# TGF-β-Induced CCR8 Promoted Macrophage Transdifferentiation into Myofibroblast-Like Cells

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**Research**

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# Abstract

**Background:** Idiopathic pulmonary fibrosis (IPF) is an unknown interstitial disease characterized by tissue fibrosis for which there currently is no effective treatment. Macrophages, as the main immune cells in lung tissue, are involved in the whole process of pulmonary fibrosis. In recent years, intercellular transformation has been widespread concerned in pulmonary fibrosis researchers. The macrophages which have flexible heterogeneity and plasticity participate in different physiological processes of the body. Cell chemokine receptor 8 (CCR8) expressed in a variety of cells plays a significant chemotactic role in inducing cell activation and migration. And it could also promote the differentiation of macrophages under certain environmental conditions. The current study is intended to explore the role of CCR8 in macrophage transdifferentiation into myofibroblast cells in idiopathic pulmonary fibrosis.

**Methods:** We conducted experiments using Ccr8-specific small interfering RNA (siRNA), autophagy inhibitor (3-methyladenine,3-MA) and agonist (rapamycin) to explore the underlying mechanisms of macrophage transdifferentiation into myofibroblast cells in TGF-β induced pulmonary fibrosis.

**Results:** The results indicated that TGF-β treatment increased the CCR8 protein level in a time- and a dose-dependent manner in MH-S, as well as macrophage transdifferentiation-related markers, including Vimentin, Collagen 1, and a-SMA, and cell migration. In addition, levels of autophagy were enhanced in macrophages treated with TGF-β. We found that 3-MA, an autophagy inhibitor decreased the expression levels of macrophage transdifferentiation-related markers and attenuated the cell migration. Furthermore, inhibition of CCR8 through using Ccr8-specific siRNA reduced the levels of autophagy and macrophage transdifferentiation-related markers, and inhibited the cell migration. Enhancing autophagy with rapamycin attenuated the inhibition effect of Ccr8-specific siRNA on macrophage migration and the increase of myofibroblast marker proteins.

**Conclusions:** Our findings showed that the macrophages exposed to TGF-β had the potential to transdifferentiate into myofibroblasts and CCR8 was involved in the process. The effect of CCR8 in TGF-β-induced macrophage transdifferentiation occurs mainly through autophagy. Targeting the CCR8 may become a novel therapeutic strategy for the treatment of IPF.


# Background

Idiopathic pulmonary fibrosis (IPF) which etiology is unknown, is a chronic progressive interstitial pneumonia, manifested as dyspnea and pulmonary dysfunction(1). Although IPF is a rarely seen fatal disease, its incidence is increasing over time. The available drug for IPF are limited, and the treatment of IPF faces many challenges and difficulties. Thus, there is an urgent need to explore the pathogenesis of IPF and develop effective therapeutic strategies.

The pathogenesis of IPF has not been interpreted completely. The investigation have shown that IPF is mainly caused by malfunctioning of repairing mechanism following lung tissue damage, then it leads to abnormal remodeling of peripheral angiogenesis which resulted in related lesions. The pathological

process of IPF is involved with oxidative stress, inflammatory response, and a series of cytokines and their associated signal transduction pathways.

Alveolar macrophages plays a vital role in the course of idiopathic pulmonary fibrosis. As the first line of defending against foreign invaders, they participates in the maintenance of tissue homeostasis in many ways(2). In the context of IPF, the macrophages secrete a variety of inflammatory molecules and cytokines affecting other cells in the lung to enhance cell proliferation and migration and exacerbate hyperblastosis and matrix deposition. The macrophages show strong flexible and heterogeneity, and are mainly differentiated into two subtypes, including proinflammatory M1 and anti-inflammatory M2 macrophages, in response to differentstimuli(3).Macrophages perform function through phenotypic transformation when receive signals from their surroundings. Macrophages also have the performance to develop into dendritic cells and foam cells under certain conditions(4, 5). The capacity for macrophages to differentiate in response to different external substances provides the foundation to take part in a variety of physiological and pathological activities. However, to the best of our knowledge, whether the macrophages could be induced to transdifferentiate into fibroblast-like cells in IPF is not previously reported.

Chemokine receptor 8 (CCR8), a G-protein-coupled receptor (GPCR), is expressed in monocytes and T lymphocytes and is the specific receptor of human CC chemokine CCL1 and viral mononuclear inflammatory proteins (vCCL1)(6). CCR8 could be selectively induced to regulate the cell activation and migration in antigen-activated neutrophils(7). CCR8 participates in the migration of monocytes to lymphatic vessels, and these monocytes could acquire the characteristics of dendritic cell in draining lymph nodes. However, the monocytes with CCR8 defect would reside in corresponding tissue and not get the properties of dendritic cell(8). The findings reported above suggest that CCR8 is involved in the activation, migration and differentiation of cells under certain physiological and pathological conditions. But, whether CCR8 could induce a transdifferentiation of macrophages to fibroblast-like cells which then led to its involvement in the development and progression of IPF was still unclear.

Autophagy is a membrane-dependent mechanism that maintains cell homeostasis. It determines the cell fate through providing the cell with energy for metabolism and removing aggregated proteins or dysfunctional mitochondria(9). It has been reported that autophagy participates in the progression of IPF(10, 11). Of note, autophagy is activated in alveolar macrophages from IPF patients and reducing the autopahgy had promoted macrophage apoptosis(12). These findings reinforces the notion that autophagy is required for pro-fibrotic macrophage. Additionally, autophagy is also associated with the increase of epithelial-mesenchymal transition (EMT) and airway remodeling in asthma(13). In view of existing studies, we investigated the impact of CCR8 on macrophage transdifferentiation to fibroblast-like cells. And we found that TGF-β induced the transdifferentiation from macrophage to fibroblast-like cells. However, inhibition of CCR8 and autophagy could attenuate the effect of TGF-β on macrophages. Furthermore, autophagy mediated the CCR8 regulation in the macrophages exposed to TGF-β. Our study revealed a novel regulatory mechanism of CCR8/autophagy in IPF and offered insights into potential therapeutic targets for IPF.

# Materials And Methods

## Reagents

TGF-β1 (HY-P7117), 3-Methyladenine (3-MA, 5142-23-4) and Rapamycin (HY-10219) were purchased from MedChem Express (MCE, Monmouth Junction, NJ, USA). RPMI 1640 medium (31800-500) was acquired from Solarbio (Beijing, China). GlutaMax Supplement (35050-061) was obtained from Gibco (New York, NY, USA), and a penicillin/streptomycin mixture (15140-122) was obtained from Fisher Scientific (Waltham, MA, USA). The antibody against GAPDH (60004-1-Ig) was purchased from Proteintech Group (Wuhan, China). The antibody against CCR8 (BS71060) and COL1A2 (BS1530) were purchased from BioWorld (St. Louis Park, MN, USA). The antibody against α-SMA(19245S) and Vimentin (5741S) were obtained from Cell Signaling, Inc (Beverly, MA, USA). The antibody against LC3B (GTX127375) used to perform western blotting was obtained from GeneTex (San Antonio, TX, USA). The antibody against LC3B (ABM40296) used to perform immunocytochemistry was obtained from Abbkine (Redlands, CA, USA).

## Cell culture and treatments

The mouse alveolar macrophage MH-S cell line (ATCC) was grown in RPMI 1640 medium containing 10% FBS with penicillin (50 U/ml) and streptomycin (100 μg/ml) at 37 °C in an incubator with 5% humidified $CO_2$ and 95% air. The cells were stored in liquid nitrogen at passages 3-7 (P3-7). For each experiment, a vial of P3-7 MH-S was thawed, plated, and passaged upon confluence; Macrophages were used between P10 and P15. For all of experiments, cells were serum-starved for 24 h prior to treatment with TGF-β. In the experiments involving pharmacological reagents, MH-S cells were pretreated with respective reagents (3-MA, rapamycin, MCE, Monmouth Junction, NJ, USA) for 1 h followed by treatment with TGF-β as described above.

## siRNA knockdown

RNA interference targeting *Ccr8* was performed to knock down the protein levels of CCR8 in MH-S cells. In brief, following MH-S cells reaching approximately 70-80% in 24-well culture plates, chemically synthesized *Ccr8*-specific siRNA was transfected into the macrophages using Lipofectamine® 3000 according to the manufacturer's instructions (Invitrogen, CA, USA); a non-specific siRNA was used as a negative control. All siRNA senses were supplied from Shanghai Jima Company (Shanghai, China). The prepared mixture containing siRNA combined with Lipofectamine 3000 was added dropwise to the culture plate. Then, the serum-free 1640 was replaced with complete medium for an additional 24-h incubation prior to subsequent experiments.

## Cell migration assays

MH-S cells were seeded in 96-well culture plates at $4\times10^4$ cells/well. When cells were approximately 80-90% confluent,IncuCyte® WoundMaker (Essen BioScience), a 96-pin mechanical device, was used to

create straight lines in cell monolayers across the centre of the well. Each well was washed twice to remove cell debris with fresh growth medium containing TGF-β and pharmacological reagents. Images of the cell gap were acquired at different time points, and the gap width was quantified using ImageJ software.

### CCK8 assays

The CCK8 assay was performed to assess the viability of MH-S cells according to the manufacturer's instructions (MCE, Monmouth Junction, NJ, USA). Briefly, cells were seeded into 96-well plates at a density of $5\times10^3$ cells/well; After treatment cells with TGF-β, 10 μl CCK8 was added to each well. Following 2 h incubation, the absorbance (OD) was measured at 450 nm on a microplate reader (Invitrogen, CA, USA).

### Western blotting

Treated cells washed with PBS were collected from culture dishes and then lysed using a mammalian cell lysis kit (cat. no. R0010; Solarbio, Beijing, China). Electrophoretic analysis of equal amounts of the proteins was performed via SDS-PAGE (12.5%) in reducing conditions. The proteins that were separated via gel electrophoresis were transferred to PVDF membranes and then blocked with 5% non-fat dry milk in TBST at room temperature for 1 h. The membranes were incubated overnight at 4 °C with antibodies recognizing CCR8 (1:1000; BioWorld ), Vimentin (1;1000; Cell Signaling), a-SMA (1:500; Cell Signaling), Beclin 1 (1:1,000; Cell Signaling), LC3B (1:2,000; GeneTex), followed by incubation with an alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody (1:5000) in TBS-T for 1 h at room temperature. A chemiluminescence detection system was used to detect the immunoreactive protein bands. Each western blot was repeated at least three times.

### Immunocytochemistry.

Immunocytochemistry was performed as previously described(14), with minor modifications. MH-S cells were placed on coverslips in 24-well plates. Treated cells with TGF-β were fixed with 4% paraformaldehyde overnight and permeabilized with 0.3% Triton X-100 in PBS. The cells were blocked at room temperature for 2 h using 10% NGS in 0.3% Triton X-100 followed by incubation with CCR8 (1:250; BioWorld ), Collagen 1 (1:250; BioWorld ) and LC3B (1:250; Abbkine) primary antibodies at 4 °C overnight. Following washes with PBS, the cells were incubated with secondary AlexaFluor 488 goat anti- rabbit IgG and AlexaFluor 569 goat anti-mouse IgG (1:250), and the cell nuclei were then stained with DAPI (4',6-diamidino-2-phenylindole). Fluorescent images were acquired using laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

### ELISA

Supernatant from macrophages was collected, and the levels of CCL1in the supernatants were determined using ELISA kits (USCN, Wuhan, China) according to the manufacturer’s instructions, with the

absorbance read at 450 nm using the microplate reader (Invitrogen, CA, USA).

### Statistics

The data are presented as the mean ± S.E.M. Significance was established using a t-test for paired values. Intergroup comparisons were made with a one-way ANOVA for more than two groups. Results were judged statistically significant if $p<0.05$ by analysis of variance.

## Results

### TGF-β induced the macrophage transdifferentiation into myofibroblasts

Macrophages play a vital role in the etiology of pulmonary fibrosis. And macrophage shows a highly phenotypic flexibility in response to different stimuli. Several lines of researches(15-17) demonstrated that epithelial- and endothlial-mesenchymal transition participated in the process of IPF followed by excessive cell proliferation, migration and collagen deposition.

In present study, to elucidate whether TGF-β treatment resulted in the macrophage transdifferentiation into myofibroblasts, we first adopted CCK8 assay to detect the cell viability of macrophages treated with TGF-β. Our results showed that both different doses of TGF-β and different time points of TGF-β treatment had no distinct effect on cell viability (Supplementary Figure S1A and B). Then, western blot was performed to detect myofibroblast marker proteins, including Vimentin and a-SMA. As shown in Fig. 1A-B, Vimentin, and a-SMA were all significantly increased in macrophages in response to TGF-β in a dose dependent manner with a peak response at 5 ng/ml (a-SMA) or 10 ng/ml (Vimentin), the dosages that have been used in previous studies of IPF(18, 19). Accordingly, we chose 10 ng/ml for all of the subsequent experiments. Moreover, the results indicated that TGF-β induced significant increases in Vimentin and a-SMA expression in a time-dependent manner. Notably, all the expression levels peaked at 24 h (Fig. 1C and D). Subsequently, scratch assay was adopted to assess the cell migration ability. As shown in Fig. 1E, The results of the scratch assay demonstrated that TGF-β induced a significant increase in the migration of MH-S.

### TGF-β induced CCR8 expression in a dose- and a time-dependent manner in MH-S

Previous studies(20, 21) have showed that CCR8 participates in the pathogenesis of several fibrosis diseases and pulmonary diseases. Therefore, we supposed that CCR8 may be involved in the development of IPF. We next measured the expression of CCR8 in MH-S cells exposed to TGF-β. CCR8 expression was found to be significantly increased in a dose- and a time-dependent manner in MH-S cells by western blot assay, which peaked at 10 ng/ml and reached its peak at 24h consistent with findings of myofibroblast marker proteins (Fig. 2A-D). Similar results were obtained via immunofluorescence analysis, which showed significantly increased expression of CCR8 in MH-S cells following TGF-β exposure (Fig. 2E). As the potent ligand for CCR8, CCL1 performed critical functions in the physiological and pathological processes involved with CCR8(22, 23). Then, we detected the level of CCL1 in MH-S cell

supernatants using ELISA kits. Surprisingly, the results showed that TGF-β significantly decreased the expression of CCL1 in a dose- and a time dependent manner which were inconsistent with the expression of CCR8 (Supplementary Figure S2A and B). Accordingly, these data demonstrated that CCL1 could not be involved in the physiological processes mediated by CCR8 in MH-S exposed to TGF-β.

### CCR8 mediated macrophage transdifferentiation induced by TGF-β

To access weather CCR8 participated in the macrophage transdifferentiation into myofibroblasts. RNAi targeting *Ccr8* in MH-S was performed. First, *Ccr8*-specific siRNA was applied and notably decreased the expression of CCR8 (Fig. 3A and B). Then, western blot results indicated that the knockdown of *Ccr8* with specific siRNA significantly reduced the expression of a-SMA and Vimentin in MH-S exposed to TGF-β compared to scramble control siRNA treatment (Fig. 3C and D). Moreover, The cell migration assay showed that CCR8 inhibition with siRNA attenuated the macrophage migration caused by TGF-β compared with that of the control (Figures 3E). These results revealed the role of TGF-β-induced CCR8 expression in modulating macrophage transdifferentiation.

### TGF-β increased autophgic protein expression in a dose- and a time-dependent manner in MH-S

Autophagy has been shown(12, 24) to be dramatically upregulated in some fibrosis diseases and involved with EMT. However, the involvement of autophagy in macrophage transdifferentiation into myofibroblasts in response to TGF-β in vitro remains to be elucidated. Therefore, we first detected the effect of TGF-β dosage on autophagy level of MH-S cells, and found that TGF-β enhanced the expression of LC3B and Beclin 1, markers of autophagy, in a dose-dependent manner (Fig. 4A and B). The maximal effects of TGF-β on autophagy marker expressions were observed at 5 ng/ml (Beclin 1) and 10 ng/ml (LC3B), which is consistent with the results above. Next, our results also showed that TGF-β induced the expression of LC3B and Beclin 1 in a time-dependent manner at 10 ng/ml, which peaked at 24h (Fig. 4C and D).

### Autophagy promoted TGF-β-induced macrophage transdifferentiation

Then, 1 mM 3-MA was applied in the pursuit of understanding the role of autophagy in macrophage transdifferentiation. The results indicated that 3-MA significantly inhibited the expression of LC3B and Beclin 1 (Supplementary Figure S3A-B). Furthermore, western blot showed that inhibiting autophagy with 3-MA dramatically dampened the α-SMA and Vimentin levels (Fig. 5A and B). And similar results in collagen 1 and LC3B were confirmed via immunocytochemistry (Fig. 5C). We also found that, as shown in Fig. 5D, the increase in macrophage migration induced by TGF-β was attenuated by pretreatment of MH-S with 3-MA. Collectively, these results suggest that increased autophagic protein expression in macrophages is causally linked to macrophage transdifferentiation.

### Autophagy facilitated CCR8-mediated macrophage transdifferentiation

In this study, both increased CCR8 and autophagic proteins promoted macrophage transdifferentiation into myofibroblasts. Considering the multi-faceted effect of autophagy in various diseases, we examined

whether autophagy was involved in CCR8-mediated macrophage transdifferentiation. Western blot results showed that *Ccr8* knockdown prominently reduced the expression of autophagic proteins, including LC3b and Beclin 1 (Fig. 6A and B). Immunocytochemistry assay was employed and showed similar results to western blot (Fig. 6C). To further verify the correlation between CCR8 and autophagy, 1 μM rapamycin, a specific activator of autophagy, was applied. As shown in Fig. 6D and E, rapamycin reversed the effect of RNAi targeting CCR8 on myofibroblast marker protein, including a-SMA and Vimentin. Moreover, rapamycin treatment reversed the migration-inhibiting effect of RNAi targeting CCR8 (Fig. 6F). Taken together, these results indicated that autophagy facilitated CCR8-mediated macrophage transdifferentiation into myofibroblasts induced by TGF-β.

# Discussion

At present, there are many difficulties for IPF treatment to be overcome, and alternative medicine is limited. The research and development of new drugs are extremely urgent. However, the etiology and pathogenesis of IPF are still unknown.

In the respiratory tract, macrophage is one of the largest quantities of cells. According to different distribution, macrophages are divided into two types, including alveolar macrophages located on the surface of the alveoli and interstitial macrophages located between alveolar epithelial cells and vascular endothelial cells. In obstructive and restrictive respiratory disease, both quantity and phenotype of pulmonary macrophage have changed. In the process of pulmonary fibrosis, macrophages play a vital role which is complicated. They are involved in many abnormal responses of tissue repair in fibrosis. Fibroblasts are the primary source of collagen secretion. However, the phenotype of fibroblasts separated from lung tissue of IPF are diverse. These cells show obvious heterogeneity which is different from normal pulmonary fibroblasts(25). Thus, it was speculated that the heterogeneous pulmonary fibroblasts may be differentiated from other cells.

A wealth of evidence(26, 27)shows that cell-mesenchymal transition including epithelial- and endothlial-mesenchymal transition plays an important role in contributing to the progression of pulmonary fibrosis. Although macrophages exhibit a much greater degree of flexibility, it is unknown whether they could be differentiated into myofibroblast-like cells. In this study, we found that alveolar macrophages exposed to TGF-β showed enhanced expression of vimentin, α-SMA and collagen 1, and migration compared with untreated group. What is noteworthy that there was no difference in cell viability of macrophages treated with TGF-β. The results indicated that macrophages were differentiated into myofibroblast-like cells following the TGF-β treatment which could contribute to the complex of fibroblast heterogeneity in IPF and underline a prominent role of macrophage transdifferentiation into fibroblasts in the pathogenesis of IPF.

CCR8 has been identified on monocytes originally(28). Impaired monocyte infiltration has been observed in a murine airway inflammation model in CCR8 KO mice. CCR8 is also expressed in macrophages in lung and liver tissue. Similarly, CCR8 KO mice has shown a significant reduction of macrophages in the lung

challenged by ovalbumin and diminished macrophage infiltration in damaged liver induced by $CCl_4$(20, 29). Studies(30)also found that CCR8 present in endothelial cells was a functional receptor of endotheliocyte and mediated the chemotaxis of endothelial cells in response to CCL1 and vCCL1. In our study, we found that CCR8 was significantly increased in macrophages exposed to TGF-β in a time- and a dose-dependent manner. CCL1 has been shown to be the only high-affinity ligand for CCR8, and involved in many physiological process mediated by CCR8(31, 32). However, the present results showd that the expression of CCL1 was decreased in a time- and a dose-dependent manner, *versus* the increased expression of CCR8, which indicated that CCL1 could not participate in the CCR8 activities in this study. Increasing evidence(33–35)suggests that other chemokines, such as CCL16, CCL18 and MIP-1 (macrophage inflammatory protein-1), have been identified as the ligands for CCR8 apart from CCL1. Whether any other cytokines took part in the macrophage transdifferentiation except CCL1 need to be further determined.

In addition to degrading and renewing intracellular protein and organelle, autophagy performs a variety of physiological and pathological functions, including cell differentiation and immune response etc. Autophagy also plays a multi-effect part in different diseases, such as cardiovascular disease, neurodegenerative diseases and pulmonary diseases. Earlier reports(12)have demonstrated that mitophagy induced by Akt1 inhibites the macrophage apoptosis that promotes pulmonary fibrosis. Emerging evidence(36) suggests that in the condition of oxygen-sugar deprivation, cancer cells undergo epithelial-mesenchymal transformation accompanied by mitophagy and the impairment of mitochondrial function, that raises the possibility that there exist a certain relation between autophagy and intercellular transformation. Interestingly, our study showed that the expression of autophagy associated proteins, like LC3B and Beclin 1, were enhanced in macrophages treated with TGF-β. Inhibiting autophagy with 3-MA attenuated the expression of vimentin and α-SMA and cell migration. These findings identify a novel function of autophagy in inducing macrophage transdifferentiation into myofibroblasts.

Although both CCR8 and autophagy were involved in the process of macrophage transdifferentiation, it was still unkonwn whether there was some sort of a link between CCR8 and autophagy in this study which was reported minimally in previous investigations. Our observation that knock-down of CCR8 with siRNA strikingly reduced the protein levels of autophagy indicated that CCR8 could promote macrophage transdifferentiation by upregulating the expression of autophagy proteins. The relation between CCR8 and autophagy was further identified by the fact that enhancing autophagy with rapamycin reversed the decreased expression of vimentin and α-SMA and macrophage migration caused by using CCR8 siRNA.

# Conclusions

In aggregate, the results of our study suggest that CCR8 significantly increased the cell migration and the expression of vimentin and α-SMA in macrophages by enhancing autophagy. It therefore comes as no surprise that CCR8 promotes the process of pulmonary fibrosis through macrophage transdifferentiation into myofibroblasts, and the discovery will facilitate the research and development of effective therapies for IPF through targeting against CCR8.

# Abbreviations

IPF: Idiopathic pulmonary fibrothersis; CCR8: Cell chemokine receptor 8; CCL1: Cell chemokine ligand 1; TGF-β: Transforming growth factor-beta; EMT: Epithelial-mesenchymal transition; 3-MA: 3-methyladenine; MH-S: Mouse alveolar macrophages; siRNA: small interfering RNA

# Declarations

### Authors' contributions

HJL designed and performed the experiments, analyzed the data and wrote the manuscript. PZ performed the experiments and analyzed the data. SJL provided laboratory space and funding and directed the project. HJL and QZG provided funding. All authors critiqued and approved the final manuscript.


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### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and material

Not applicable.

### Competing interests

The authors declare no conflicts of interest.


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None


### Supplementary data

All Supplementary data are freely available in the attachment.

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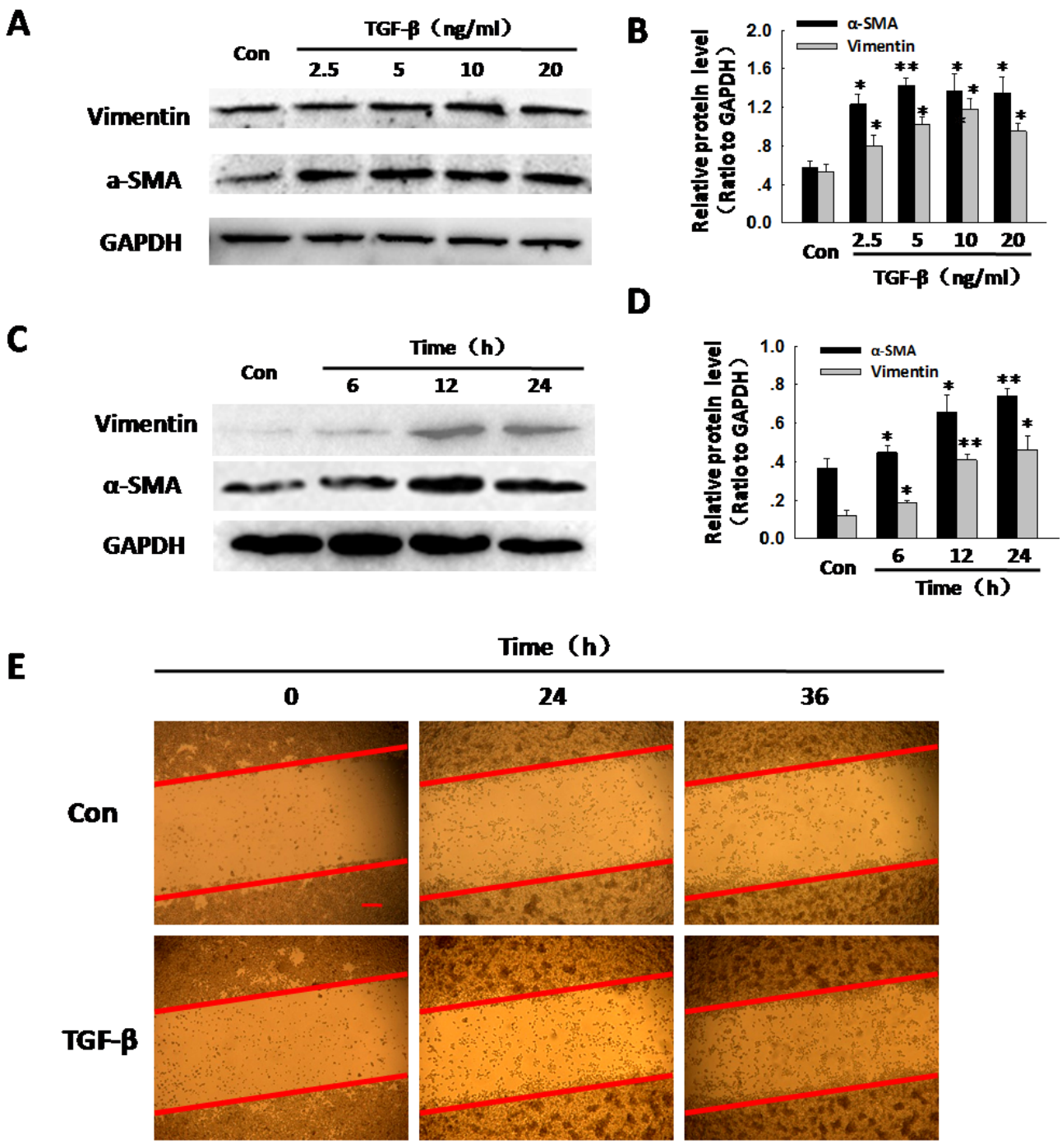
# Figures

# Figure 1

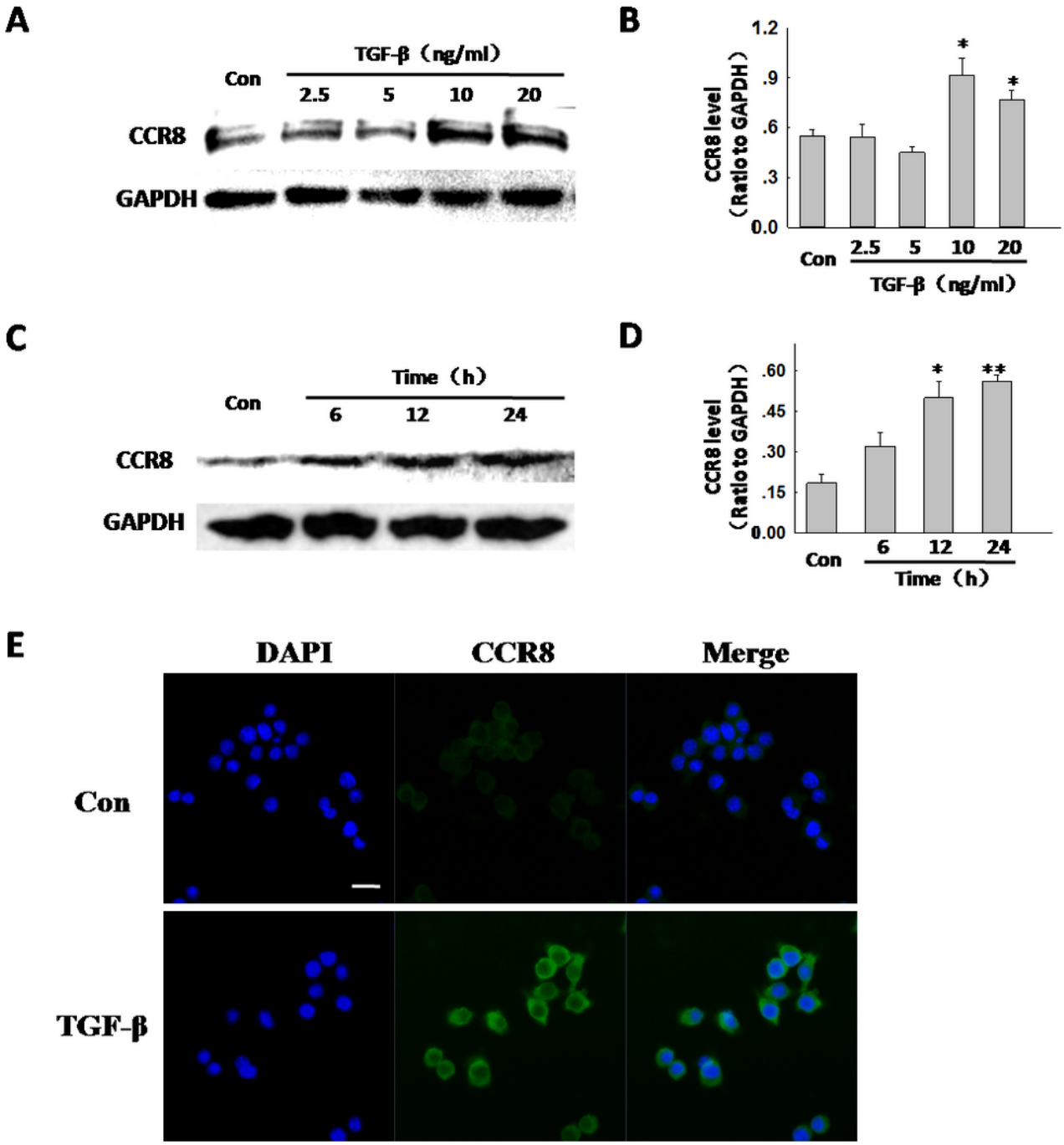



## Figure 1

TGF-β induced the macrophage transdifferentiation into myofibroblasts. (A-D) Representative Western blot and densitometric analyses showing the effects of TGF-β on the expression of the myofibroblast marker Vimentin and a-SMA in a dose-dependent manner (A and B)and a time-dependent manner (C and D) in MH-S cells. Data are presented as the mean ± S.E.M. (n=5); *P<0.05; **P<0.01 versus the control group (Student's t-test). (E) Representative images of TGF-β-induced migration of MH-S cells cultured as

a monolayer. The results indicated that TGF-β significantly increased the migration of MH-S cells. Scale bar=100 μm. Quantification of the scratch gap distance from six separate experiments. *p<0.05; **P<0.01 versus the control group at the corresponding time points (Student's t-test).

# Figure 2




## Figure 2

TGF-β mediated an increase in CCR8 expression in MH-S cells. (A-D) Representative Western blot and densitometric analyses showing the effects of TGF-β on the expression of CCR8 in a dose-dependent

manner (A and B)and a time-dependent manner (C and D) in MH-S cells. Data are presented as the mean ± S.E.M. (n=5); *P<0.05; **P<0.01 versus the control group (Student's t-test). (E) Representative immunocytochemical images showing that TGF-β increased the expression of CCR8 (green) in MH-S cells. Scale bar= 20 μm. Images are representative of three independent experiments.

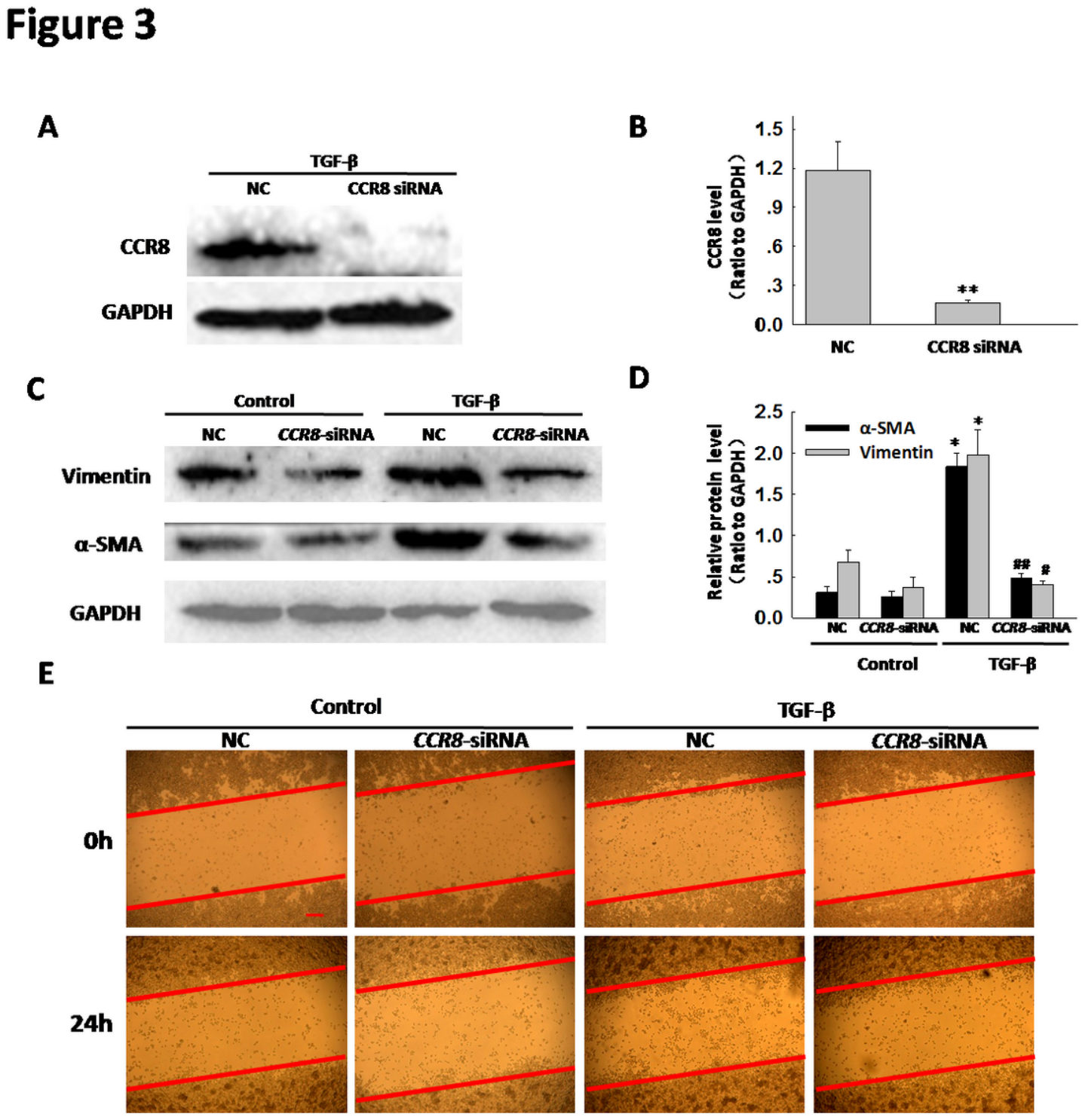
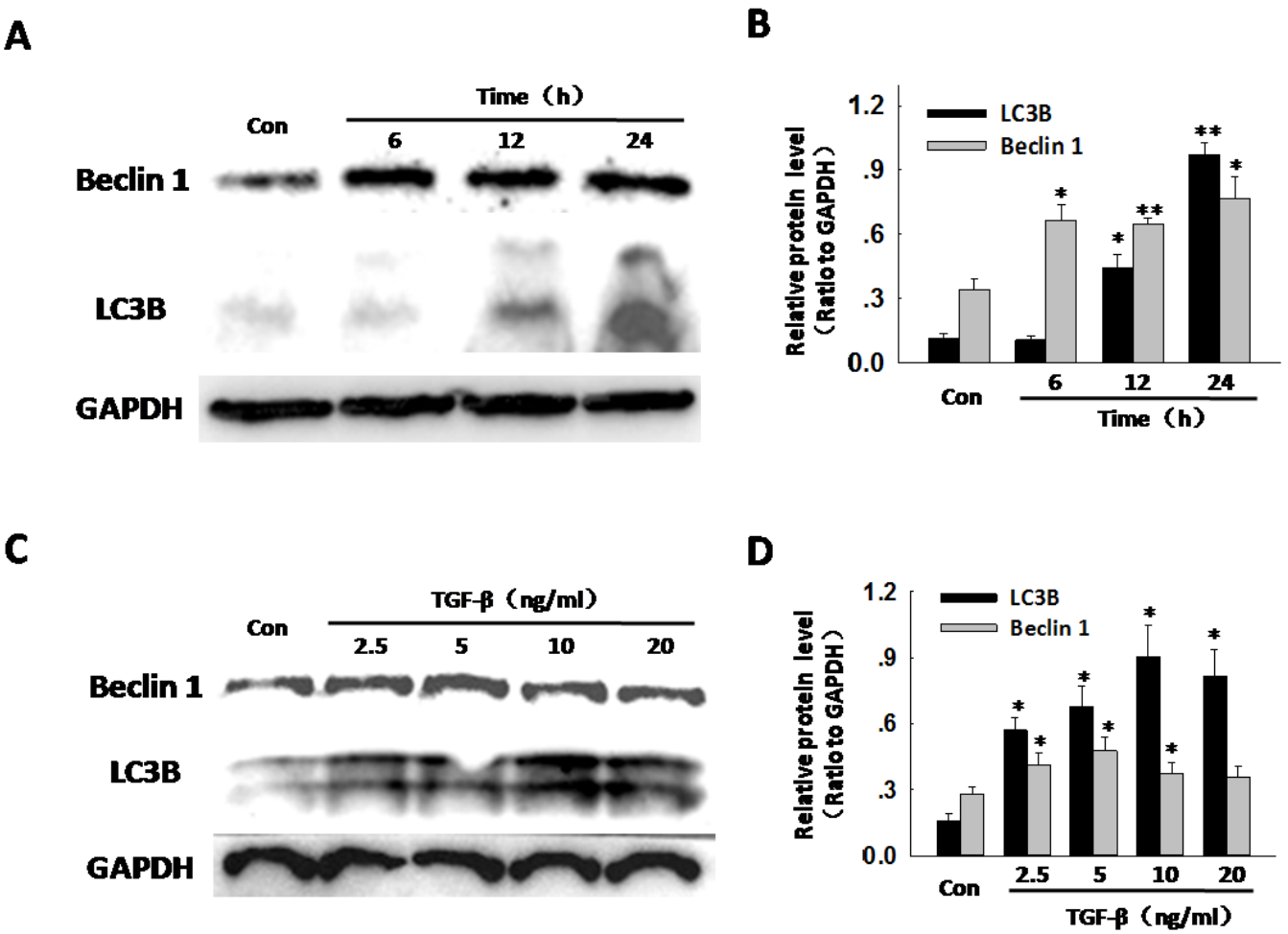



Figure 3

TGF-β-induced CCR8 was involved in macrophage transdifferentiation into myofibroblasts. (A and B) Representative Western blot and densitometric analyses showing Ccr8-specific siRNA significantly

inhibited the CCR8 expression. Data are presented as the mean ± S.E.M (n =3); **P<0.01 versus the con-siRNA + TGF-β group (Student's t-test); (C and D) Representative Western blot and densitometric analyses showing the effects of Ccr8-specific siRNA on the expression of a-SMA and Vimentin induced by TGF-β. The results suggested that TGF-β induced a-SMA and Vimentin expression, which were reversed by the Ccr8-specific siRNA. Data are presented as the mean ± S.E.M (n =3); *P<0.05 versus the con-siRNA group; #P<0.05; ##P<0.01 versus the con-siRNA+ TGF-β group (one-way ANOVA). (E) Representative images and data showing the effects of TGF-β on the migration of MH-S cells. The results indicated that TGF-β induced an increase in MH-S cell migration. However, Ccr8-specific siRNA reduced the effects of TGF-β on MH-S cells. Scale bar=80 μm. Data are representative of at least 3 independent experiments.

# Figure 4

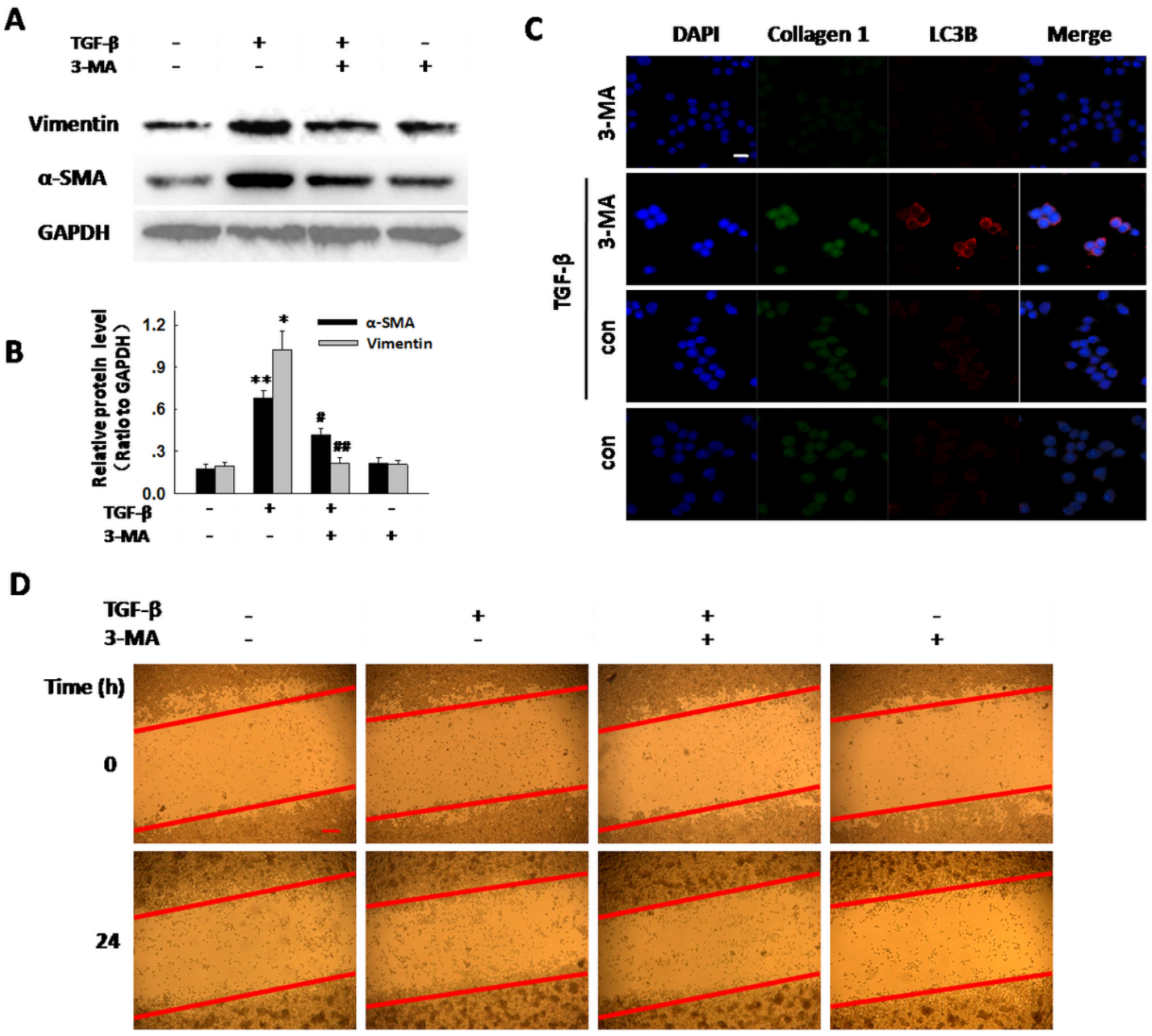



## Figure 4

TGF-β promoted autophagy protein expression in MH-S cells. (A and B) Representative Western blot densitometric analyses demonstrating that the TGF-β significantly enhanced the expression of the autophagic proteins, includingLC3B and Beclin 1 in a dose-dependent manner (A and B)and a time-

dependent manner (C and D) in MH-S cells. Data are presented as the mean ± S.E.M. (n=5); *P<0.05; **P<0.01 versus the control group (Student's t-test).

# Figure 5




## Figure 5

Autophagy is responsible for macrophage transdifferentiation in response to TGF-β. (A and B) Representative western blot and densitometric analyses showing the effects of 3-MAon the expression of a-SMA and Vimentin in MH-S cells treatment with TGF-β. The results suggested that pretreatment of MH-S cells with 3-MA attenuated the the increases in a-SMA and Vimentin expression induced by TGF-β. Data are presented as the mean ± S.E.M. (n=5); *p < 0.05; **P<0.01 vs the control group; #p < 0.05; ##P<0.01

vs the TGF-β group (one-way ANOVA). (C) Immunocytochemical staining results showing that 3-MA significantly alleviated pulmonary fibrosis compared with control group treated with TGF-β. Scale bar= 20 μm. Images are representative of several individuals from each group (n= 4). (D) Representative images and data showing the effects of 3-MA on the migration of MH-S cells. The results indicated that pretreatment of MH-S cells with 3-MA attenuated the pro-migration effects induced by TGF-β on MH-S cells. Scale bar=100 μm. Data are representative of at least 3 independent experiments.

## Figure 6

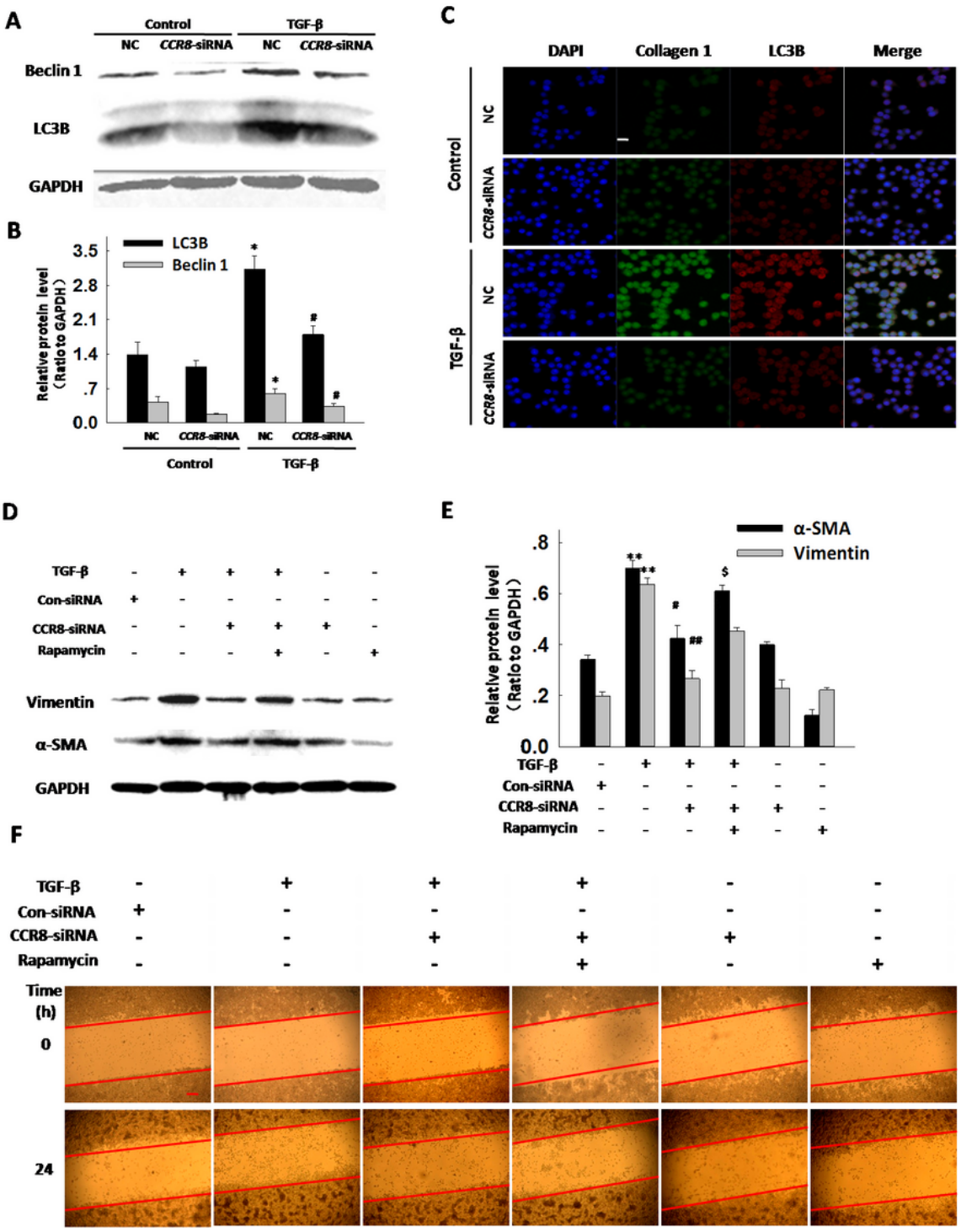



Figure 6

Autophagy was involved in CCR8-mediated macrophage transdifferentiation. (A and B) Representative Western blot and densitometric analyses showing the effects of Ccr8-specific siRNA on the expression of Beclin 1 and LC3B induced by TGF-β. The results suggested that the Beclin 1 and LC3B expression induced by TGF-β were reversed by Ccr8-specific siRNA. Data are presented as the mean ± S.E.M (n =3); *P<0.05 versus the con-siRNA group; #P<0.05 versus the con-siRNA+ TGF-β group (one-way ANOVA). (C) Representative images and data of immunocytochemical staining demonstrating that the autophagic protein induced by TGF-β was attenuated by Ccr8-specific siRNA. Scale bar= 20 μm. Images are representative of three independent experiments. (D and E) Representative Western blot and densitometric analyses showing the effects of rapamycin and RNAi targeting CCR8 on TGF-β-induced expression of a-SMA and Vimentin in MH-S cells. The results suggested that rapamycin reversed the effects of RNAi targeting CCR8 on TGF-β-induced a-SMA and Vimentin expression. Data are presented as the mean ± S.E.M (n =3); *p < 0.05; **P<0.01 vs the con-siRNA group; #p < 0.05; ##P<0.01 vs the corresponding TGF-β+ con-siRNA group; $p < 0.05 vs the corresponding TGF-β+ Ccr8-siRNA group (one-way ANOVA). (F) Representative images and data demonstrating rapamycin reversed the effects of RNAi targeting CCR8 on TGF-β-induced MH-S cell migration. Scale bar=100 μm. Data are representative of at least 3 independent experiments.

## Figure 7

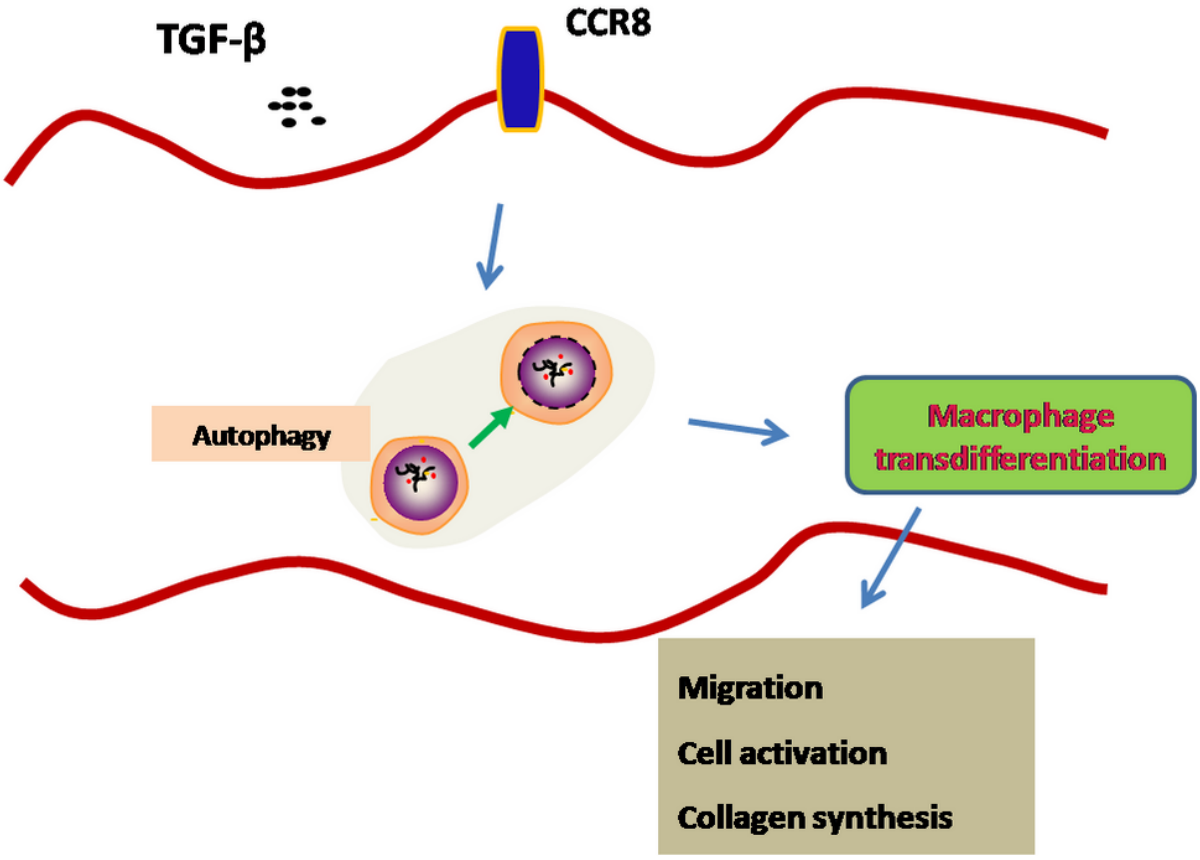


**Figure 7**

Schematic diagram showing the mechanisms by which CCR8 mediates TGF-β-induced macrophage transdifferentiation in IPF. CCR8 expression was increased in macrophages treatment with TGF-β, leading to the subsequent enhancement of autophagy. CCR8-mediated autophagy further promoted macrophage migration and increased expression of myofibroblast marker proteins.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- suppdata.pdf