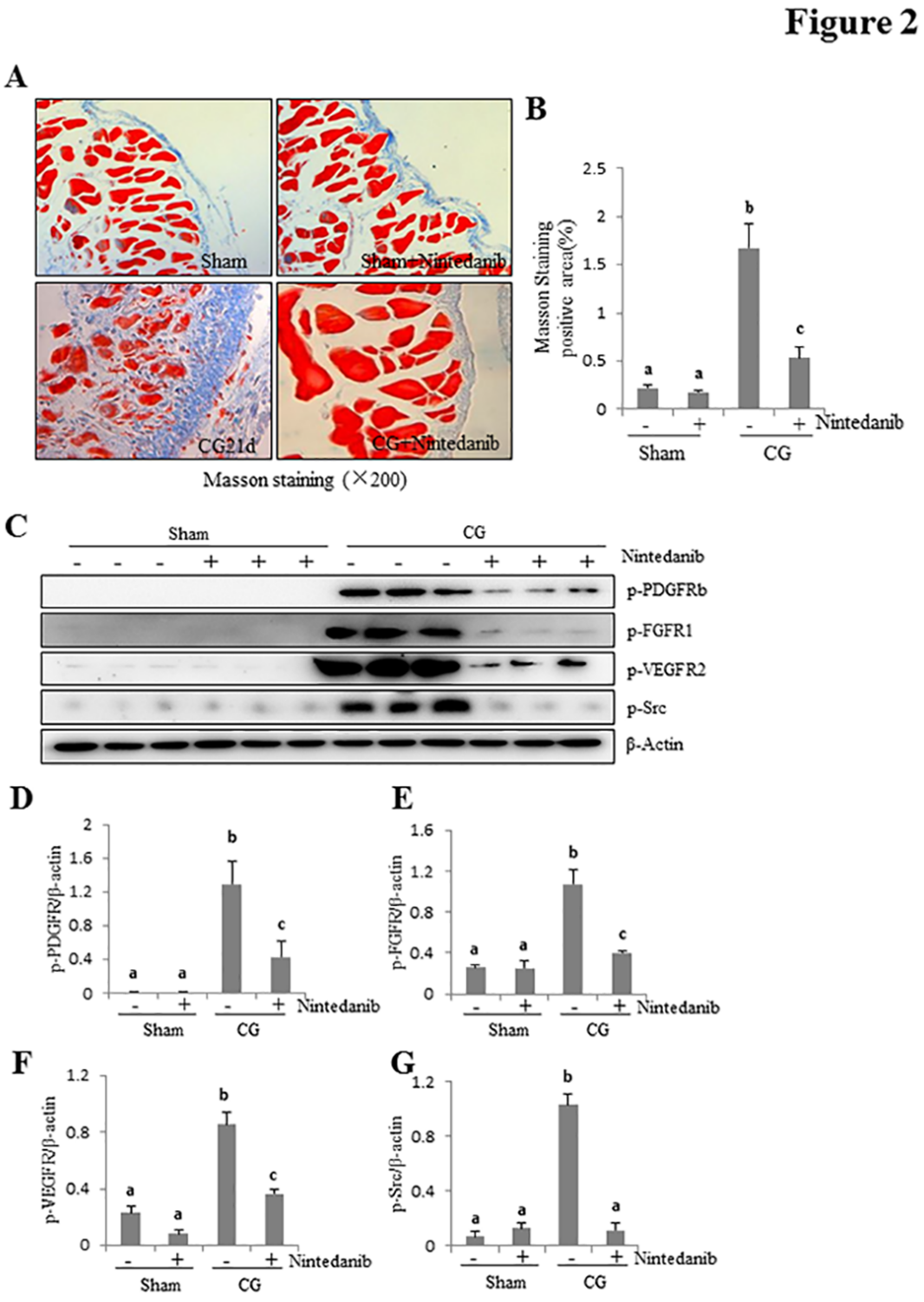

Figure 3

Nintedanib attenuates the development of peritoneal fibrosis and inhibits phosphorylation of multiple RTKs and Src in mice induced by CG injury. A. Photomicrographs illustrate Masson trichrome staining of

the peritoneum with or without nintedanib treatment (×200). B. The graph shows the score of the Masson-positive submesothelial area (blue) from 10 random fields (200 ×) (means±S.E.M.)(n=6). Means with different superscript letters are significantly different from one another (P <0.05). C. The peritoneum was taken for immunoblot analysis of phospho–PDGFRβ (p-PDGFRβ), phospho–FGFR1(p-FGFR1), phospho–VEGFR2 (p-VEGFR2), phospho–Src (p-Src) and β-Actin as indicated. Representative immunoblots from 3 experiments are shown. Expression levels of p-PDGFRβ (D), p-FGFR1 (E), p-VEGFR2 (F) and p-Src (G) were quantified by densitometry and normalized with β-Actin as indicated. Data are means±S.E.M. (n=6). Means with different superscript letters are significantly different from one another (P <0.05).

Figure 3

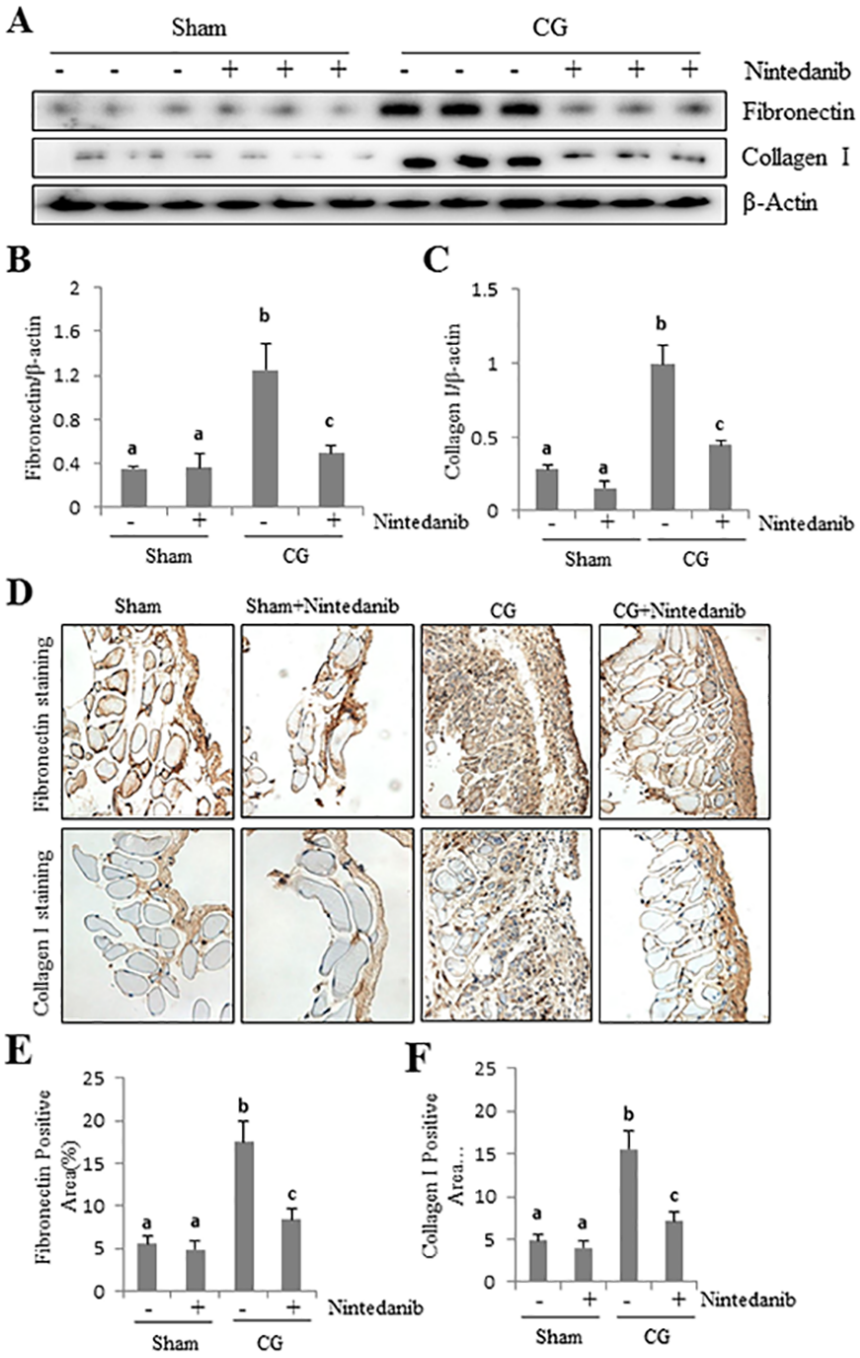

Figure 6

Nintedanib reduces the expression of collagen 1 and fibronectin in mice following CG injection. A. The peritoneum was taken for immunoblot analysis of fibronectin, collagen I and β-Actin as indicated. Representative immunoblots from 3 experiments are shown. Expression levels of fibronectin (B) and collagen I (C) were quantified by densitometry and normalized with β-Actin as indicated. Data are means±S.E.M. (n =6). Means with different superscript letters are significantly different from one another

(P <0.05). Photomicrographs illustrating immunohistochemistry staining of fibronectin or collagen I (D) in the peritoneum treated with or without nintedanib for 21 days. The graph shows the percentage of the immunohistochemistry-positive area (brown) for fibronectin (E) or collagen I (F) relative to the whole area from 10 random cortical fields (×200) (means±S.E.M.) (n=6).

Figure 4

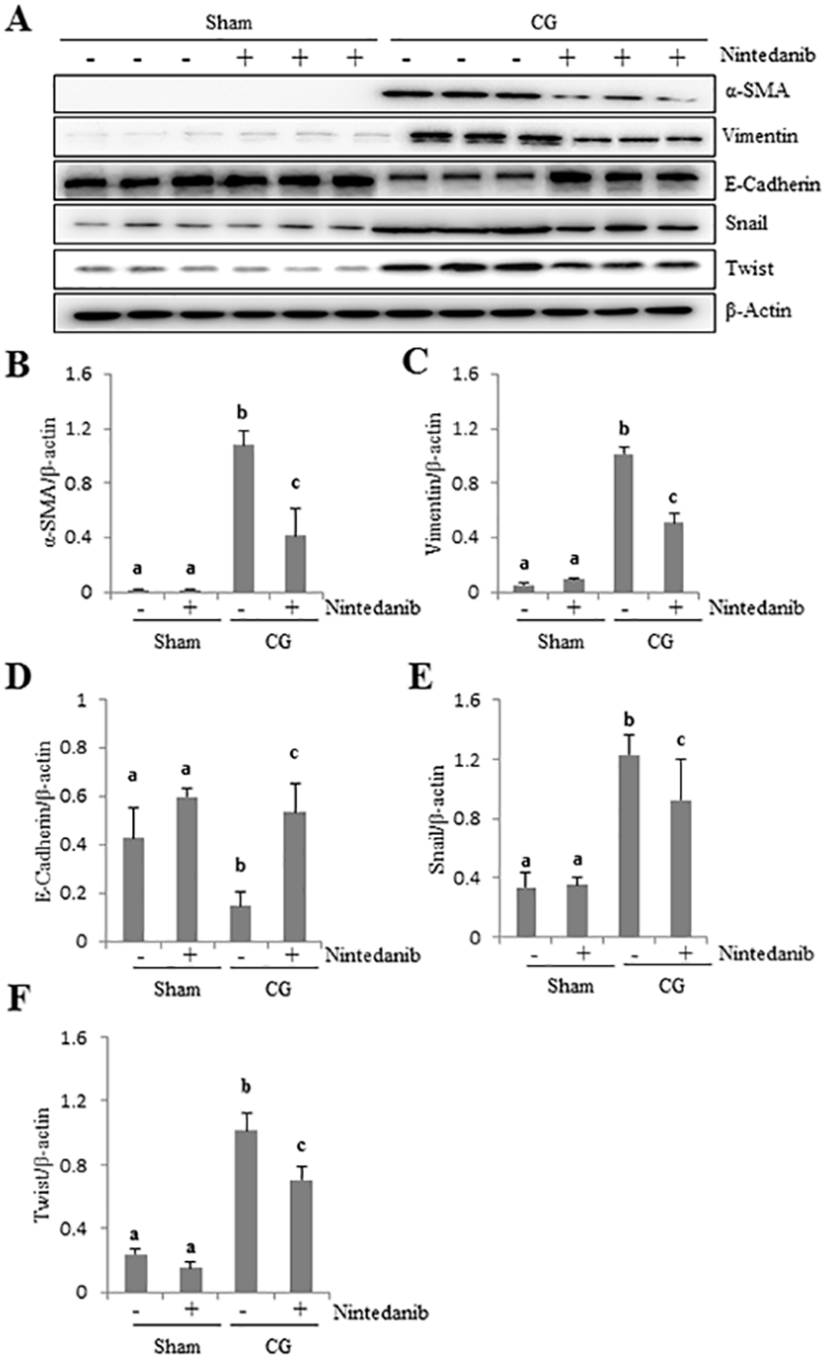

Figure 7

Nintedanib inhibits MMT in the peritoneum after CG injury. A. The peritoneum was taken for immunoblot analysis of α-SMA, vimentin, E-cadherin, Snail, Twist and β-Actin as indicated. Representative immunoblots from 3 experiments are shown. Expression levels of α-SMA (B), vimentin(C), E-cadherin (D), Snail (E), Twist (F) were quantified by densitometry and normalized with β-Actin as indicated. Data are means±S.E.M. (n =6). Means with different superscript letters are significantly different from one another (P <0.05).

Figure 5

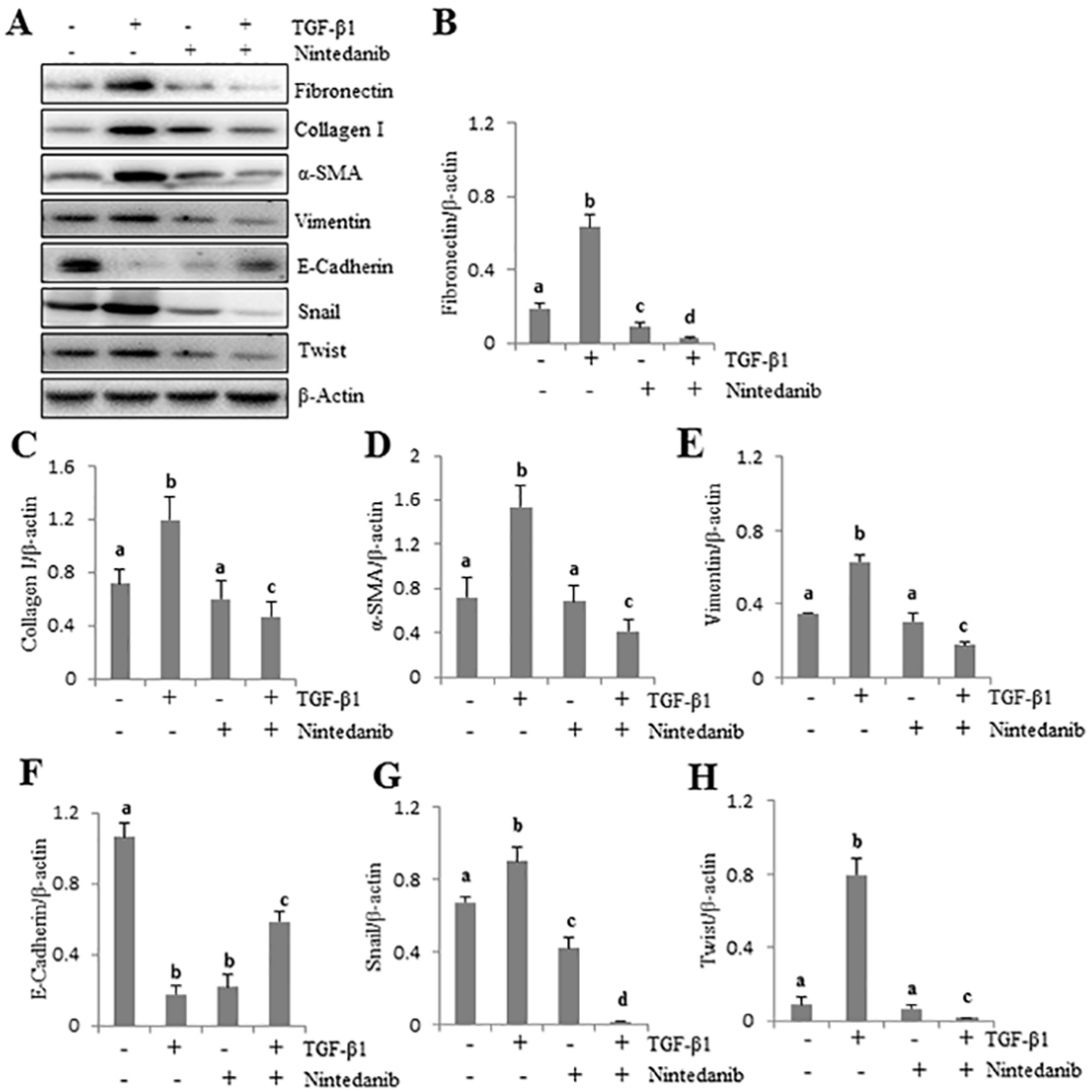

Figure 9

Nintedanib inhibits TGF1-induced MMT and expression of ECM proteins in cultured HPMCs. A, Immunoblot analysis shows the levels of fibronectin, collagen I,α-SMA, vimentin, E-cadherin, Snail, Twist and β-Actin in HPMCs after treatments by TGF-β1 in the presence or absence of nintedanib. Expression levels of fibronectin (B), collagen I (C), α-SMA (D), vimentin(E), E-cadherin (F), Snail (G) and Twist (H) were quantified by densitometry and normalized with β-Actin. Data are means± S.E.M. (n =6). Means with different superscript letters are significantly different from one another (P < 0.05).

Figure 6

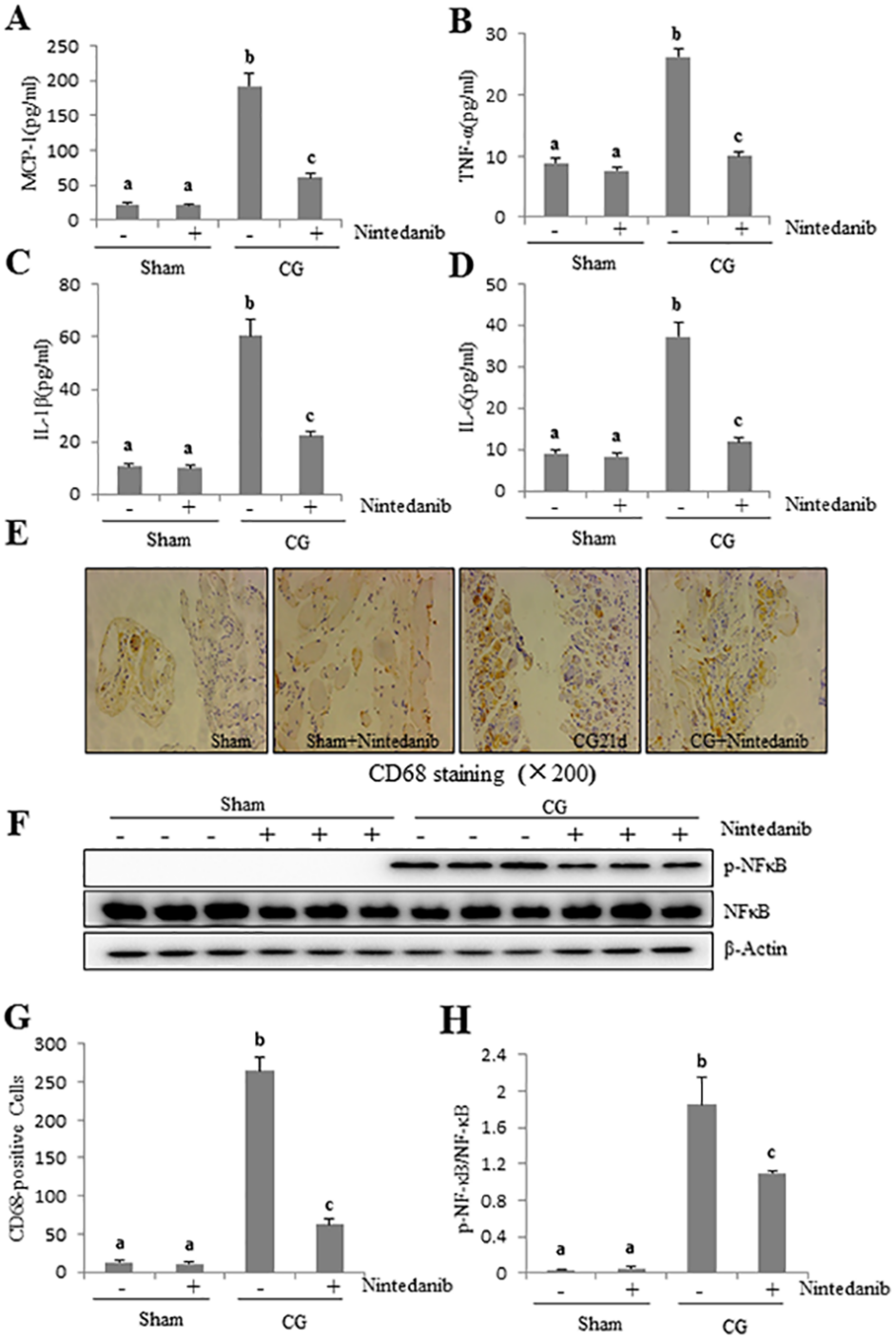

Figure 11

Nintedanib suppresses production of multiple proinflammatory cytokines/chemokines and infiltration of macrophages in the peritoneum after CG injury. Protein was extracted from the peritoneum of the mouse after CG injury with or without nintedanib administration, and subjected to the ELISA assay for MCP-1 (A), TNF-α (B), IL-1β (C), IL-6 (D). Photomicrographs illustrating immunohistochemistry staining of CD68-positive cells in the kidney tissue treated with or without nintedanib for 21 days (E). The peritoneum was taken for immunoblot analysis of phospho–NF-κB (p-NF-κB), NF-κB and β-Actin as indicated (F). Representative immunoblots from 3 experiments are shown. The graph shows the percentage of immunohistochemistry-positive area relative to the whole area from 10 random cortical fields (×200) (G). Expression levels of p-NF-κB/NF-κB were quantified by densitometry as indicated (H). Data are represented as the means± S.E.M. (n =6).Means with different superscript letters are significantly different from one another ($P<0.05$).

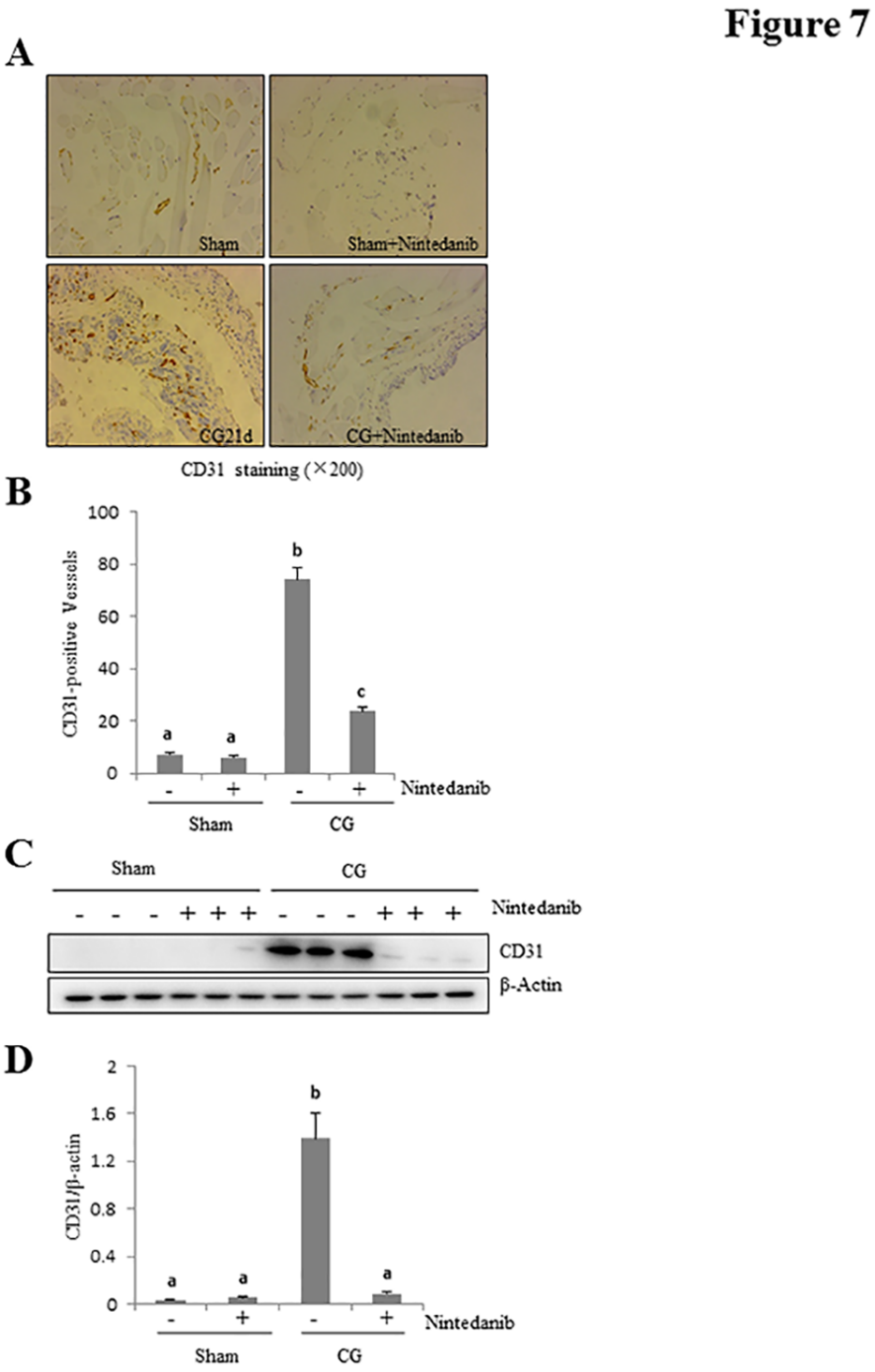

Figure 13

Nintedanib reduces angiogenesis in the peritoneum after CG injury. A. Photomicrographs illustrating immunohistochemistry staining of CD31-positive vessels in the kidney tissue treated with or without nintedanib for 21 days. B. The graph shows the number of CD31-positive vessels was calculated from ten random fields (original magnification, ×200) of six mouse peritoneal samples. (×200). C. The peritoneum was taken for immunoblot analysis of CD31 and β-Actin as indicated. Representative immunoblot from 3

experiments are shown. D. Expression levels of CD31 were quantified by densitometry and normalized with β-Actin as indicated. Data are represented as the means± S.E.M. (n =6). Means with different superscript letters are significantly different from one another (P<0.05).

Figure 8

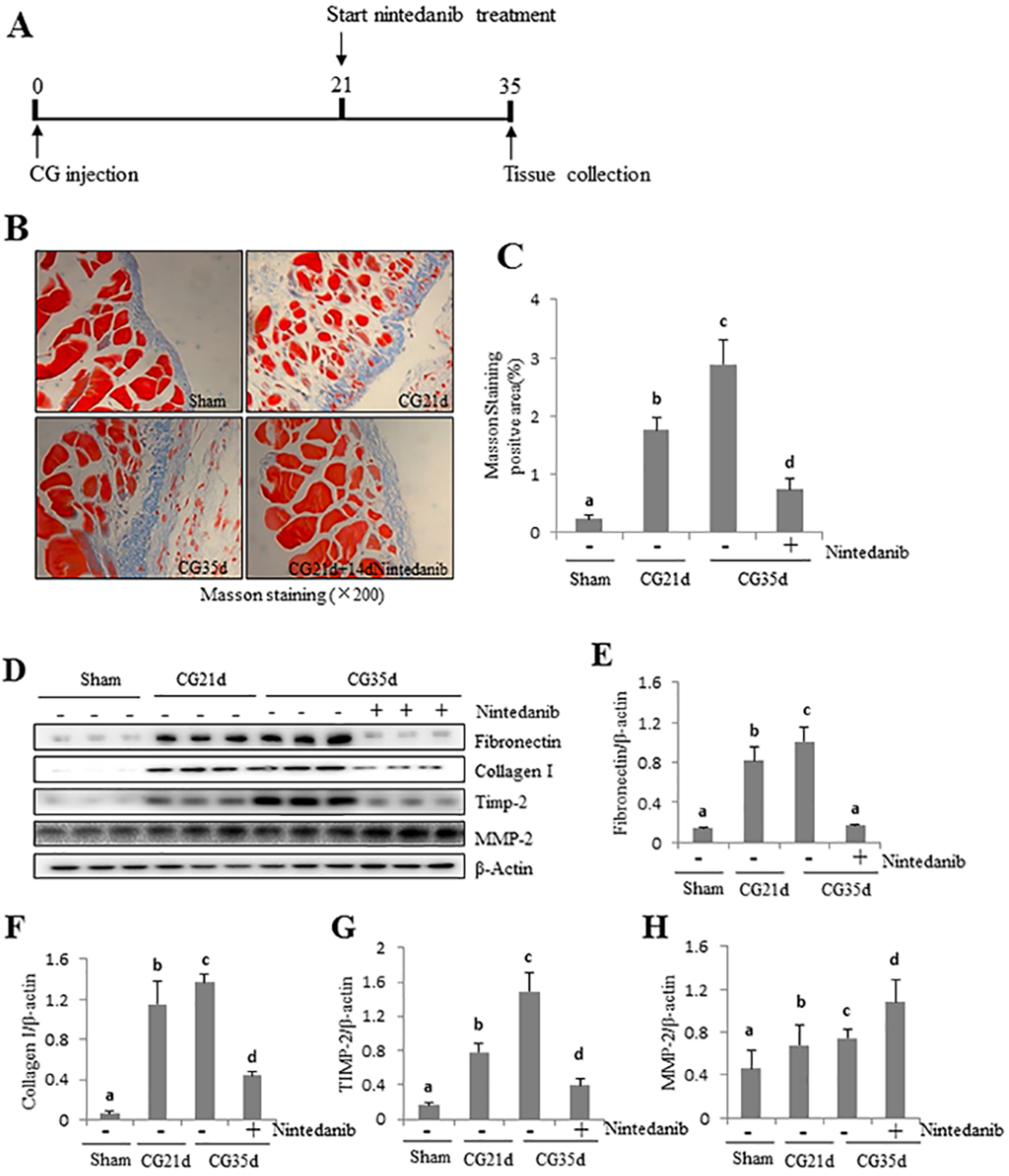

Figure 15

Delayed administration of nintedanib attenuates progression of peritoneal fibrosis and metabolism of ECM induced by CG injury. Mice received CG-injection and nintedanib treatment as described in Materials

and Methods (A). Photomicrographs illustrate Masson trichrome staining of the peritoneum with or without nintedanib treatment (×200) (B). The graph shows the score of the Masson-positive submesothelial area (blue) from 10 random fields (200 ×) (means±S.E.M.)(n=6) (C). The peritoneum was taken for immunoblot analysis of fibronectin, collagen I, Timp-2, MMP-2 and β-Actin as indicated. Representative immunoblots from 3 experiments are shown (C). Expression levels of fibronectin (E), collagen I (F), Timp-2 (G) and MMP-2 (H) were quantified by densitometry and normalized with β-Actin as indicated. Data are means±S.E.M. (n=6). Means with different superscript letters are significantly different from one another ($P < 0.05$).

Figure 9

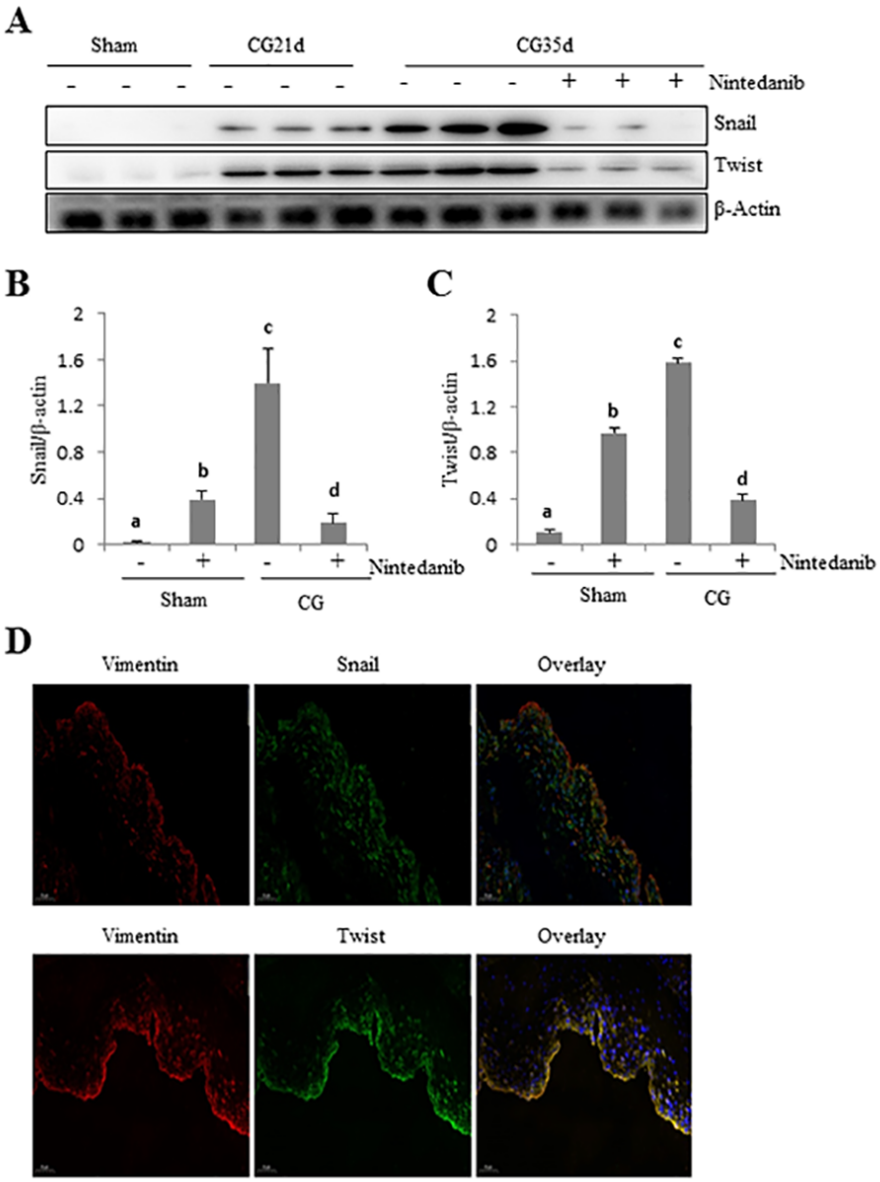

Figure 17

Delayed administration of nintedanib inhibits expression of Snail and Twist in the peritoneum after CG injury. The peritoneum was taken for immunoblot analysis of Snail, Twist and β-Actin as indicated (A). Representative immunoblots from 3 experiments are shown. Expression levels of Snail (B) and Twist(C) were quantified by densitometry and normalized with β-Actin as indicated. Data are means±S.E.M. (n =6). Means with different superscript letters are significantly different from one another (P <0.05). D.

Immunofluorescence staining shows the costaining of vimentin either Snail or Twist in the peritoneum at day 21 after CG injury.

Figure 10

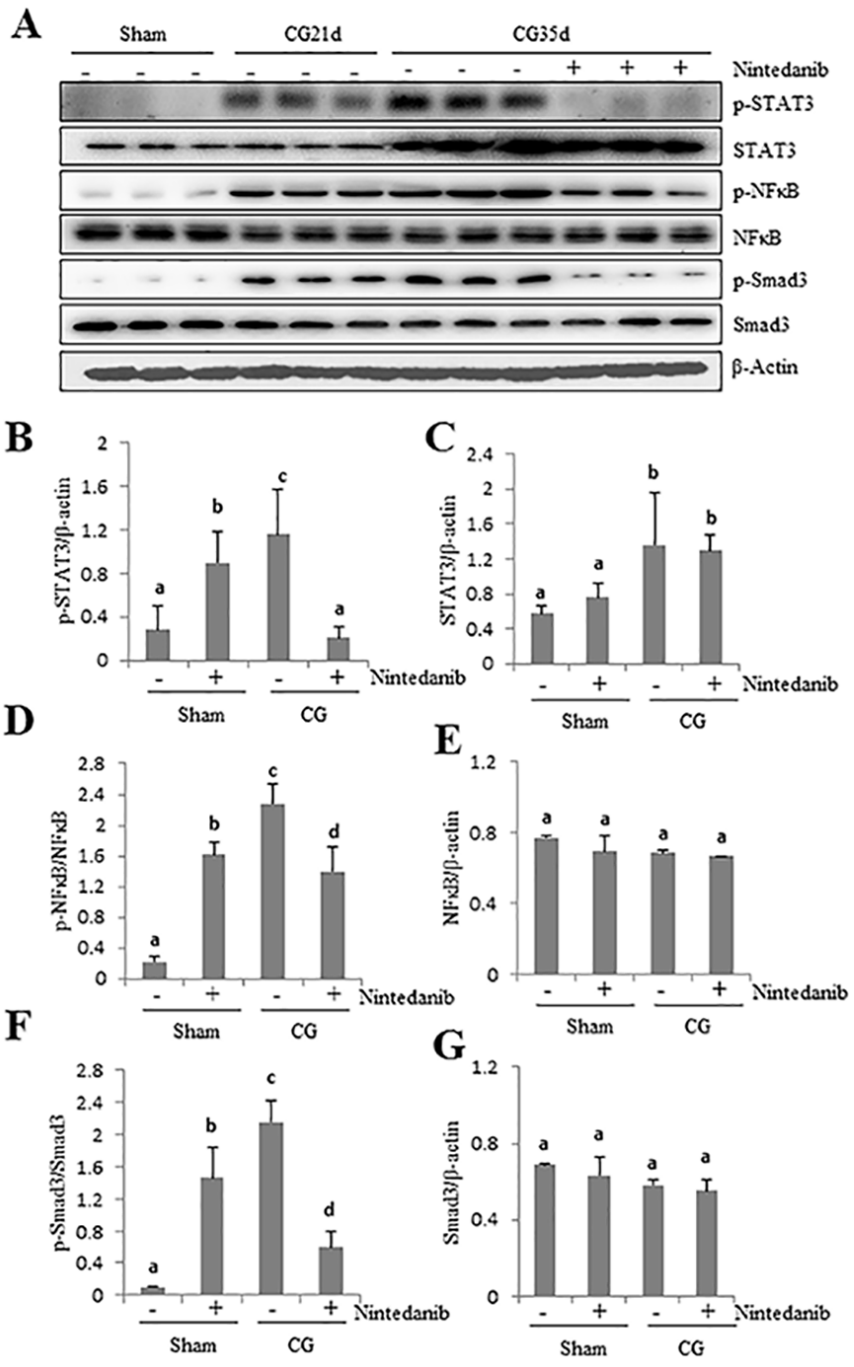

Figure 20

Delayed administration of nintedanib blocks phosphorylation of STAT3, NF-κB and Smad3 after CG injury. A, The peritoneum was taken for immunoblot analysis of phospho-STAT3 (p-STAT3), phospho-NF-κB (p-NF-κB) and phospho-Smad3 (p-Smad3), and total STAT3, NF-κB, Smad3 and β-Actin as indicated.

Representative immunoblot from 3 experiments are shown. Expression levels of p-STAT3 (B), p-NF-κB (D) and p-Smad3 (F) were quantified by densitometry and normalized with total STAT3, NF-κB, and Smad3 as indicated, respectively. Expression levels of total STAT3 (C), NF-κB (E) and Smad3 (G) were quantified by densitometry and normalized with β-Actin as indicated, respectively. Data are means± S.E.M. (n =6). Means with different superscript letters are significantly different from one another (P < 0.05).

Figure 11

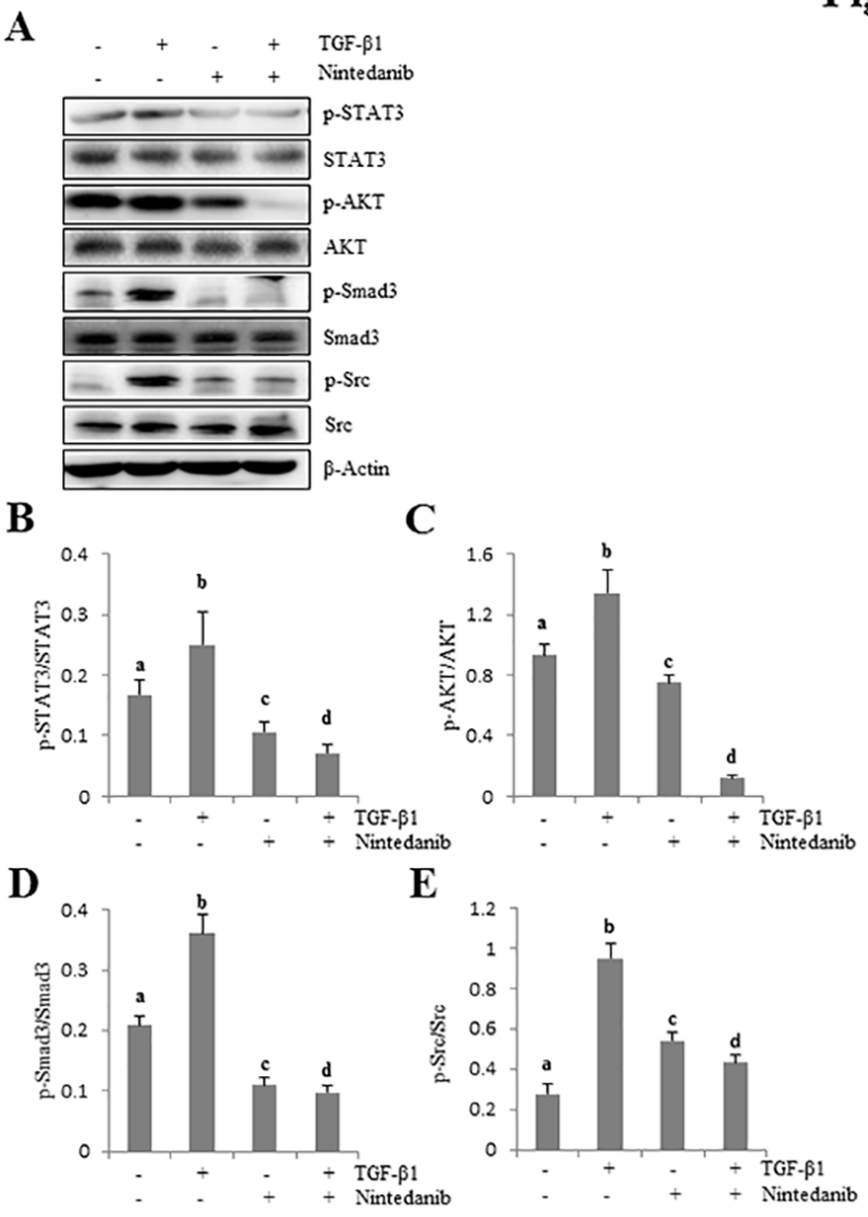

Figure 21

Nintedanib blocks TGF-β1-induced phosphorylation of STAT3, AKT, Smad3 and Src in cultured HPMCs. A, Immunoblot analysis shows the levels of phospho-STAT3 (p-STAT3), phospho-AKT (p-AKT), phospho-Smad3 (p-Smad3), phospho-Src (p-Src) and total STAT3, AKT, Smad3 and Src as indicated. Representative immunoblot from 3 experiments are shown. Expression levels of p-STAT3 (B), p-AKT (C), p-Smad3 (D) and p-Src (E) were quantified by densitometry and normalized with total STAT3, AKT, Smad3, and Src as indicated, respectively. Data are means± S.E.M. (n =6). Means with different superscript letters are significantly different from one another ($P < 0.05$).