CXCR4 antagonist alleviates proliferation and collagen synthesis of cardiac fibroblasts induced by TGF-β1

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Abstract. We aimed to investigate the effects of CX-C chemokine receptor type 4 (CXCR4) on transforming growth factor (TGF)-β1-induced cardiac fibrosis in Human cardiac fibroblasts (HCFs). HCFs were stimulated with TGF-β1, and the level of α-smooth muscle actin (α-SMA) was assessed by immunofluorescence assay. The expression of CXCR4 was detected by Western blotting. Then the cells were incubated with CXCR4 antagonist AMD 3465. Cell viability was measured by CCK-8 assay. The expression of α-SMA, proliferating cell nuclear antigen (PCNA) and Ki67 were examined. Collagen synthesis was detected by sirius red staining. Moreover, the expression of phpspho-Smad2 (p-Smad2) and p-Smad3 were determined. We found that the level of α-SMA was increased after induction with TGF-β1. The expression of CXCR4 was upregulated in TGF-β1-treated HCFs. Following treatment with AMD 3465, cell proliferation was inhibited coupled with a decrease in PCNA and Ki67 expression. Additionally, the expression of α-SMA was decreased after being intervened with AMD 3465. Concurrently, the levels of collagen were reduced accompanied by downregulation of Collagen I and III. Furthermore, AMD 3465 treatment decreased the expression of p-Smad2 and p-Smad3. Our findings suggested that CXCR4 antagonist AMD 3465 could alleviate cardiac fibrosis *via* blocking TGF-β1-induced activation of Smad2/3 in HCFs.

Key words: CX-C chemokine receptor type 4 — AMD 3465 — TGF-β1 — Cardiac fibrosis — Collagen

Introduction

Characterized by the excessive proliferation of cardiac fibroblasts and overproduction of collagen, myocardial fibrosis has been identified as a critical event in physiological and pathological cardiac remodeling, thus contributing to many cardiac dysfunctions, such as cardiac arrhythmia, myocardial infarction and heart failure (Kong et al. 2014; Rathod et al. 2016). To date, however, there is still no effective diagnosis and treatment for cardiac fibrosis. Therefore, it is of great clinical and scientific significance to explore the molecular mechanism and find out potential drug targets of myocardial fibrosis.

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CX-C chemokine receptor type 4 (CXCR4) is known as a typical G-protein coupled seven-fold transmembrane receptor and one of a CXCR chemokine receptor. Accumulating evidence suggests that CXCR4 plays a significant role in a number of physiological processes (Schneider et al. 2015; Luo et al. 2016). Recent researches have confirmed that CXCR4 might be implicated in the process of fibrosis. For instance, the expression level of CXCR4 is closely related to pulmonary fibrosis in Adenosine A2a receptor (A2aR) gene-knockout mice (Chen et al. 2017). The activation of CXCR4 contributed to kidney fibrosis and genetic ablation of CXCR4 prevented unilateral ureteral obstruction induced fibrosis *via* inhibiting transforming growth factor (TGF)-β1 pathways (Yuan et al. 2015). In addition, emerging evidence supports the notion that inhibition of CXCR4 could treat pulmonary fibrosis (Ding et al. 2018). Importantly, a previous study reported that activation of CXCR4 pathway leads to cardiac fibrosis in dilated cardiomyopathy (Chu et al. 2019). However, the effect of

CXCR4 and the underlying mechanisms in human cardiac fibroblasts (HCFs) of myocardial fibrosis remains to be further elucidated.

Numerous studies unveiled that TGF-β1 is one of the most powerful profibrogenic mediators and a dominant stimulator of collagen production (Chen et al. 2005). Therefore, in the current study, we aimed to investigate the effects of CXCR4 on TGF-β1-induced cardiac fibrosis. As a well-known highly selective CXCR4 antagonist, AMD 3465 was used to block CXCR4 in HCFs (Hartimath et al. 2014). And our data demonstrated that CXCR4 inhibition alleviates proliferation and collagen secretion of cardiac fibroblasts in HCFs through blocking TGF-β1-induced activation of Smad2/3.

Materials and Methods

Cell culture and treatment

HCFs were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in DMEM (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS. All cells were incubated in an incubator containing 5% CO_2. HCFs were stimulated with TGF-β1 (20 ng/ml) for 24 h, then the cells were incubated with various concentrations of age-related macular degeneration (AMD) 3465 (2.5, 5 and 10 μM) for 48 h. Cells that cultured in normal conditions were used as control.

Immunofluorescence assay

The levels of α-smooth muscle actin (α-SMA) and Ki67 were detected by an immunofluorescence assay. Briefly, after being fixed in 4% formaldehyde for 15 min and permeabilized with 0.1% Triton™ X-100 for another 15 min, the cells were blocked in 5% goat serum for 1 h. Subsequently, cells were probed overnight at 4°C with α-SMA (#19245, cell signaling technology) or Ki67 (#12075, cell signaling technology), followed by incubation with secondary antibodies for 1 h. Finally, after being stained with DAPI (D9542, Sigma) for 5 min, the samples were imaged using a fluorescence microscope (IX73-A12FL/PH; Olympus, Japan).

Quantitative real-time polymerase chain reaction (qPCR)

RNA in cells is collected using TRizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in line with the supplier's instructions. And cDNA was obtained using SuperScript II reverse transcriptase (Thermo Fisher Scientific, Inc.). Then quantitative PCR reactions were performed using PowerUp™ SYBR Green (Thermo Fisher Scientific, Inc.) with ABI 7500 thermocycler (Thermo Fisher Scientific,

Inc. USA). Primers used were as follows: α-SMA, Forward 5'-GTGTTGCCCCTGAAGAGCAT-3' and Reverse 5'-GCTGGGACATTGAAAGTCTCA-3'; GAPDH, Forward 5'-CTGGGCTACACTGAGCACC-3' and Reverse 5'-AA-GTGGTCGTTGAGGGCAATG-3'. GAPDH was used as control for normalization. The expression was calculated using the $2^{-\Delta\Delta Cq}$ method.

Cell counting kit-8 (CCK-8) assay

Cell proliferation was measured by a CCK-8 kit. HCFs were plated into 96-well plates (3×10^3 cells/well). The cells were treated with TGF-β1 for 24 h and then incubated with AMD 3465 for 48 h. Then, 10 μl CCK-8 (Beyotime, Beijing, China) was added into each experimental well for 2 h. Absorbance of each well was read at a wavelength of 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA), and the viability of the control group was assumed to be 100%.

Western blotting

Total proteins were extracted from HCFs using RIPA lysis buffer (Beyotime, Shanghai, China). The concentration of protein was detected using a BCA protein assay kit (Beyotime, Shanghai, China). Subsequently, protein (20 ug/lane) was isolated by SDS-polyacrylamide gels (PAGE), and electrophoretically transferred onto polyvinylidene fluoride membranes. Then, all membranes were blocked by 5% skimmed milk. After being probed with primary antibodies, membranes were subsequently incubated with secondary antibodies (sc-358914, Santa Cruz). Finally, the signals of immunoblots were visualized using an enhanced chemiluminescence system (Amersham Bioscience, USA) and analyzed with ImageJ software. The protein expression was normalized to GAPDH levels.

Sirius red staining

The degree of myocardial fibrosis of HCFs was determined using Sirius red staining. Briefly, 4% paraformaldehyde was employed to fix with cells for 10 min, followed by incubation with 0.1% Sirius Red F3B for 30 min. Then the cells were stained with hematoxylin and dehydrating thrice in 100% ethanol.

Statistical analysis

All quantitative data were expressed as the mean ± standard error of the mean (mean ± SD). Statistical analyses were carried out by using Student's unpaired *t*-test and one-way ANOVA followed by Dunnett's post-hoc test. A value of $p < 0.05$ was considered statistically significant and each experiment was repeated at least three times.

Results

AMD 3465 treatment inhibited the expression of CXCR4 induced by TGF-β1 in HCFs

TGF-β1 was employed to treat HCFs, and the level of α-SMA was determined using immunofluorescence assay in our study. As presented in Figure 1, there was almost no expression of α-SMA in the control group. On the contrary, TGF-β1 treatment increased the level of α-SMA, which is a marker of myofibroblast. As a novel antagonist of CXCR4, AMD 3465 was applied to treat cells with different doses (2.5, 5 and 10 μM). Proliferation of cells was measured by a CCK-8 kit. As exhibited in Figure 2A, AMD 3465 significantly reduced the proliferation of HCFs in a dose-dependent manner. Moreover, when the concentration of AMD 3465 was 5 μM, the cell activity of HCFs was close to 50% compared with the control group. Take it into account, 5 μM AMD 3465 treatment was chosen for the following experiment. It was found that the expression of CXCR4 was enhanced in TGF-β1-induced HCFs and AMD 3465 decreased the expression of CXCR4 induced by TGF-β1 (Fig. 2B). These results indicated that AMD 3465 treatment inhibited the expression of CXCR4 induced by TGF-β1 in HCFs.

AMD 3465 treatment relieved cell proliferation of HCFs induced by TGF-β1

To explore the effect of AMD 3465 on cell proliferation of HCFs induced by TGF-β1. The expression of PCNA and Ki67 were assessed in the current study. We found that TGF-β1 up-regulated the level of PCNA, whereas AMD 3465 treatment decreased this increase obviously (Fig. 3A). Concurrently, the level of Ki67 was detected by immunofluorescence assay.
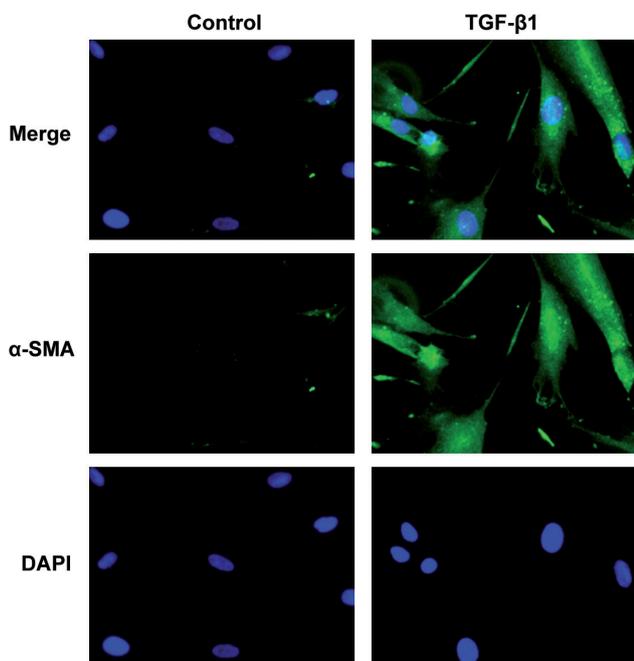
Figure 1. The expression of α-SMA in TGF-β1-induced human cardiac fibroblasts. Immunofluorescence staining assay was applied to measure the level of α-SMA in each group. 200× magnification. α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor -β1.
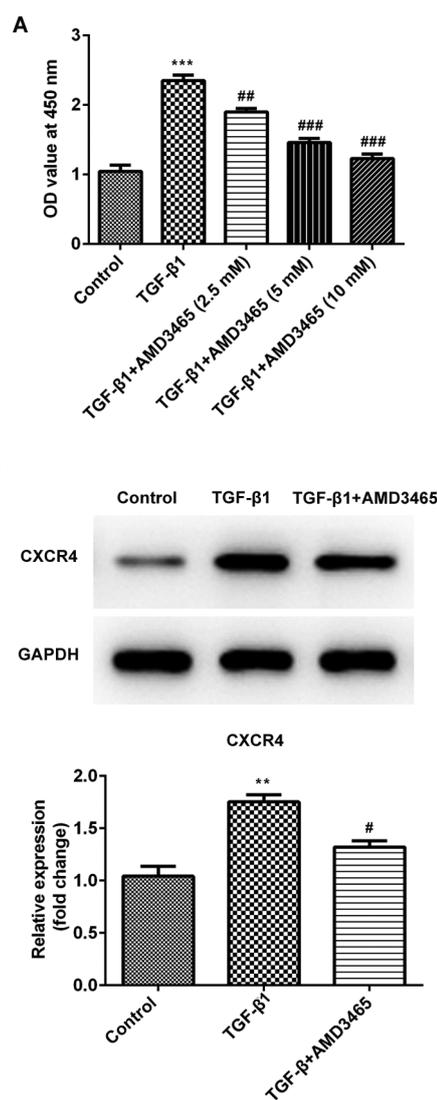
Figure 2. AMD 3465 treatment reduced cell proliferation and decreased the level of CXCR4 in TGF-β1-treated human cardiac fibroblasts (HCFs). **A.** Cell proliferation was assessed by CCK-8 assay after treatment with AMD 3465 in TGF-β1-induced HCFs. **B.** AMD 3465 treatment reduced the level of CXCR4 in TGF-β1-treated HCFs. ** $p < 0.01$, *** $p < 0.001$ *vs.* Control; $^{#}$ $p < 0.05$, $^{##}$ $p < 0.01$, $^{###}$ $p < 0.001$ *vs.* TGF-β1. TGF-β1, transforming growth factor-β1; CXCR4, CX-C chemokine receptor type 4.

As presented in Figure 3B, AMD 3465 treatment reversed the upregulation of Ki67 induced by TGF-β1 stimulation in HCFs. These findings suggested that AMD 3465 treatment relieved cell proliferation of HCFs induced by TGF-β1.

AMD 3465 treatment alleviated TGF-β1-induced differentiation of HCFs

To explore the effect of AMD 3465 on TGF-β1-induced differentiation of HCFs, the level of myofibroblast marker

α-SMA was assessed using Western blotting and immuno-fluorescence assay, respectively. As exhibited in Figure 4A, the level of α-SMA was increased notably in comparison with the control group, whereas AMD 3465 treatment markedly reduced TGF-β1-induced α-SMA expression. And the results of analysis by immunofluorescence assay was in accordance with the western blotting (Fig. 4B). These observations revealed that AMD 3465 treatment reversed the upregulated expression of α-SMA induced by TGF-β1 stimulation in HCFs.
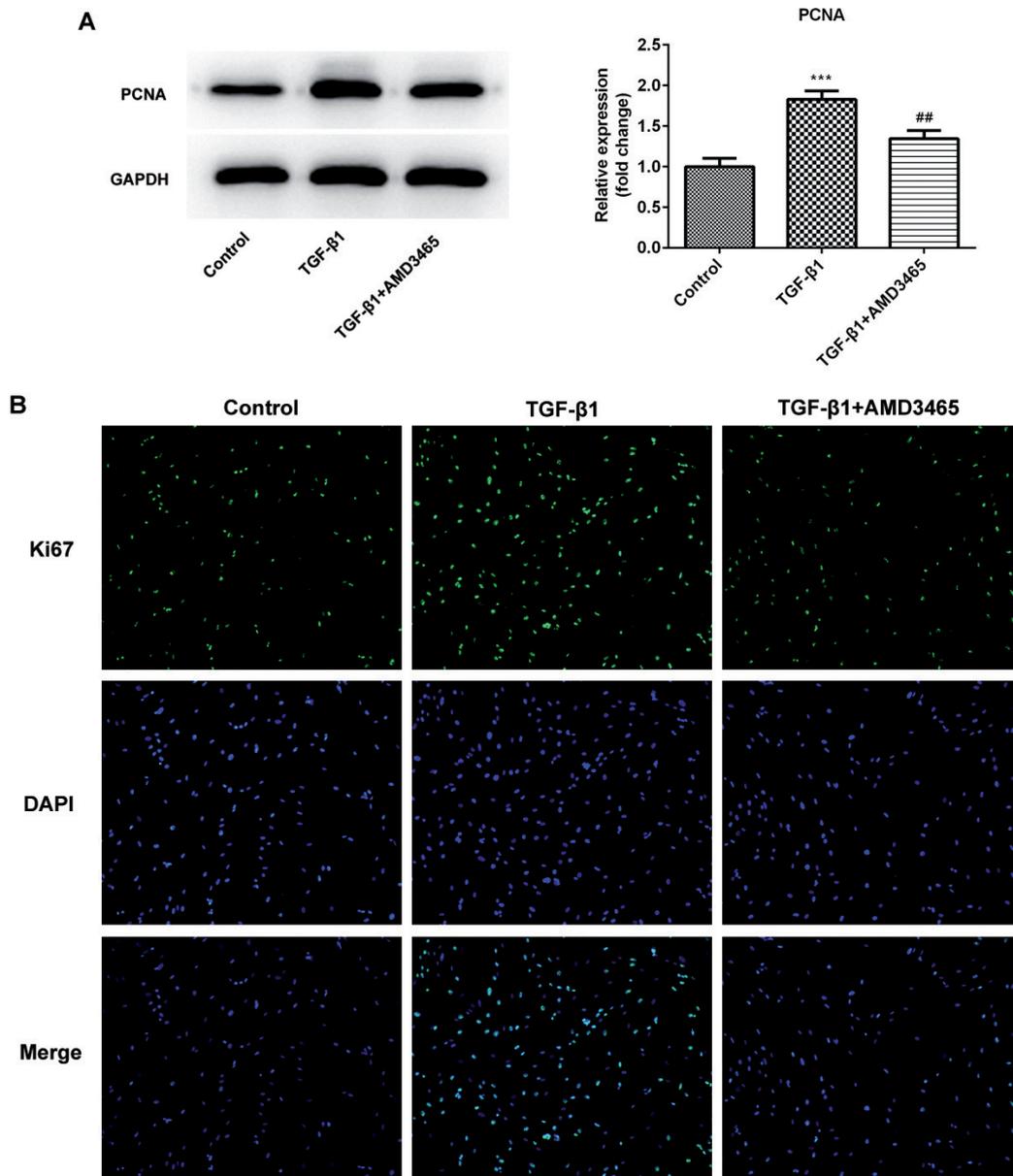
Figure 3. AMD 3465 treatment downregulated the expression of PCNA and Ki67 in TGF-β1-induced human cardiac fibroblasts. **A.** The expression of PCNA was assessed by western blotting. **B.** The level of Ki67 was determined by immunofluorescence assay. $^{***}p < 0.001$ *vs.* Control; $^{\#\#}p < 0.01$ *vs.* TGF-β1. 100× magnification. TGF-β1, transforming growth factor-β1; PCNA, proliferating cell nuclear antigen.

AMD 3465 treatment inhibited TGF-β1-induced collagen synthesis in HCFs

Collagen synthesis is crucial in the process of myocardial fibrosis, therefore, the expression of Collagen I and III was detected using Western blotting. As expected, the expression of Collagen I and Collagen III were increased obviously in TGF-β1-induced HCFs. And AMD 3465 treatment dramatically suppressed both Collagen I and III expression induced by TGF-β1 (Fig. 5A). Consistent with that, the results of sirius red staining showed that AMD 3465 markedly inhibited collagen synthesis of HCFs (Fig. 5B and C).

AMD 3465 treatment blocked TGF-β1-induced activation of Smad2/3 in HCFs

To further investigate the potential molecular mechanism of AMD 3465 functions in myocardial fibrosis, western blotting assay was applied to detect the expression level of
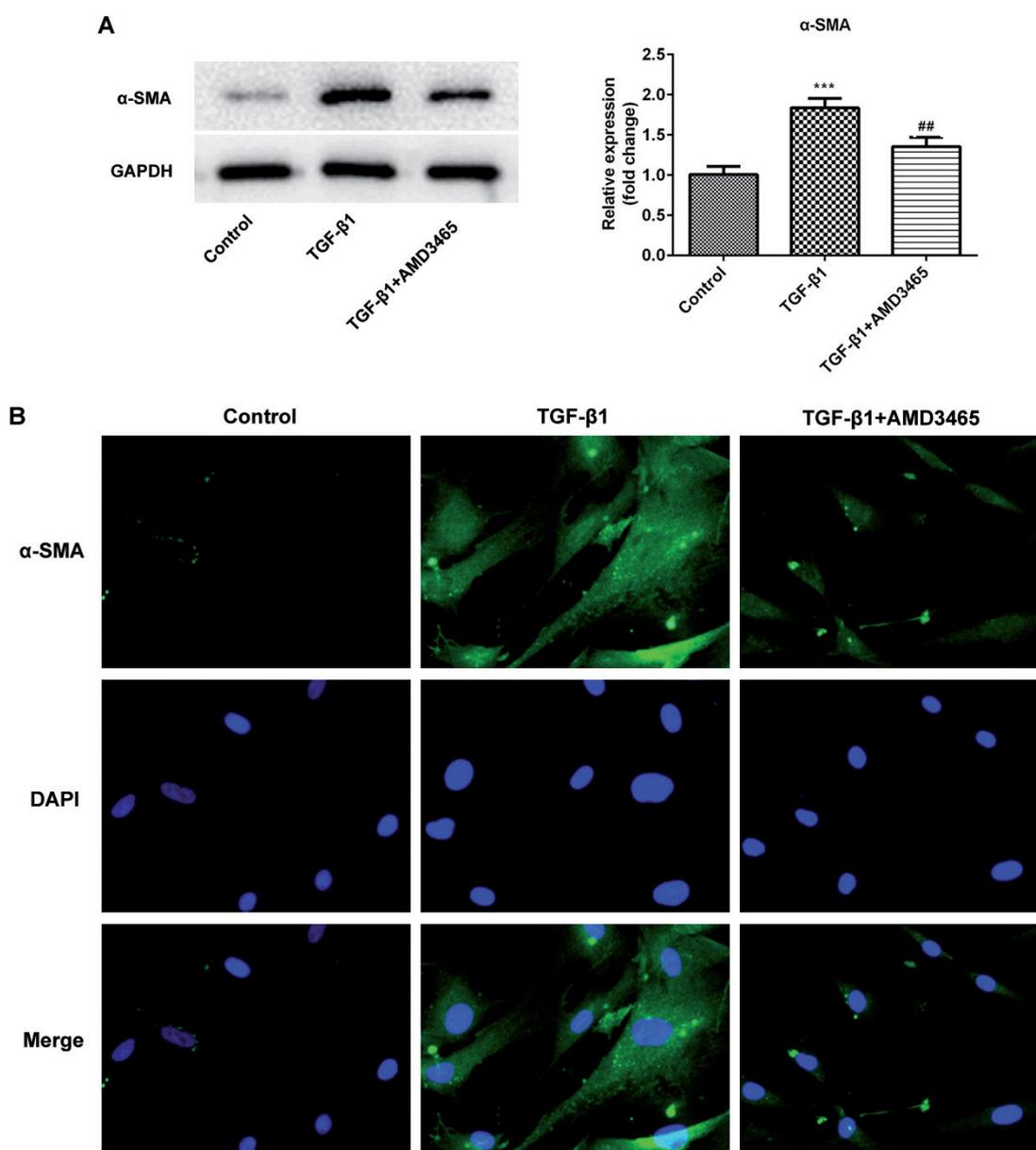
Figure 4. AMD 3465 treatment decreased the level of α-SMA in TGF-β1 treated human cardiac fibroblasts. The expression of α-SMA was detected by (**A**) Western blotting and (**B**) immunofluorescence assay. $^{***} p < 0.001$ *vs.* Control; $^{##} p < 0.01$ *vs.* TGF-β1. 200× magnification. α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1.

A

B

C
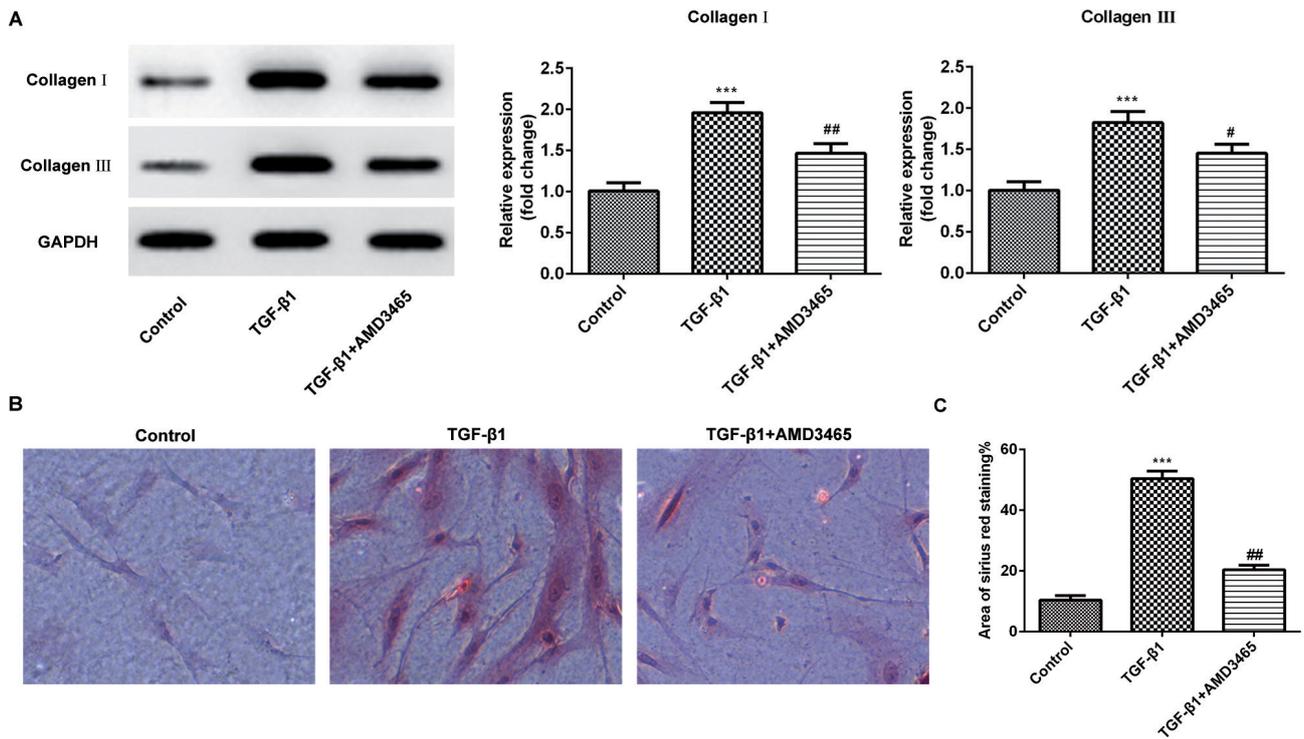
Figure 5. AMD 3465 treatment decreased the collagen secretion in TGF-β1-treated human cardiac fibroblasts. **A.** The expression of Collagen I and Collagen III were evaluated by Western blotting. **B.** Representative images of Sirius red staining from each experimental group. **C.** Quantification of Sirius red staining. $^{***}p < 0.001$ *vs.* Control; $^{#}p < 0.05$, $^{##}p < 0.01$ *vs.* TGF-β1. TGF-β1, transforming growth factor-β1.

the key proteins of TGF-β1/Smad signaling pathway. Our results suggested that the expression of both phpspho-Smad2 (p-Smad2) and p-Smad3 were markedly upregulated in TGF-β1-treated HCFs (Fig. 6). And AMD 3465 treatment markedly downregulated the levels of p-Smad2 and p-Smad3 induced by TGF-β1 in HCFs. Overall, above data suggested that AMD 3465 treatment could block TGF-β1-induced activation of Smad2/3 in HCFs.
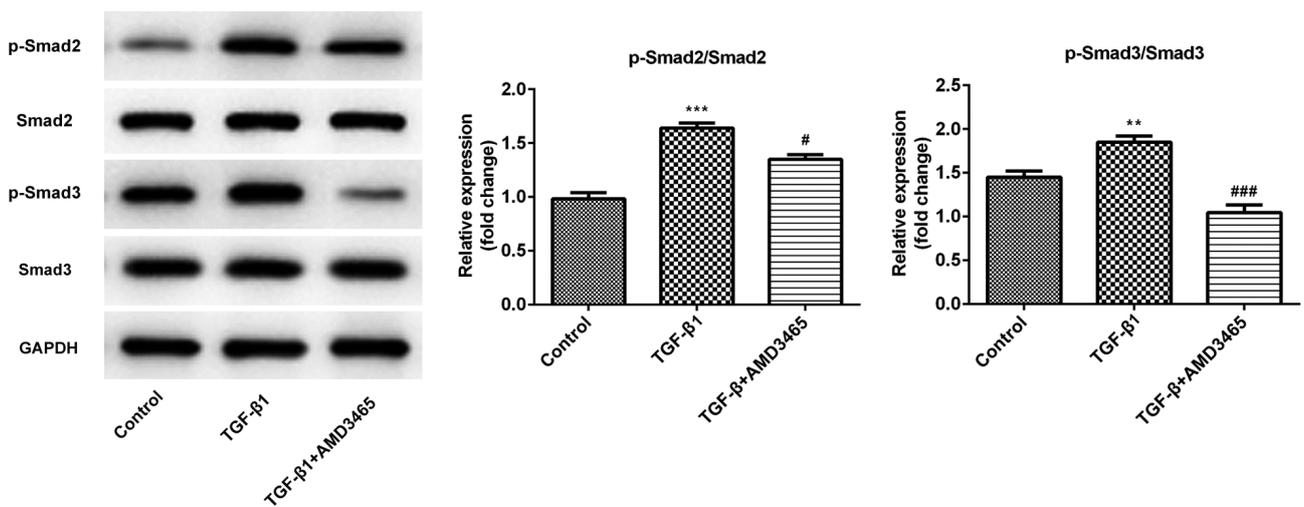
Figure 6. AMD 3465 treatment inhibited the expression of p-Smad2 and p-Smad3. The levels of p-Smad2 and p-Smad3 were assessed by Western blotting. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* Control; $^{#}p < 0.05$, $^{###}p < 0.001$ *vs.* TGF-β1. TGF-β1, transforming growth factor-β1; p-Smad2, phospho-Smad2.

Discussion

CXCR4 is involved in many physiological and pathological processes including occurrence and development of cardiovascular disease (Segret et al. 2007; Chatterjee et al. 2017). Cardiac fibrosis is one of the important pathophysiological mechanisms underlying cardiovascular disease. However, little is known about the role of CXCR4 in the process of myocardial fibrosis. Recently, a study in myocardial infarction showed that CXCR4 blockade could reduce fibrosis and enhance cardiac function and survival after myocardial infarction, suggesting that CXCR4 may participate in myocardial fibrosis (Jujo et al. 2010). Herein, we aimed to investigate the effects of CXCR4 on cardiac fibroblasts. Our results showed that the expression of CXCR4 was upregulated notably in TGF-β1-stimulated HCFs, which suggested that CXCR4 may be involved in the TGF-β1-induced cardiac fibrosis. To further investigate the potential role of CXCR4 and reveal the underlying molecular mechanisms, the CXCR4 antagonist AMD 3465 was used to perform. We found that AMD 3465 alleviates TGF-β1-induced collagen synthesis and myofibroblasts differentiation of HCFs *via* suppression of TGF-β1/Smad pathway. These findings suggested that CXCR4 is crucial for cardiac fibrosis and it could be a potential target in cardiac fibrosis treatment.

It has been documented previously that CXCR4 could promote the differentiation of fibroblasts to myofibroblasts (Abu El-Asrar et al. 2008; Gharaee-Kermani et al. 2012; Marts et al. 2019). For example, a study involving idiopathic pulmonary fibrosis (IPF) revealed that the expression of CXCR4 mRNAs were decreased in IPF patients compared with the healthy control (Antoniou et al. 2010). In addition, prior report has demonstrated that CXCR4 inhibition could treat the activation of HSC-T6 cells induced by TGF-β1 in liver fibrosis (Ullah et al. 2019). Moreover, it has been well reported that CXCL4 promotes the process of cell fibrosis by inducing the α-SMA production (Gillen et al. 2013). In our study, it was found that AMD 3465 treatment decreased the expression of α-SMA, which was in accordance with the previous study (Xu et al. 2016).

Excessive proliferation of cardiac fibroblasts contributes to the pathological changes of fibrotic diseases. It has been reported that endogenous SO_2 could inhibit myocardial fibroblast proliferation *via* inhibiting ERK signaling pathway (Zhang et al. 2018). Relaxin is able to reduce the expression of PCNA and reverses cardiac and renal fibrosis in rats (Lekgabe et al. 2005). In addition, a previous study reported that Dasatinib could inhibit Ki67 level and relieve cardiac fibrosis in mice induced by pressure overload (Balasubramanian et al. 2015). Consistent with these data, the present study revealed that AMD 3465 treatment decreased the proliferation of HCFs induced by TGF-β1 accompanied by

downregulation of PCNA and Ki67 expression, which are the proliferation associated genes. Our results suggested that AMD 3465 treatment relieved cell proliferation of HCFs induced by TGF-β1. Moreover, TGF-β1 is a multifunctional cytokine and one of the most powerful stimuli of fibroblast activation and fibrosis identified to date (Fix et al. 2019). A growing body of evidence confirms that suppression of Collagen I and III attenuates cardiac fibroblasts (Wang et al. 2018; Song and Ren 2019). We found that AMD 3465 treatment decreased the level of collagen deposition, coupled with downregulation of Collagen I and III expression. These observations revealed that AMD 3465 treatment inhibited TGF-β1-induced collagen deposition in HCFs.

The activation of CXCR4 can induce a range of physiological responses and gene expression by activating downstream pathways (Ding et al. 2019). To investigate the precise mechanism of CXCR4 blocking in fibrosis inhibition, we assessed the level of key proteins in TGF-β1/Smad signaling pathway. Our data showed that AMD 3465 markedly downregulated the levels of p-Smad2 and p-Smad3 induced by TGF-β1 in HCFs. Those results suggested that there was a crosstalk between CXCR4 and TGF-β1/Smad pathway. Based on the above results, we concluded that AMD 3465 treatment could block TGF-β1-induced activation of Smad2/3 in HCFs.

In conclusion, our data demonstrated that CXCR4 antagonist AMD 3465 alleviates cardiac fibrosis induced by TGF-β1 in HCFs *via* blocking, at least partly, Smad dependent signaling pathway. TGF-β1 obviously increases the level of CXCR4, and AMD 3465 blocks TGF-β1-induced activation of Smad2/3 in HCFs. Interference of CXCR4 may be a potential treatment for myocardial fibrosis.

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