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Delayed Administration of Nintedanib Ameliorates Fibrosis Progression in CG-Induced Peritoneal Fibrosis Mouse Model

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Keywords
Nintedanib · Receptor tyrosine kinases · Peritoneal fibrosis · Epithelial-to-mesenchymal transition · Inflammation · Chlorhexidine gluconate

Abstract
Background: A multiple-target tyrosine kinase inhibitor, nintedanib, which is approved for treatment of interstitial pulmonary disease, has been demonstrated to have anti-fibrotic activity outside of the lungs. We explored its therapeutic effect in a murine model of peritoneal fibrosis. ***Methods:*** Daily intraperitoneal injections of chlorhexidine gluconate (CG) induced peritoneal fibrosis in mice. The effects of delayed administration of nintedanib (given at day 21 after CG injection and then given daily for 14 days) were determined by immunohistochemical staining, ELISA, and immunoblot analysis. ***Results:*** Delayed administration of nintedanib significantly inhibited peritoneal fibrosis progression as indicated by decreasing deposition and expression of extracellular matrix (ECM) proteins (fibronectin and type I collagen). Treatment with nintedanib also upregulated MMP-2 and reciprocally downregulated TIMP-2, along with reducing expression of α-SMA, β-vimentin, and two transcription factors (Snail and Twist), and retaining E-cadherin expression. Nintedanib also inhibited co-expression of β-vimentin with Snail or Twist as shown by immunofluorescent staining. Moreover, nintedanib decreased the number of CD31-positive blood vessels and CD31 expression in the injured peritoneum. Moreover, delayed application of nintedanib inhibited the expression of several cytokines/chemokines, including monocyte chemoattractant protein-1, tumor necrosis factor-α, interleukin-1β (IL-1β), and IL-6, and infiltration of $CD68^+$ macrophages to the injured peritoneum. Finally, nintedanib blocked phosphorylation of STAT3, NF-κB, and Smad3 during the development of peritoneal fibrosis. ***Conclusions:*** Delayed administration of nintedanib inhibits progression of peritoneal fibrosis and partially reverses established peritoneal fibrosis by attenuating epithelial-mesenchymal transition, inflammation, and angiogenesis, as well as promoting ECM degradation. We conclude that nintedanib has a therapeutic potential to treat peritoneal fibrosis.

Introduction

Nintedanib is a small multiple-target tyrosine kinase inhibitor (TKI) that abrogates activation of platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), and Src family kinases [1]. Because of its anti-fibrotic properties, nintedanib was one of the first drugs approved for use in idiopathic pulmonary fi-

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brosis (IPF) and has recently further been approved for treatment of progressive fibrosing interstitial lung diseases (ILDs) and systemic sclerosis-associated ILD [2, 3]. Inspired by this breakthrough, many researchers, including our team, have examined the anti-fibrotic effect of nintedanib in other tissues and organs and found its anti-fibrotic effects in the liver, skin, kidney, and peritoneum [4–9].

Regardless of the disease type, there is currently no effective treatment for the established tissue fibrosis [1]. Fortunately, nintedanib offers a glimmer of hope because of its efficacy and safety in treating IPF or ILDs in clinical studies [3], as well as its therapeutic potential in other animal models of organ fibrosis. In the lung, fibroblasts isolated from IPF patients and murine models, Wollin et al. [10] demonstrated that nintedanib significantly reduced tissue inhibitors of metalloproteinases-2 (TIMP-2) and increased matrix metalloproteinases-2 (MMP-2), leading to reduction of total collagen production. Öztürk Akcora et al. [5] showed that nintedanib ameliorated inflammation, angiogenesis, and fibrosis in carbon tetrachloride-induced liver fibrogenesis in a mouse model. Juhl et al. [6] revealed that nintedanib halts skin fibrosis induced by transforming growth factor-β (TGF-β) or PDGF. Our recent study verified that delayed administration of nintedanib could inhibit renal fibrosis and inflammation induced by unilateral ureteral obstruction in mice [4, 8]. We also found that nintedanib can attenuate peritoneal fibrosis in a murine model of peritoneal fibrosis induced by chlorhexidine gluconate (CG) when given before peritoneal damage. However, the effect of this drug on established peritoneal fibrosis remains unknown and is examined here.

Compared with hemodialysis, peritoneal dialysis is a renal replacement therapy with superior quality-of-life measures and cost savings [11–14]. However, long-term usage and continuous exposure to hyperglycemic and acidic dialysis solutions lead to peritoneal fibrosis and dysfunction [15] due to accumulation of extracellular matrix (ECM), thickening of the sub-mesothelial cell layer, and vasculopathy [15]. Therefore, we explored the therapeutic effect and mechanisms of delayed administration of nintedanib on CG-induced peritoneal fibrosis in a mouse model.

Materials and Methods

Chemicals and Antibodies

Antibodies to type I collagen and fibronectin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to α-SMA, CD31, MMP-2, TIMP-2, E-cadherin, β-vimentin, Snail, Twist, MCP-1, TNF-α, interleukin-1β (IL-1β), IL-6, and ELISA assay kits were purchased from Abcam Inc (Cambridge, UK). Antibodies to p-STAT3, STAT3, p-Smad3, Smad3, p-NF-κB, NF-κB, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody to CD68 was purchased from Servicebio (Wuhan, China). Nintedanib was purchased from Cayman (Arbor, MI, USA). CG and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

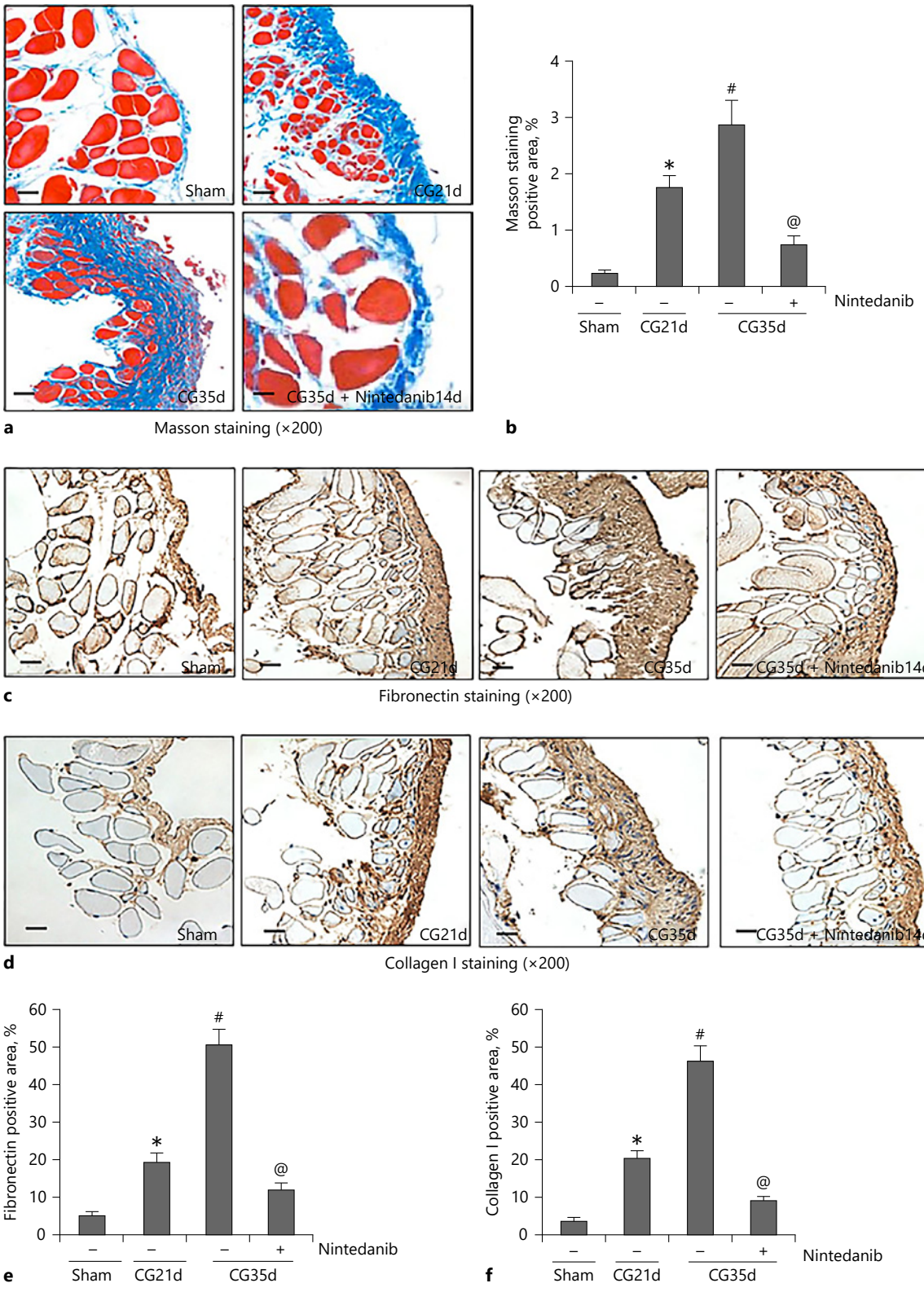
Establishment of Mouse Peritoneal Fibrosis Models and Nintedanib Administration

The peritoneal fibrosis model was established in male C57/BL6 mouse that weighed 24–28 g (Shanghai Super–B&K Laboratory Animal Corp. Ltd.), as described in our previous study [9]. Briefly, peritoneal fibrosis in mice was generated by intraperitoneal injection of 0.1% CG dissolved in 0.9% saline every other day for 21 days or 35 days. To examine the effect of delayed nintedanib administration on peritoneal fibrosis, nintedanib at 50 mg/kg was given by gavage on day 21 after CG injection and then daily for 14 days. Mice were randomly divided into four groups with 6 mice per group: mice were administered with an equivalent amount of saline and DMSO (Group 1); mice were administered with 0.1% CG and an equivalent amount of DMSO for 21 days (Group 2) or 35 days (Group 3); mice were administered with 0.1% CG for 35 days, and nintedanib (50 mg/kg/daily) for 14 days, starting at 21 days after first injection of CG (Group 4). At 21 (Group 2) or 35 (Group 1, 3, 4) days, mice were euthanized and the parietal peritoneum apart from the injection points was harvested for further analysis. All the animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee at Tongji University and Shanghai East Hospital.

Histochemical and Immunofluorescent Staining

Formalin-fixed samples of peritoneum were embedded in paraffin and processed to prepare 3-μm-thick sections. Immunohistochemical staining was conducted as described in our previous study [9]. To evaluate peritoneal fibrosis, Masson trichrome staining was performed according to the protocol provided by the manufacturer (Sigma-Aldrich). The collagen tissue area (blue color) was quantitatively measured using Image Pro-Plus software (Media-Cybernetics, Silver Spring, MD, USA) by drawing a line around the perimeter of the stained area, and the average ratio to each microscopic field (×200) was calculated and graphed. The thickness of the sub-meso-

Fig. 1. Delayed administration of nintedanib attenuates progression of peritoneal fibrosis, deposition 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 sub-mesothelial area (blue) from 10 random fields (×200) (means ± SEM) ($n = 6$). Photomicrographs illustrating immunohistochemistry staining of fibronectin (**c**) or collagen I (**d**) in the peritoneum treated with or without nintedanib. The graph shows the percentage of immunohistochemistry-positive area (brown) for fibronectin (**e**) or collagen I (**f**) relative to the whole area from 10 random cortical fields (×200) (means ± SEM) ($n = 6$). $^{*,\#,@}p < 0.05$ versus sham control. Means with different superscript symbols ($^{*,\#,@}$) are significantly different from one another. *(For figure see next page.)*


Sham
CG21d
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a
Masson staining (×200)
Masson staining positive area, %
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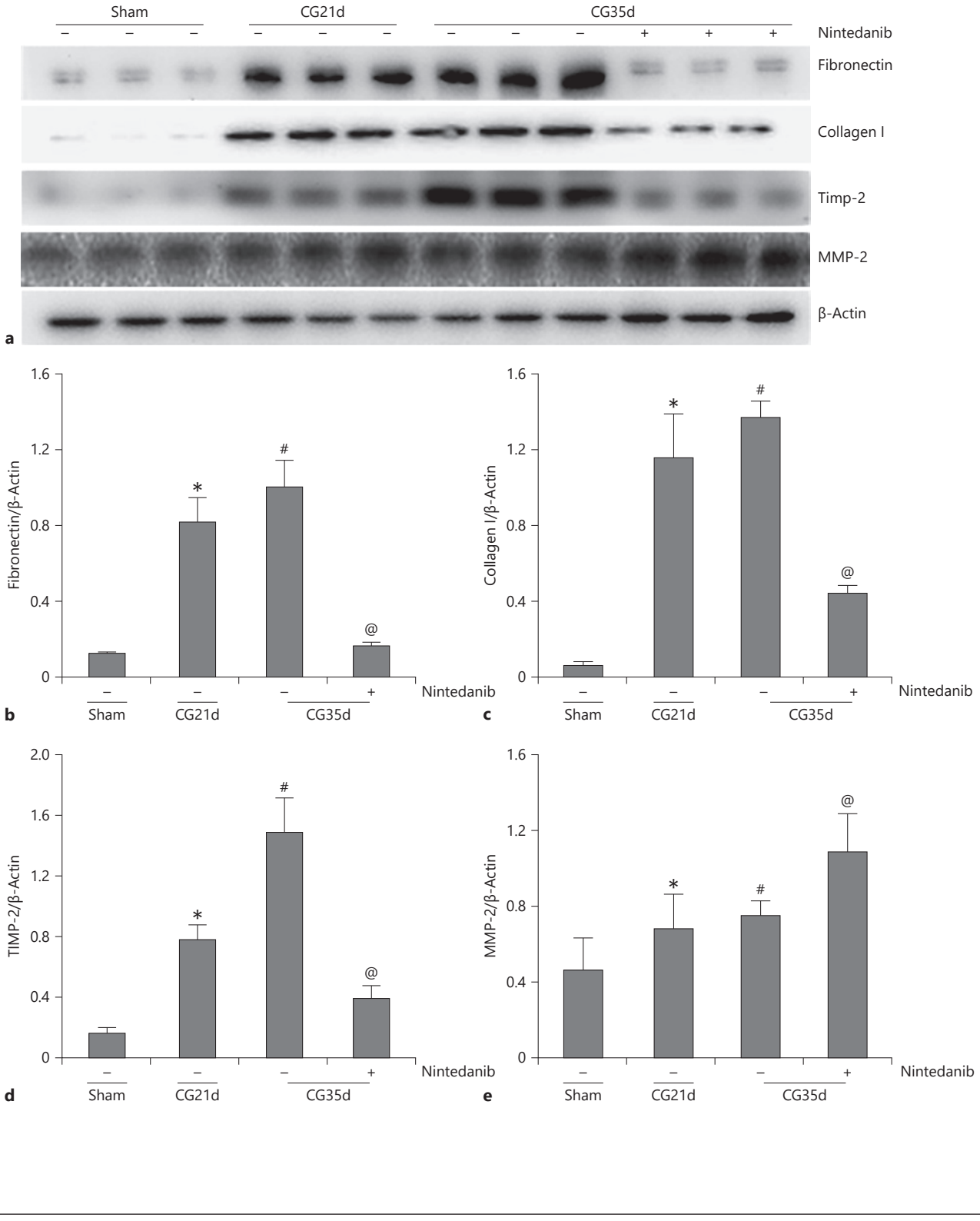

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thelial tissue was evaluated (in micrometers), and the average of ten independent measurements was calculated for each section (original magnification, ×200). Fibronectin, collagen I, CD31, and CD68 expression in peritoneum tissue were assessed by immunohistochemical staining. Co-expression of β-vimentin and Snail or Twist in peritoneum tissue was assessed by co-focal immunofluorescent staining using a Zeiss 710 Duo microscope (Zeiss, Germany).

Immunoblot Analysis

Immunoblot analysis of peritoneum tissue samples was conducted as described previously [9]. The densitometry analysis of immunoblot results was conducted using Image J software developed at the National Institute of Health. The quantification data are given as the ratio between target protein and loading control.

ELISA Analysis

To examine the expression of MCP-1, TNF-α, IL-1β, and IL-6, mouse peritoneum was homogenized in an extraction buffer. The supernatant recovered after centrifugation was used for determination of these chemokine/cytokines by commercial Quantikine ELISA kits in accordance with the protocol specified by the manufacturer (Abcam Inc, 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 ± SEM for each group. For all the experiments, the differences between two groups were made using one-way ANOVA followed by the Tukey test. Statistically significant difference between mean values was marked in each graph. $p < 0.05$ was considered a statistically significant difference between mean values. The statistical analyses were conducted by using IBM SPSS Statistics 20.0.

Results

Delayed Administration of Nintedanib Attenuates Progression of Peritoneal Fibrosis and Deposition of ECM Induced by CG Injury

To explore the therapeutic effect of nintedanib on peritoneal fibrosis, nintedanib at 50 mg/kg was given late at day 21 after CG injection, when peritoneal fibrosis had already developed to an advanced stage. Following additional 14 days of treatment, peritoneum was collected to assess the degree of peritoneal fibrosis and deposition of ECM. As shown in Figure 1a, b, our results from Masson trichrome staining demonstrated that the increased thickness of the sub-mesothelial compact zone and Masson-positive areas were observed at 21 days and were further increased at 35 days after CG injection; delayed administration of nintedanib significantly decreased Masson-positive blue areas in the peritoneum.

The deposition of ECM proteins (mainly including fibronectin and collagen I) in the peritoneum are the hallmark of peritoneal fibrosis. As shown in Figure 1c–f, the results from immunohistochemical staining demonstrated that the expression levels of fibronectin and collagen I increased in the sub-mesothelial compact zone at 21 days and were elevated at 35 days after CG injury; delayed administration of nintedanib significantly reduced their deposition induced by CG injury.

Delayed Administration of Nintedanib Reduces Expression and Metabolism of ECM Induced by CG Injury

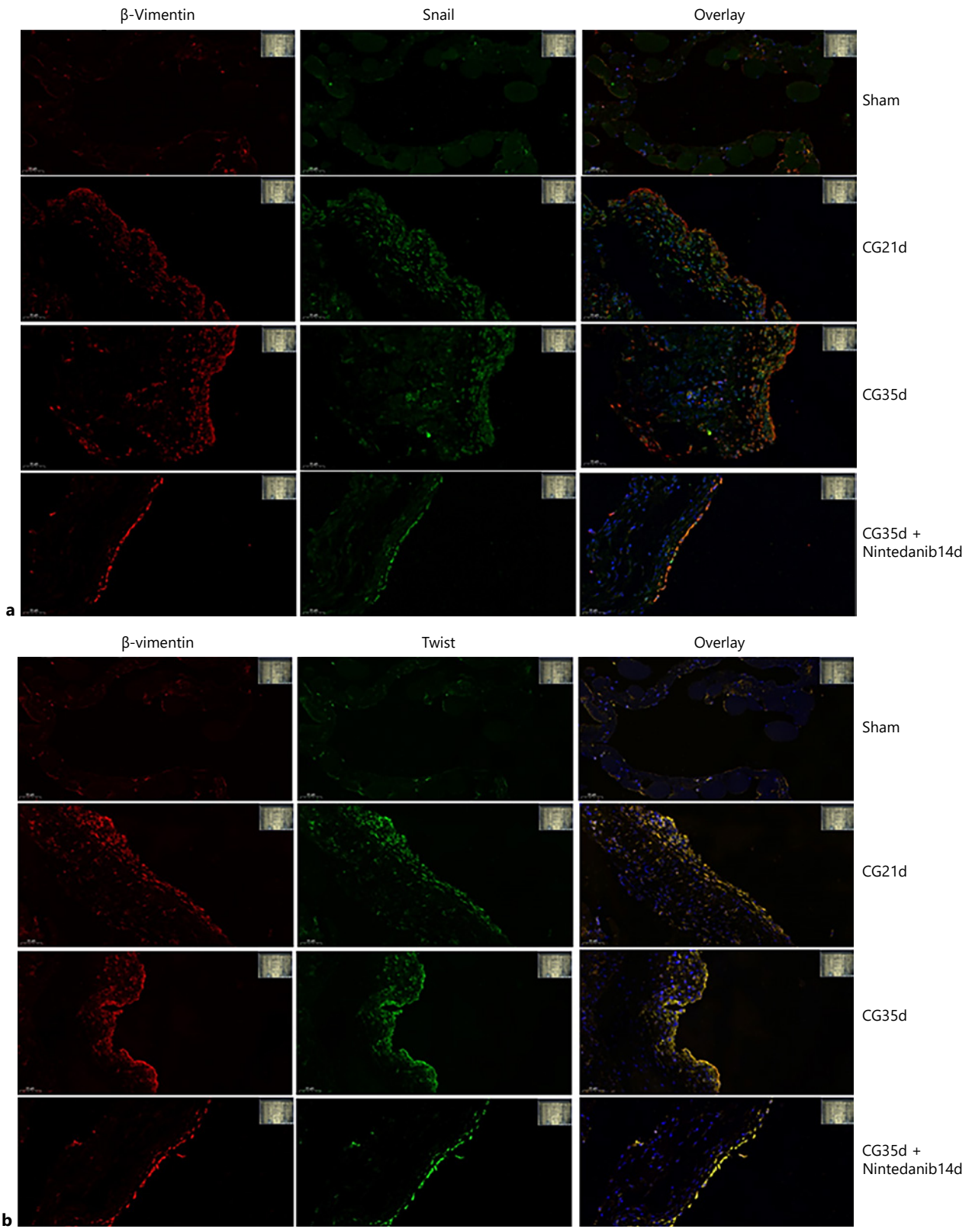
Consistent with the results of immunohistochemical staining, our immunoblot analysis results showed that expression of fibronectin and collagen I increased after CG injury, and delayed administration of nintedanib significantly reduced their expression to a level below that observed on day 21 after CG injection (Fig. 2a–c).

The metabolism of ECM proteins is regulated by MMPs and TIMPs. Figure 2a, d, e showed that MMP-2 and TIMP-2 expression levels increased after CG injury; delayed administration of nintedanib inhibited TIMP-2 expression but increased MMP-2 expression. Hence, these data demonstrated that nintedanib not only prevents ECM overproduction and deposition but also partially promotes ECM degradation in the injured peritoneum through a mechanism regulating expression of TIMP-2 and MMP-2.

Delayed Administration of Nintedanib Inhibits EMT of PMCs after CG Injury

The epithelial transition to mesenchymal cells (EMT) of PMCs has been identified as a major mechanism contributing to peritoneal fibrosis [15]. As shown in Figure 3, co-staining of β-vimentin (markers of mesenchymal cell) and Snail or Twist (transcription factors) indicated that β-vimentin is expressed mostly in Snail- or Twist-positive cells, and the number of double-positive cells increased in CG21d and CG35d groups and decreased in

Fig. 2. Delayed administration of nintedanib reduces expression and metabolism of ECM induced by CG injury. **a** 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. Expression levels of fibronectin (**b**), collagen I (**c**), Timp-2 (**d**) and MMP-2 (**e**) were quantified by densitometry and normalized with β-actin as indicated. Data are means ± SEM ($n = 6$). $^{*,\#,@}p < 0.05$ versus sham control. NS means no significance versus sham control. Means with different superscript symbols ($^{*,\#,@}$) are significantly different from one another.



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the CG35d treated with nintedanib. Moreover, our results from immunoblot analysis showed an inhibitory effect of nintedanib on the development of EMT. Following additional 14 days of treatment, beginning at day 21 after CG injection, nintedanib significantly decreased expression of α-SMA, β-vimentin, Snail and Twist, while expression of E-cadherin was preserved (Fig. 4a–f). These results suggest delayed treatment of nintedanib inhibits the EMT in PMCs exposed to CG.

Delayed Administration of Nintedanib Reduces Angiogenesis in the Peritoneum after CG Injury

Angiogenesis in the fibrotic sub-mesothelial zone is another feature of peritoneal fibrosis, which leads to peritoneal functional decline [16]. We examined the expression of CD31, a marker of endothelial cells, in the peritoneum by immunohistochemical staining and immunoblot analysis. As shown in Figure 5a, b, CG injection for 21 days induced an increase of CD31 (+) vessels increased to the peak at 35 days; delayed administration of nintedanib significantly suppressed this response. Our immunoblot analysis results (Fig. 5c, d) confirmed the inhibitory effect of nintedanib on CD31 expression. This suggested that nintedanib may reduce the formed angiogenesis in the peritoneum after CG injury.

Delayed Administration of Nintedanib Suppresses Production of Multiple Proinflammatory Cytokines/Chemokines and Infiltration of Macrophages in the Peritoneum after CG Injury

Nintedanib had been proved to possess a strong anti-inflammation effect during the process of respiratory, liver, and renal fibrosis [4, 5, 10]. Hence, we examined the effect of delayed administration of nintedanib on the inflammation response induced by CG in the peritoneum. As shown in Figure 6a–d, delayed administration of nintedanib reduced the elevation of inflammatory cytokines/chemokines, including MCP-1, TNF-α, IL-1β, and IL-6 after CG injury by ELISA assay. Immunohistochemical staining showed that the number of CD68 (a marker of macrophages)-positive macrophages increased in the sub-mesothelial layer after CG injury, while delayed administration of nintedanib significantly reduced this macrophage infiltration (Fig. 6e, f). Therefore, nintedanib may also inhibit the progression of peritoneal fibrosis via its anti-inflammation effect.

Fig. 3. Delayed administration of nintedanib decreased co-staining expression of β-vimentin and Snail in the peritoneum after CG injury. **a** Double (Con-focal) immunofluorescence staining shows the co-staining of β-vimentin and Snail in the peritoneum after CG injury (21 or 35 days) treated with or without nintedanib for another 14 days. **b** Double immunofluorescence staining shows the co-staining of β-vimentin and Twist in the peritoneum after CG injury (21 or 35 days) treated with or without nintedanib for another 14 days.

Delayed Administration of Nintedanib Blocks Phosphorylation of STAT3, NF-κB, and Smad3 after CG Injury

Because of multiple fibrotic signaling pathways, including STAT3, NF-κB, and Smad3, activated and involved in peritoneal fibrosis [9], we explored the effect of nintedanib on the CG-induced activation of these pathways in the peritoneum. A significant increase of STAT3, NF-κB, and Smad3 phosphorylation was observed at 21 days and increased to a peak at 35 days after CG injury (Fig. 7a–g). Delayed administration of nintedanib resulted in reducing phosphorylation of these signaling molecules. Notably, nintedanib did not affect expression levels of total STAT3, NF-κB, and Smad3 (Fig. 7a, c, e, g). Thus, delayed administration of nintedanib can suppress CG-induced phosphorylation of STAT3, NF-κB, and Smad3 in the fibrotic peritoneum.

Discussion

Peritoneal fibrosis is a serious complication for patients undergoing long-term peritoneal dialysis, which is closely associated with EMT, deposition of ECM components, angiogenesis, and inflammation [17]. Despite multiple factors and mechanisms identified contributing to peritoneal fibrosis, so far available therapeutic treatments are scarce. In the current study, we demonstrated that delayed administration of nintedanib, multiple tyrosine kinase receptors (RTKs) inhibitor, significantly attenuated peritoneal fibrosis as indicated by decreasing accumulation of collagen fibrils, inhibiting EMT phenotype, and diminishing inflammatory responses and angiogenesis in the peritoneum of mice exposed to CG. Given that nintedanib has been approved to treat human IPF, progressive fibrosing ILDs, and systemic sclerosis-associated ILD, we believe that our findings have potential to be translated to clinical application for patients with peritoneal fibrosis.

Previous studies have demonstrated that multiple TKIs contribute to peritoneal fibrosis. Among the RTKs targeted by nintedanib [18], PDGFR activation can stimulate ECM deposition in an animal model of peritoneal

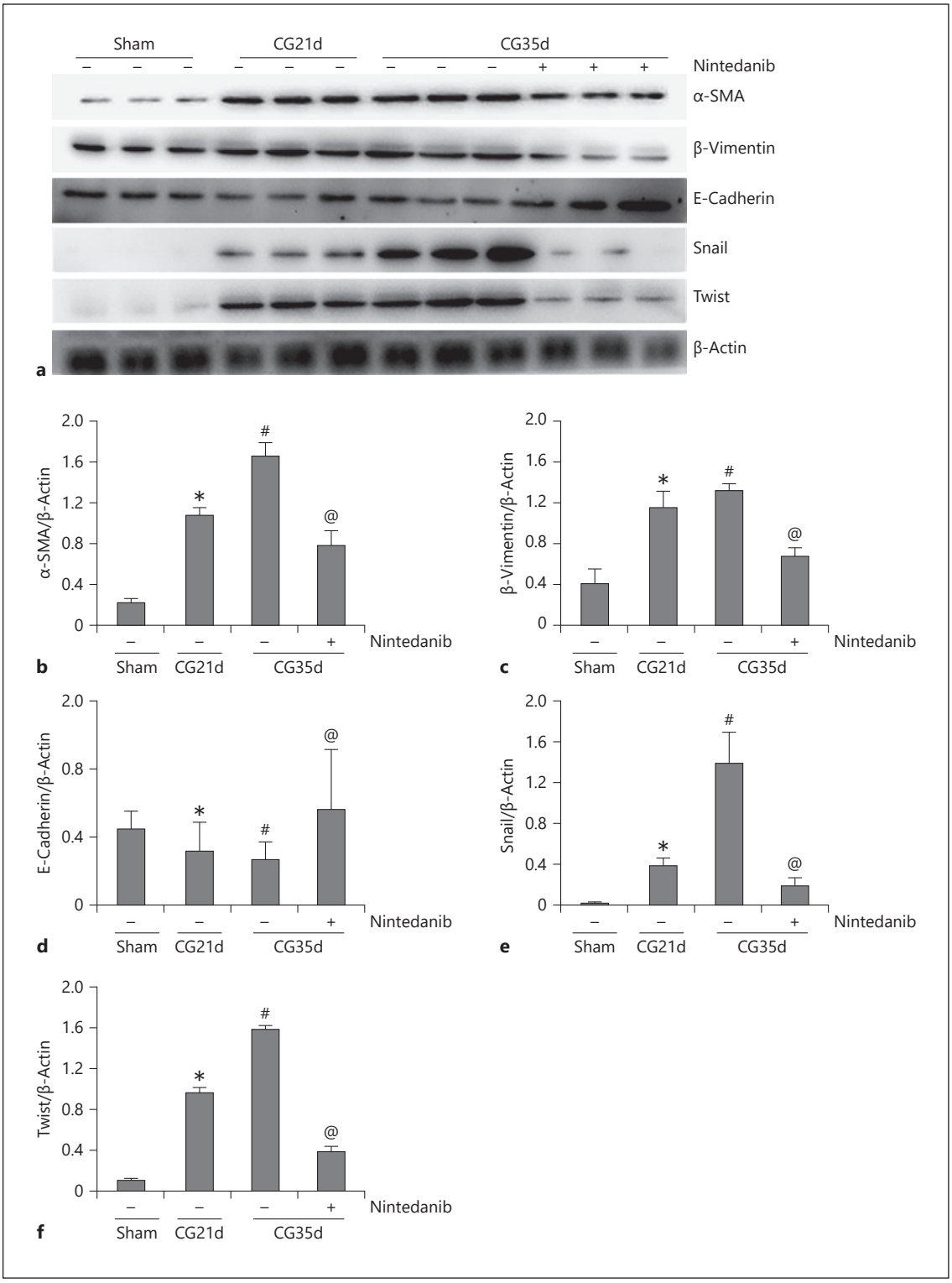

Fig. 4. Delayed administration of nintedanib inhibits EMT of PMCs after CG injury. **a** The peritoneum was taken for immunoblot analysis of α-SMA, β-vimentin, E-cadherin, Snail, Twist, and β-actin as indicated. Representative immunoblots from 3 experiments are shown. Expression levels of α-SMA (**b**), β-vimentin (**c**), E-cadherin (**d**), Snail (**e**), and Twist (**f**) were quantified by densitometry and normalized with β-actin as indicated. Data are means ± SEM ($n = 6$). $^{*,\#,@}p < 0.05$ versus sham control. Means with different superscript symbols ($^{*,\#,@}$) are significantly different from one another.

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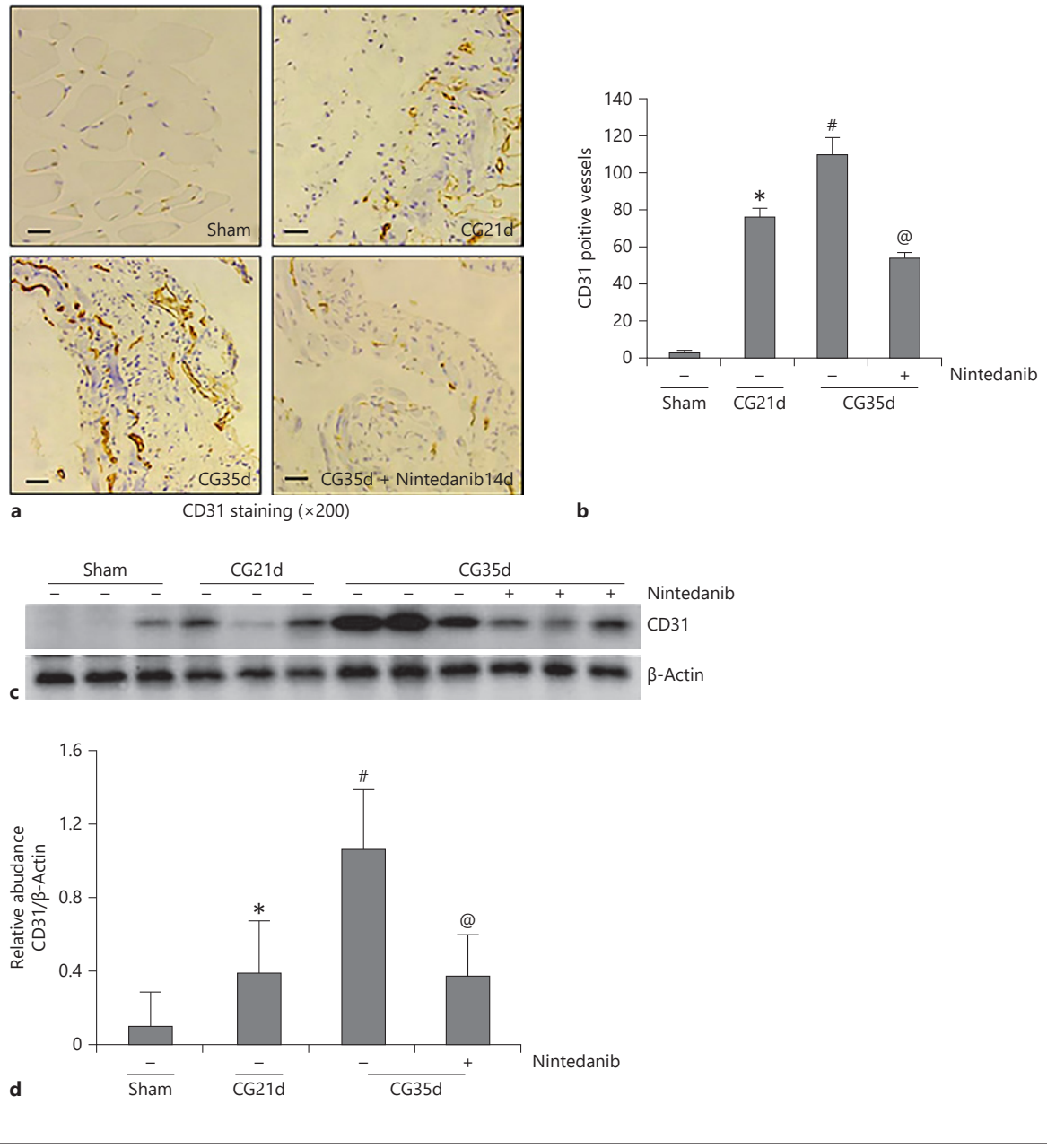
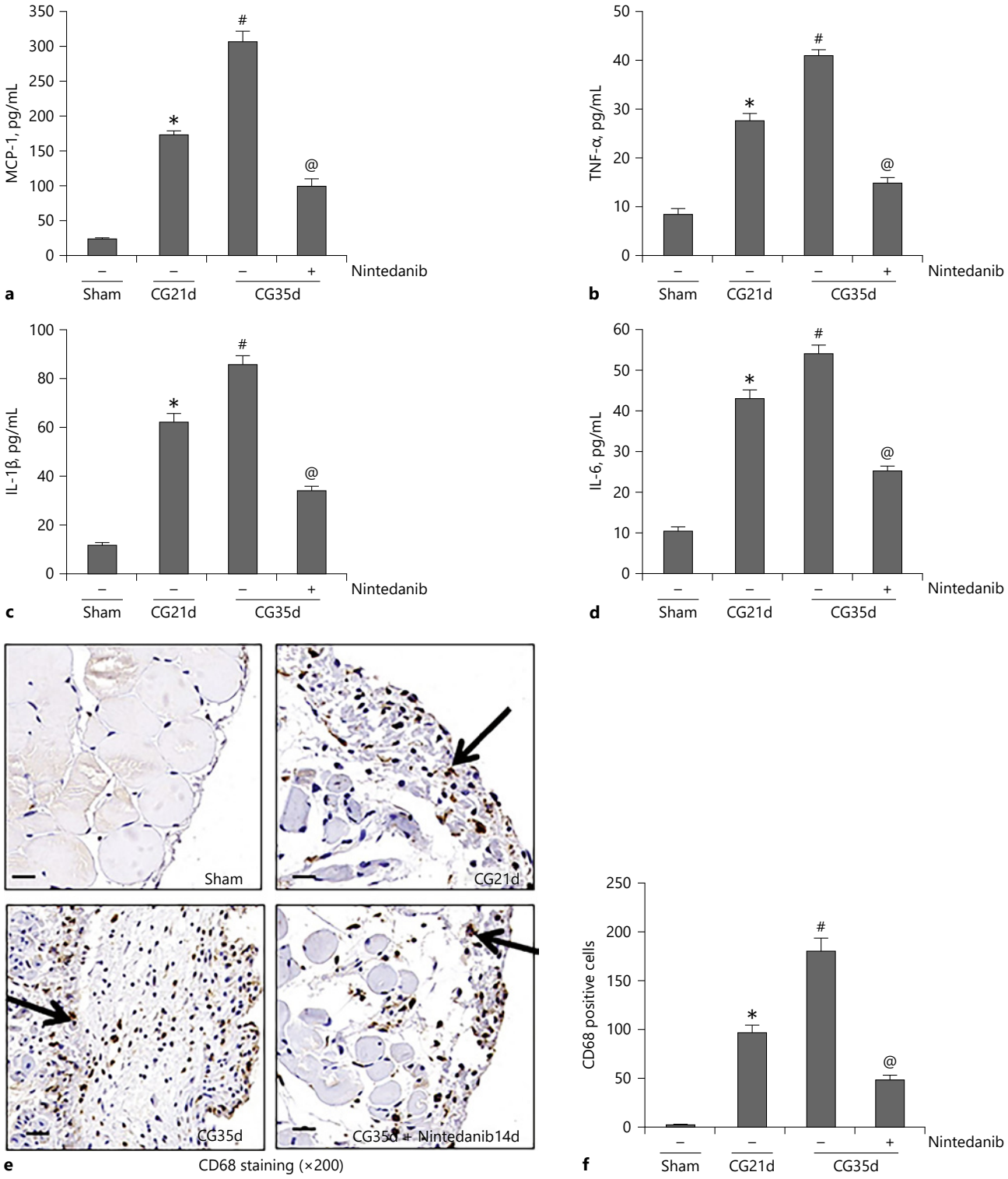


Fig. 5. Delayed administration of nintedanib reduces angiogenesis in the peritoneum after CG injury. **a** Photomicrographs illustrating immunohistochemical staining of CD31-positive vessels in the peritoneum treated with or without nintedanib. **b** The graph shows the number of CD31-positive vessels was calculated from 10 random fields (×200) (means ± SEM) (n = 6). **c** The peritoneum was taken for immunoblot analysis of CD31 and β-actin as indicated. Representative immunoblots from 3 experiments are shown. **d** Expression levels of CD31 was quantified by densitometry and normalized with β-actin as indicated. Data are means ± SEM (n = 6). $^{*,\#,@}p < 0.05$ versus sham control. Means with different superscript symbols ($^{*,\#,@}$) are significantly different from one another.



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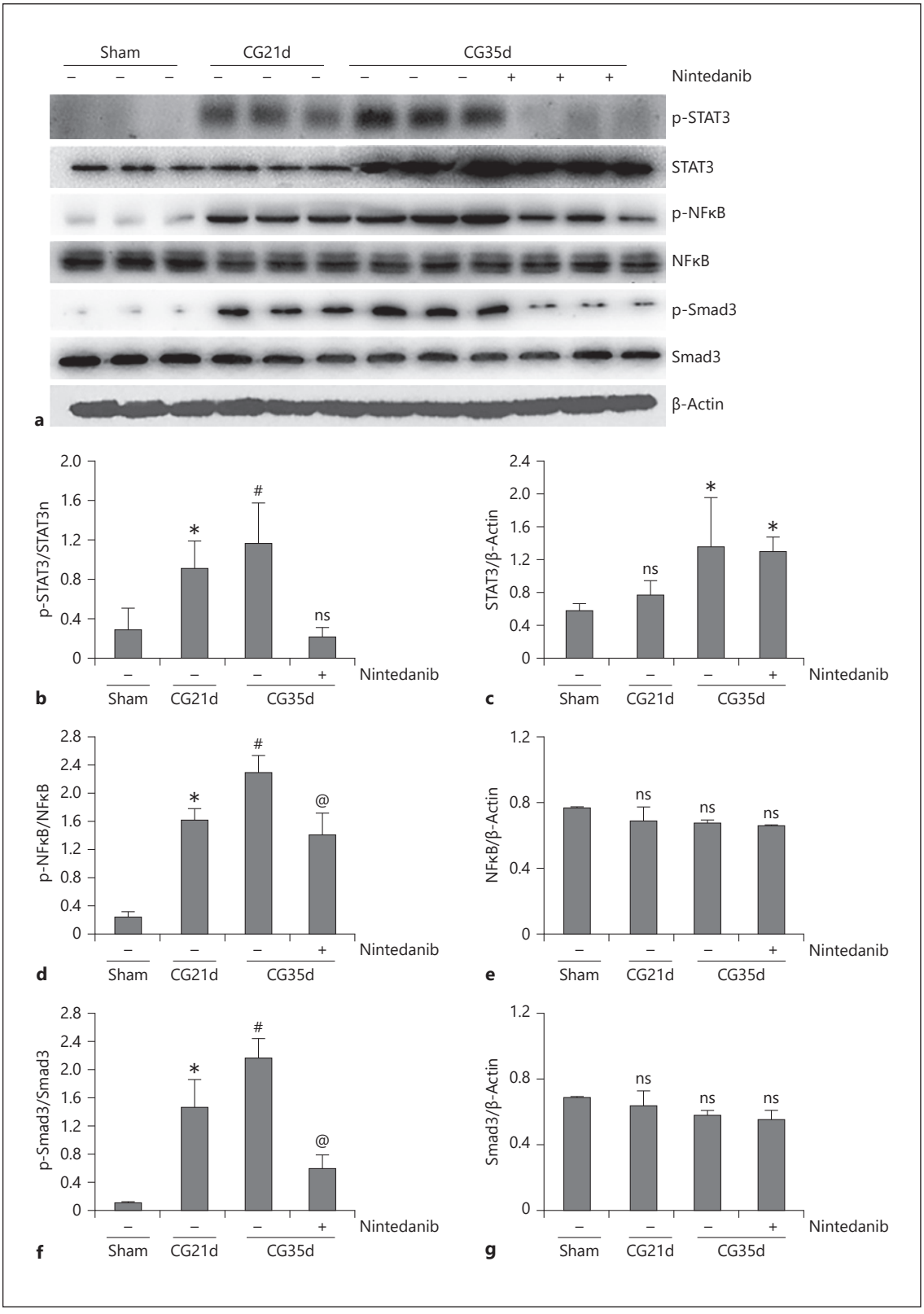
fibrosis and the EMT of PMCs in culture [19]. FGFR activation is required for interstitial fibrosis of the peritoneum [20], and VEGFR activation is associated with angiogenesis and increased permeability of peritoneal capillaries [21, 22]. Moreover, Src, an nRTK, is involved in the pathogenesis of peritoneal fibrosis [23]. As a potent inhibitor of all these kinases, nintedanib was shown to inhibit the progression of tissue fibrosis in lung and other organs, including the liver, skin, and kidney [4–6]. In agreement with findings in these organs, our previous study showed that nintedanib was able to prevent the development of peritoneal fibrosis when it was given at the beginning of CG injection [9]. Our current study extended this observation by illustrating that delayed administration of nintedanib not only halted the progression of peritoneal fibrosis but also in part reversed established peritoneal fibrosis. This was evidenced by the finding that application of nintedanib at 21 days after CG injection for additional 14 days reduced deposition of collagen fibrils and expression of fibronectin and collagen I to the levels below that seen at 21 days.

The mechanism by which nintedanib reverses established peritoneal fibrosis remains elusive, but may be associated with its regulation on the expression of TIMPs and MMPs. It is known that a balance between matrix production and degradation determines the degree of peritoneal fibrosis under various pathological conditions [24]. MMPs, a family of zinc-containing endopeptidases, play a critical role in the degradation of various ECM components including collagens, while TIMPs is a family of endogenous inhibitors of MMPs [24]. On this basis, increased expression/activation of MMPs and decreased expression/activation of TIMPs could lead to reduce deposition of ECM and less peritoneal fibrosis. In the present study, we observed that delayed administration of nintedanib increased MMP-2 expression, along with reducing TIMP-2 expression, suggesting that nintedanib-elicited-upregulation of MMP2 and/or downregulation of TIMP-2 contributes to reversal of the established peritoneal fibrosis. In line with our observations, other researchers also found in the lung fibroblasts isolated from IPF patients and IPF murine models that nintedanib significantly decreased TIMP-2 but increased MMP-2, which is correspond to reducing total collagen [10, 25]. In addition, delayed administration of nintedanib was also reported to suppress TIMP-1 expression in an animal model of CCL4-induced liver fibrosis [5]. Further studies are needed to investigate the detailed mechanism(s) by which nintedanib differentially regulates the expression/activation of MMPs/TIMPs in tissue fibrosis.

The EMT is an important mechanism by which PMCs contribute to peritoneal fibrosis. In response to many stimuli such as high glucose and inflammatory factors, PMCs can undergo EMT that is characterized by loss of cell adhesion, decreased expression of E-cadherin, and increased expression of α-SMA and β-vimentin [15, 26]. Twist and Snail, the two major transcription factors, play a critical role in driving EMT process [9, 27]. In this study, we found that delayed administration of nintedanib inhibited CG-stimulated upregulation of Snail or Twist, along with downregulation of β-vimentin, α-SMA, and retained E-cadherin expression. These results are similar to what have been observed in animal models of IPF and liver fibrosis treated with nintedanib [5, 10]. Therefore, nintedanib-mediated suppression of the EMT might serve as one important mechanism to inhibit peritoneal fibrosis and even to reverse established peritoneal fibrosis.

Angiogenesis is another important event in the pathogenesis of peritoneal fibrosis [16]. Angiogenesis and fibrosis are closely connected through initiating growth factors and inflammatory cytokines as well as the EMT process [15]. In the current study, we found that delayed administration of nintedanib was effective in suppressing CD31 (a marker of vascular endothelial cells) expression and the number of CD31-positive cells in the thickened peritoneum area induced by CG injury. This suggests that nintedanib is a powerful inhibitor of angiogenesis in the peritoneum. The mechanism of nintedanib-mediated suppression of angiogenesis may be due to its inhibition of VEGFR or other signaling molecules. In this context, peritoneal effluent derived mesothelial cells with typical EMT were shown to be the main source of VEGF, and exposure PMCs to TGF-β promotes VEGF production [28]. Furthermore, TGF-β and its downstream signaling

Fig. 6. Delayed administration of 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 mouse after CG injury with or without delayed administration of nintedanib, and subjected to the ELISA assay for MCP-1 (**a**), TNF-α (**b**), IL-1β (**c**), and IL-6 (**d**). **e** Photomicrographs illustrating immunohistochemical staining of CD68-positive cells in the peritoneum treated with or without nintedanib. **f** The graph shows the number of CD68-positive macrophages was calculated from 10 random fields (×200) (means ± SEM) ($n = 6$). $^{*,\#,@}p < 0.05$ versus sham control. Means with different superscript symbols ($^{*,\#,@}$) are significantly different from one another.



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partner, Smad3, was able to activate VEGFR and Src to stimulate peritoneal angiogenesis [9].

Inflammation plays an essential role in the development of peritoneal fibrosis, including monocytes/macrophages filtration, proinflammatory cytokines production by inflammatory cells, fibroblasts, and mesenchymal cells via EMT from PMCs, and this process can promote ECM protein synthesis, EMT, angiogenesis, and inflammation [29]. In this study, we observed that CG injury induced a significant elevation of multiple proinflammatory factors, including MCP-1, TNF-α, IL-6, and IL-1β, as well as CD68-positive macrophage infiltration in the peritoneum, and delayed administration of nintedanib inhibited these inflammatory cytokines. Here, we speculate that the anti-inflammatory effect of nintedanib could aid in alleviating progression of peritoneal fibrosis.

RTKs and Src may induce activation of multiple intracellular signaling pathways including Akt and STAT3 pathways [1]. The activation of STAT3 dramatically in-

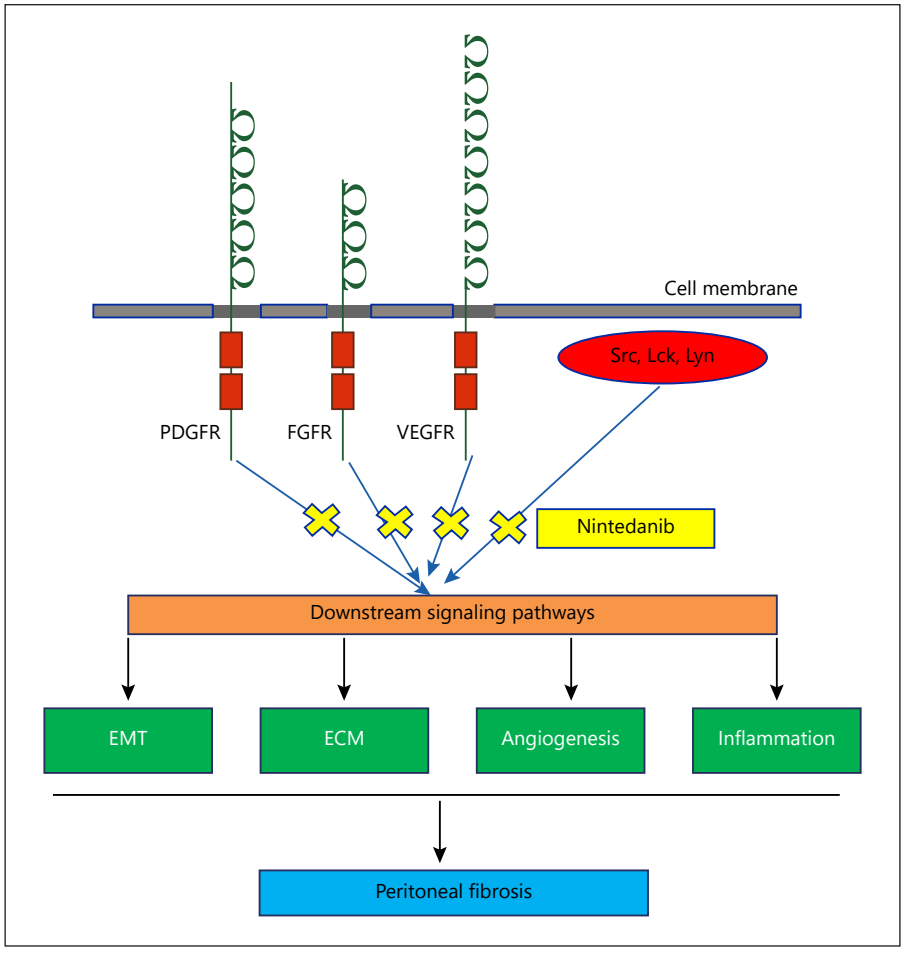

Fig. 8. Mechanism of delayed administration of nintedanib on peritoneal fibrosis. Nintedanib can block both RTKs (PDGFR, FGFR, VEGFR) and non-RTKs receptors, such as Src superfamily (Src, Lck, Lyn) activation simultaneously, resulting in inhibiting downstream signaling pathways (STAT3, NF-κB, Smad3, etc.). Therefore, it may inhibit the EMT of peritoneal mesenchymal cells, regulate the deposition and degradation of ECM, decrease angiogenesis, reduce inflammation, and ultimately ameliorates the progression of peritoneal fibrosis.

Fig. 7. Delayed administration of nintedanib blocks phosphorylation of STAT3, NF-κB and Smad3 after CG injury. **a** The peritoneums were 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 immunoblots 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 ± SEM $n = 6$). $^{*,\#,@}p < 0.05$ versus sham control. ns, no significance versus sham control. Means with different superscript symbols ($^{*,\#,@}$) are significantly different from one another.

duces the expression of Twist and Snail, leading to the initiation of EMT programs [30]. Akt, STAT3, and NF-κB activation also promote macrophage infiltration and release of proinflammatory factors [15]. Along with its downstream signaling partner, Smad3, TGF-β is recognized as the most important fibrogenic factor involved in the whole process of peritoneal fibrosis [15]. In addition, there exists "cross-talk" between various fibrotic signaling pathways. In this study, we found that delayed administration of nintedanib suppressed phosphorylation of STAT3, NF-κB, and Smad3 induced by CG injection, suggesting that nintedanib could simultaneously block the transduction of fibrotic signals initiated from RTKs and Src, suppressing and partially reversing established peritoneal fibrosis. Several clinical trials have provided evidence for the effectiveness, safety and tolerability of nintedanib in patients with IPF. Therefore, our data support that nintedanib may be a suitable choice to treat peritoneal fibrosis clinically.

Conclusion

Our current study supports the application of nintedanib for treatment of peritoneal fibrosis. The underlying mechanism may be related to blocking multiple RTKs, Src, and downstream signaling pathways simultaneously, inhibiting EMT, regulating ECM synthesis and degradation, and suppressing inflammation and angiogenesis (Fig. 8). Our preclinical findings may be helpful to promote nintedanib for clinical application to treat peritoneal fibrosis.

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Statement of Ethics

The animal experiments conform to internationally accepted standards and have been approved by the Ethics Committee of Tongji University (reference number 81670623).

Conflict of Interest Statement

We declare that Professor Shougang Zhuang, the corresponding author of this article, is one of the editorial board members of Kidney Diseases. We promise that this article has not been affected by this situation in the writing and submission process.

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Author Contributions

F.L. and S.Z. designed the research; B.C., F.L., C.Y., S.Z., X.H., Y.W., and J.W. performed experiments; B.C. and F.L. analyzed the data; F.L. and S.Z. contributed to reagents; B.C., F.L., and S.Z. wrote the manuscript. All the authors read and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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