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Human Umbilical Cord Mesenchymal Stem Cells Combined with Pirfenidone upregulates the Expression of RGS2 in the Pulmonary Fibrosis in Mice

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Research Article

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Abstract

Objective

The therapeutic effect of umbilical cord-derived mesenchymal stem cells(hUC-MSCs) in combination with pirfenidone(PFD) on pulmonary fibrosis in mice and its possible mechanism were investigated.

Methods

C57BL/6 mice were randomly divided into six groups: control group, model group, P_{10} group, P_{30} group, P_{100} group and P_{300} group. Modeled by tracheal intubation with 3 mg/kg bleomycin drip, each dose of PFD was given daily by gavage from day 7 onwards. Mice were observed continuously for 21 days and survival was recorded. Lung tissues were collected on day 21, and HE and Masson staining were performed to assess morphological changes and collagen deposition in the lungs. Collagen content was measured by Sircol method, and fibrosis marker levels were detected by PCR and Western Blot. Another batch of C57BL/6 mice was then randomly divided into five groups: hUC-MSCs control group, model group, P_{100} group, hUC-MSCs treatment group and hUC-MSCs + P_{30} group. 5×10^5 hUC-MSCs were injected into the tail vein on day 7, and the mice were given PFD gavage daily from day 7 onwards, and their survival was recorded. Lung tissues were collected on day 21 to detect pathological changes and the expression of collagen content and regulator of G protein signaling 2(RGS2). Pulmonary myofibroblasts were divided into MFB group and MFB + hUC-MSCs group, and different doses of PFD were added to each group, and the levels of RGS2 and fibrosis markers were detected in each group.

Results

Compared with other doses of PFD groups, the P_{100} group significantly improved mouse survival and lung pathology, and significantly reduced collagen and fibrosis marker levels ($p < 0.05$). The hUC-MSCs + P_{30} group significantly improved mouse survival and lung pathology, significantly reduced collagen and fibrosis marker levels ($p < 0.05$), and the efficacy was better than that of the P_{100} and hUC-MSCs groups ($p < 0.05$). RGS2 expression was significantly higher in the MSCs + P_{30} group compared with the P_{100} and hUC-MSCs groups ($p < 0.05$). PFD increased RGS2 expression in MFB ($p < 0.05$) in a dose-dependent manner. Fibrosis markers were more significantly reduced in the hUC-MSCs + PFD relative to the PFD alone group and the hUC-MSCs group.

Conclusion

The study suggested that hUC-MSCs combined with low-dose PFD showed a therapeutic effect better than that of the two treatments used separately. Its effect attenuating bleomycin-induced pulmonary fibrosis in mice is related with the increase of RGS2.

Background

Idiopathic pulmonary fibrosis(IPF), a diffuse inflammatory disease of the lower respiratory tract with unknown etiology, is a chronic interstitial lung disease characterized by progressive dyspnea and progressive deterioration of lung function[1]. Its incidence is increasing year by year, the median survival is only 2.5–3.5 years, and the prognosis is worse than that of many types of cancer[2, 3].

The 2015 edition of the clinical practice guidelines for the treatment of IPF states that the use of prednisone, azathioprine, N-acetylcysteine monotherapy, anticoagulants, platelet-derived growth factor receptor antagonists, endothelin receptor antagonists, and 5-phosphodiesterase inhibitors is strongly discouraged in patients with IPF, and that there are only four conditional recommended drugs, including pirfenidone(PFD), nidanib, N-acetylcysteine combination therapy, and antacid therapy[1]. The mechanism of PFD for IPF is still unclear, but the latest test confirmed that RGS2 is the basis of the anti-fibrotic effect of PFD which provides an a new direction to study the mechanism of PFD for IPF[4]. Although the emergence of PFD has brought some benefits to IPF patients, in the 2015 edition of the clinical practice guidelines for the treatment of IPF, PFD is defined as a conditional recommended drug for use due to its high effective dose, numerous adverse effects, and effectiveness only in mild to moderate IPF[1, 5, 6]. Therefore, the effect of monotherapy for PFD is not satisfactory, so it is important to find a reasonable treatment.

Cell therapy based on stem cell technology has been a hot trend in recent years[7]. Human umbilical cord-derived mesenchymal stem cells(hUC-MSCs) are a type of adult stem cells with multi-directional differentiation potential derived from the mesoderm, and are widely used because of their simplicity of extraction, lack of ethical restrictions, strong immunomodulatory ability and low immunogenicity[8, 9]. Clinical trials of hUC-MSCs for the treatment of various systemic diseases have been conducted worldwide, confirming the safety of hUC-MSCs in clinical applications[10, 11, 12]. Recent studies have shown that hUC-MSCs achieve immune modulation and inflammation control through paracrine secretion of multiple factors and microvesicles, which can reduce pro-fibrotic factors and collagen deposition, thus having therapeutic effects in animal models of bleomycin-induced early pulmonary fibrosis[13, 14], but limited therapeutic effects in advanced pulmonary fibrosis[15].

Therefore, in this study, we established a mouse model of pulmonary fibrosis using bleomycin to investigate the effects of hUC-MSCs combined with low-dose PFD through observing the survival rate, lung pathological changes and detected the expression level of pulmonary fibrosis markers in mice. PFD and hUC-MSCs were combined to act on myofibroblasts to observe the effect of their combination on the expression of RGS2, which has antifibrotic effect, to preliminarily explore the possible mechanism of the combined treatment and provide a theoretical basis for the treatment of IPF with hUC-MSCs combined with PFD.

Methods

Reagents and cells

PFD was purchased from Shanghai Adamas Reagents Co. Ltd.; hUC-MSCs were donated by Chongqing Stem Cell Therapy Engineering Technology Research Center; β-actin, type I collagen a1(Col1a1), type I collagen a2(Col1a2), α-smooth muscle actin(α-SMA), RGS2, calcium adhesion protein E(E-cad) and fibronectin(FN) primers were synthesized by Chengdu Kengke Zixi Biotechnology Co. Ltd.; ultrafiltration tubes were purchased from Millipore; other reagents were all domestic analytical purity. The mouse embryonic fibroblasts (NIH3T3) and human Embryonic Lung Fibroblasts(HLF-9) were purchased from Cell Resource Center, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. Cell culture and drug treatments Cells were routinely cultured at 37 °C with 5 % CO2 in 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Nutrient Mixture (F12) supplemented with 10 % fetal bovine serum(FBS) and were used at < passage 6(P_6) for experiments. For dose–response experiments, human lung fibroblast cells were seeded into 12-well plates, starved in serum-free DMEM/F12 medium for 24 h, and then treated with the indicated concentrations of PFD for the indicated times. 6-well plate co-culture dishes were purchased from Thermo Fisher Scientific.

Animal grouping and treatment

SPF-grade 7-week-old C57BL/6 male rats were purchased from the Animal Experiment Center of Chongqing Medical University and housed in an SPF-grade breeding room at 22-26 °C, 55-60% humidity, and 12 h/day light rotation, and modeling was started after 1 week of adaptation.

Thirty C57BL/6 were divided into 6 groups using the random number method: control group (N), bleomycin model group (B), P_{10} group (10 mg/kg PFD, P_{10}), P_{30} group (30 mg/kg PFD, P_{30}), P_{100} group (100 mg/kg PFD, P_{100}), and P_{300} group (300 mg/kg PFD, P_{300}). Pulmonary fibrosis model was established by dripping bleomycin 3mg/kg into the lungs of mice at an equal rate through tracheal intubation, and the control group was given an equal amount of saline at an equal rate. After successful modeling, each PFD dose group was given 10 mg/kg, 30 mg/kg, 100 mg/kg, 300 mg/kg PFD suspension by gavage from day 7 after modeling, and the control group was given equal volume of saline once a day until day 21 after modeling.

Another batch of 30 C57BL/6 was divided into 6 groups using the random number method: control group (N), hUC-MSCs control group (N+M), bleomycin model group (B), hUC-MSCs treatment group (B+M), P_{100} group(100 mg/kg PFD, P_{100}) and hUC-MSCs+P_{30} group (hUC-MSCs+30 mg/kg PFD, B+M+P_{30}). Modeling was performed by tracheal intubation with 3 mg/kg bleomycin drip, and on day 7 after modeling, hUC-MSCs control and hUC-MSCs treatment groups were injected with $5×10^5$/200 μL P4 generation hUC-MSCs via the tail vein of mice, and the non-MSCs group was given an equal amount of saline. Starting from day 7 after modeling, the P_{100} groups and the hUC-MSCs+P_{30} group (B+M+P_{30}) were given 100 mg/kg, and 30 mg/kg PFD suspension by gavage, respectively, and the control group was given an equal volume of saline once daily until day 21 after modeling. The survival of mice was observed and recorded, and lung tissues were collected on day 21. The mice survival curves, analysis of lung histopathology,

determination of collagen content in the lungs, detection of mRNA, the markers of lung fibrosis, and detection of RGS2 expression will be briefly described in the following.

Survival curves

The survival of each group of mice was observed and recorded, and survival curves were plotted using Graphpad Prism 5.0.

Lung histological analysis

Paraformaldehyde-fixed mouse lung sections were analyzed with hematoxylin-eosin (HE) or Masson`s trichrome staining to assess fibrotic changes in the lungs. Three anterior, middle and posterior sections of each lung specimen of mice were taken for HE and Masson staining, respectively, and then five high magnification views of each section were selected for observation and scored separately using the modified Ashcroft method (scale range 0 to 8)[16]. The histopathological score of pulmonary fibrosis in each mouse was expressed as the mean score.

Determination of intrapulmonary collagen content

80mg of lung tissue from the right lung was taken and determined according to the instructions of the Sircol method for measuring soluble collagen kit. A standard curve was made using collagen standards, and then the collagen concentration was calculated from the standard curve. Soluble collagen content was calculated according to the following formula: soluble collagen content = calculated collagen concentration × total volume of hydrolysate (1 mL)/80 mg × total wet weight of right lung tissue (mg).

Fluorescence quantitative PCR

Total RNA was extracted from each group of lung tissues using the TRIZOL method, and the RNA was reverse transcribed into cDNA using a reverse transcription kit. cDNA obtained was used for fluorescence quantitative PCR, and the expression of Col1a1, Col1a2 and α-SMA/ACTA2, RGS2, E-cad, and FN was detected using the β-actin gene as the internal reference gene levels. The genes, mRNA or protein are referred to using offcial gene symbols as provided by Te National Center for Biotechnology Information (NCBI;https:// www.ncbi.nlm.nih.gov/).

Western Blot

The expression of G protein signaling regulator 2 in lung tissues was detected in each group. Samples were electrophoresed and subjected to western blot using primary antibodies against RGS2 and β-actin.

Acquisition and identification of hUC-MSCs

P_2 generation hUC-MSCs were obtained from Chongqing Stem Cell Therapy Engineering Technology Research Center. hUC-MSCs were cultured and identified according to the methods reported in the literature[17]. hUC-MSCs were grown to P4 generation, and cell suspensions were collected after trypsin

digestion, centrifuged and resuspended with PBS and counted to a final cell concentration of 2.5×10^6/mL, placed on ice for use.

Acquisition, grouping and treatment of myofibroblasts

P_4 generation NIH3T3 and HLF-9 were spread in six-well plates at 1×10^6, respectively, and when the cell fusion reached approximately 80%, they were replaced with fresh DMEM medium containing 1% fetal bovine serum and treated with 4ng/ml transforming growth factor-β1(TGF-β1) and cultured for 24 hours, i.e. myofibroblasts of both cell lines.

Different concentrations of PFD were added in MFB: 0, 2, 4, 6, 8 and 10 mM, respectively, and the cells in each group were collected after 2h of incubation, and RGS2 mRNA expression was detected byRT- PCR to find out the effective concentration of RGS2 mRNA elevation caused by PFD.The effective concentration of PFD was added to MF separately, and the cells in each group were collected after 24h of culture separately, and the mRNA expression of fibrosis markers was detected by RT-PCR.

The cells were divided into three groups according to different culture methods: NIH3T3 group (N) or HLF-9 group(H), hUC-MSCs group (M), and NIH3T3+hUC-MSCs group (N+M) or HLF-9+hUC-MSCs group (H+M). NIH3T3 or HLF-9 were cultured in the lower chamber of one well of the co-culture six-well plate alone (N or H). The P_4 generation hUC-MSCs were cultured in the upper chamber of the other well of the co-culture six-well plate alone (M). MFB were cultured in the lower chamber of the co-culture six-well plate while P4 generation hUC-MSCs were cultured in the upper chamber of the co-culture six-well plate (N+M or H+M). All were cultured using DMEM/F12 containing 5% fetal bovine serum. When the cell fusion of each group reached approximately 80%, the effective concentration of PFD or equal amount of PBS buffer

was added, and each group of cells was collected after 24 h of culture respectively, and the mRNA expression of RGS2 and fibrosis markers were detected by PCR.

The hUC-MSCs were divided into three groups: hUC-MSCs group (M), hUC-MSCs+TGF-β1 group (M+T), and hUC-MSCs+TGF-β1+PFD group (M+T+P). hUC-MSCs were cultured using DMEM/F12 containing 5% fetal bovine serum, and when the fusion of cells in each group reached approximately 80%, they were replaced with fresh DMEM containing 1% DMEM medium with fetal bovine serum, treated with 4ng/ml TGF-β1 and cultured for 24h. Cells in the M+T group were collected and detected by PCR for E-cad, FN,α-SMA mRNA expression; in the M+T+P group, PFD or equal amount of PBS buffer was added after 24h of TGF-β1 treatment and cells were collected after 24 h of culture and detected by PCR for E-cad, FN, α-SMA mRNA expression.

Statistical analysis

Data were expressed as mean±SEM. Comparisons between groups were made using Student's t test for unpaired observations or two-factor ANOVA and Bonferroni correction for multiple comparisons. $p < 0.05$

was considered statistically significant.

Results

Therapeutic effects of different doses of PFD in BLM induced pulmonary fibrosis mice

Compared with the survival time in the model group(B), the low dose of PFD(10 mg/kg, P_{10}) alone could not prolong the survival time of mice with pulmonary fibrosis, and there was a trend to prolong the survival time in the low dose of PFD(30 mg/kg, P_{30}), the medium dose group (100 mg/kg, P_{100}) and the high dose PFD (300 mg/kg, P_{300}) alone, but the differences were not statistically significant (P was 0.38, 0.08, 0.19, respectively) (Fig. 1A).

The Sircol method could detect the content of soluble collagen in the lungs of mice, and the collagen content of the right lung of mice in the model group was significantly higher compared with the control group ($p < 0.001$). Compared with the model group, the collagen content of the P_{10} group was not significantly changed; the collagen content of the P_{30} group was reduced, and the difference was statistically significant ($p < 0.05$); the right lung collagen of the P_{100} group was significantly reduced ($p < 0.001$); and the right lung collagen content of the P_{300} group was reduced ($p < 0.001$), but the reduction was not as great as that of the P_{100} group ($p < 0.05$) (Fig. 1B) .

The pathological changes in the mice of the model group on day 21 of modeling were: inflammatory cell exudation, widening of alveolar septa, formation of ground glass like, strip like, grid like structure at the base of both lungs, and formation of cystic changes of varying sizes in some areas, i.e., honeycomb lung. Compared with the lung pathology of the model group, there was no significant improvement in the P_{10} group, a trend of improvement in the P_{30} group ($p = 0.055$), a decrease in the areas of lattice-like and honeycomb shape in the lungs of the P_{100} group, and a statistical difference in the improvement of Ashcroft score ($p < 0.001$). There was some improvement in the lung pathology of the P_{300} group ($p < 0.05$), but not as much as that of the P_{100} group ($p < 0.05$) (Fig. 1C, 1D). Masson staining stained the collagen in the lungs blue, which could reflect the severity of fibrosis in the lungs. A large amount of collagen deposition in the lung was seen microscopically in the model group. Compared with the model group, there was no significant change in intrapulmonary collagen deposition in the P_{10} group; collagen deposition was significantly reduced in the P_{30}, P_{100}, and P_{300} groups, but the collagen reduction in the P_{30} and P_{300} groups was not as great as that in the P_{100} group ($p < 0.05$) (Fig. 1E, 1F).

Col1a1, Col1a2 and α-SMA are considered to be the main pulmonary fibrosis markers, and their levels reflect the degree of pulmonary fibrosis [18]. Col1a1 and Col1a2 mRNA levels were significantly higher in the model group compared with the control group. Compared with the model group, there was no significant change in Col1a1 mRNA in the P_{10} group, and the expression of Col1a1 mRNA was

significantly reduced in the P_{30}, P_{100} and P_{300} groups, and the difference was statistically significant (Fig. 1G). Col1a2 mRNA levels were reduced in all treatment groups except for the low-dose PFD P_{10} group ($p < 0.05$), and the most significant reduction in Col1a2 mRNA expression was observed in the P_{100} group ($p < 0.001$). Compared with the α-SMA mRNA level in the model group, there was no significant change in the P_{10} group, a group decrease trend in P_{30} and P_{300} ($p = 0.07$, $p = 0.056$), and a significant decrease in the P_{100} group ($p < 0.05$) (Fig. 1I). It can be seen that low-dose PFD (30 mg/kg) was the lowest effective dose for anti-fibrosis, medium-dose PFD (100 mg/kg) had the best efficacy among the groups with PFD alone, and high-dose PFD (300 mg/kg) was not as effective as medium-dose PFD for anti-fibrosis.

Culture and characterization of hUC-MSCs

hUC-MSCs cultured in DMEM/F12 medium at 37°C in a 5% CO2 incubator were assayed for surface-specific antigens of P_4 generation hUC-MSCs using flow cytometry. The results showed that the surface molecules CD34, CD45, and HLA-DR positivity of hUC-MSCs were less than 2%, and CD73, CD90, and CD105 positivity were higher than 95% (Fig. 2A), and this result was in accordance with the standards published by the International Stem Cell Therapy Association in 2006. In addition, we also examined the multidirectional differentiation potential of hUC-MSCs, and our results showed that hUC-MSCs were differentiated to chondrogenic (Fig. 2B), osteogenic (Fig. 2C) and adipogenic cells (Fig. 2D).

Therapeutic effects of hUC-MSCs combined with P30 in BLM induced pulmonary fibrosis mice

Studies have shown that hUC-MSCs can attenuate acute lung injury and early pulmonary fibrosis, but the therapeutic effect on established pulmonary fibrosis is controversial[10, 11]. Preliminary experimental data from our group showed that hUC-MSCs alone by transcatheter tail vein injection were less effective in treating a mouse model of pulmonary fibrosis[19, 20], therefore the therapeutic effect of hUC-MSCs in combination with PFD on middle and late-stage pulmonary fibrosis is unclear. In contrast, the efficacy of too high doses of PFD is also less satisfactory and has significant adverse effects. Therefore, we next explored the therapeutic effect of hUC-MSCs combined with the lowest effective dose of PFD (30 mg/kg, P_{30}) on pulmonary fibrosis in mice. The results showed that the hUC-MSCs combined with P_{30} group significantly prolonged the survival time of mice (Fig. 3A), and the difference was statistically significant ($p < 0.01$), and the survival time of the hUC-MSCs combined with P30 group longer than the P_{100} group. The combination group significantly reduced the collagen content in the lungs ($p < 0.001$), and the collagen content was significantly lower than that in the P_{100} group. The collagen content in the hUC-MSCs combined with P_{30} group was significantly lower than that in the P_{100} group (Fig. 3B, $p < 0.001$)). Moreover, the combination group significantly improved bleomycin-induced pulmonary lesions with significantly better Ashcroft scores ($P < 0.001$) and significantly reduced intrapulmonary collagen deposition, all with better improvement than P_{100} (Fig. 3C-3F, $p < 0.01$). For the expression of pulmonary fibrosis markers, Col1a1, Col1a2, and α-SMA mRNA levels were significantly decreased in the hUC-MSCs combined with P_{30} group relative to the model group (Fig. 3G-3I, $p < 0.01$), where the reduced levels of

Col1a1 and Col1a2 were statistically different compared to the P_{100} group alone, while the reduced levels of α-SMA compared to the P_{100} group tended to be statistically different ($p = 0.09$).

hUC-MSCs combined with low-dose P_{30} elevated RGS2 mRNA and protein expression levels in mouse lung tissue

Studies have shown that RGS2 is a novel mechanism for the antifibrotic effect of PFD[4]. Quantitative RT-PCR analysis in this study confirmed that, as expected, the treatment of pulmonary fibrosis in mice with the optimal effective dose of PFD (100 mg/kg) increased RGS2 mRNA levels in mouse lung tissues (Fig. 4A, $p < 0.05$), and RGS2 mRNA levels in the hUC-MSCs combined with P_{30} group were more elevated than those in the P_{100} group ($p < 0.05$). Western blot confirmed that RGS2 protein levels in lung tissue were also significantly increased after PFD treatment in mice (Fig. 4B), and RGS2 protein levels in the hUC-MSCs combined with P_{30} group were also more than those in the P_{100} group.

hUC-MSCs can affect the expression of RGS2 and the markers of pulmonary fibrosis of myofibroblasts by PFD treatment

Studies have shown that MFB are the main cells of tissues after fibrosis and that TGF-β1 induces the conversion of fibroblasts to myofibroblasts[21]. In this experiment, two fibroblast model cells, MFB, were obtained after stimulation of two fibroblasts, NIH3T3 and HLF-9, for 24h using 4 ng/mL TGF-β1. hUC-MSCs and PFD for fibroblasts in subsequent experiments were added after TGF-β1 stimulation.

Quantitative RT-PCR analysis confirmed that RGS2 mRNA was elevated in a concentration-dependent manner after PFD treatment of both MFB (Fig. 5B, 5C), and the elevation of RGS2 mRNA in both MFB treated with ≥ 6 mM PFD was statistically significant ($p < 0.05$). As shown in Fig. 5D, 5E, the mRNA of fibrosis markers in both MFB decreased significantly after 24 h of PFD treatment, and the difference was statistically significant.

The effect of PFD (8 mM PFD) on the expression of RGS2 and lung fibrosis markers within MFB were explored after co-culture of hUC-MSCs with MFB. The results showed that the differences in RGS2 mRNA levels were statistically significant in the H + M + P group compared with the H + P group (Fig. 5F, $p < 0.01$), as well as in the N + M + P group compared with the N + P group (Fig. 5G, $p < 0.05$), indicating that hUC-MSCs further elevated RGS2 mRNA after co-action of MFB with PFD. As shown in Fig. 5H and 5I, hUC-MSCs co-treatment of MFB with PFD resulted in a statistically significant decrease in mRNA for fibrosis markers in both MFB.

We also examined the changes in mRNA levels of endothelial mesenchymal transition markers in hUC-MSCs. The results showed that E-cad mRNA was significantly increased and both FN and ACTA2 mRNA expression were significantly decreased after 8 mM PFD treatment of hUC-MSCs (Fig. 5J, $p < 0.05$).

Discussion

Although PFD has been approved by the Food and Drug Administration(FDA) for the treatment of IPF, its therapeutic effects are limited and its adverse effects are large. In the present study, we used hUC-MSCs combined with low-dose PFD for the treatment of pulmonary fibrosis in mice. We found combined with hUC-MSCs, the low-dose PFD could exert antifibrotic effects superior to its 3-fold or even 10-fold dose alone. Therefore, it can be speculated that the combination of hUC-MSCs and PFD can enhance the antifibrotic efficacy of PFD, reduce the dosage of PFD, and decrease the incidence of adverse effects, in order to enhance the tolerability and compliance of IPF patients.

Hisashi O et al. used three concentrations of 10, 30, and 100 mg/kg PFD to treat a mouse model of bleomycin-induced pulmonary fibrosis, and the results of this study showed that the antifibrotic effect of PFD was positively correlated with the dose[22]. However, the efficacy of PFD containing higher dose gradients on pulmonary fibrosis in mice has not been reported in this literature. In the present study, four dose gradients of 10, 30, 100, and 300 mg/kg PFD were applied to explore the therapeutic effects on pulmonary fibrosis, and contrary to expectations, the highest dose (300 mg/kg) PFD did not achieve better antifibrotic effects than the medium dose PFD (100 mg/kg). In clinical trials of PFD for IPF conducted by Azuma A et al. and Wijsenbeek MS et al. adverse effects of PFD were predominantly gastrointestinal reaction and were positively correlated with dose, with an incidence of 40–60%, and some patients discontinued the drug due to intolerance[23, 24]. Combined with the observation that the appetite of mice in the high-dose PFD group was poorer than that in the medium and low-dose PFD groups, it can be assumed that the PFD dose exceeded the appropriate range, resulting in gastrointestinal adverse reactions and reducing the therapeutic effect of PFD. Therefore, we cannot expect to achieve better anti-fibrotic effect by increasing the dose of PFD in clinical practice. For this reason, in order to avoid the adverse effects of higher doses of PFD, the combination of the low dose, 30 mg/kg PFD, with hUC-MSCs was used in this study instead of 100 mg/kg PFD with hUC-MSCs.

RGS2 is a known negative regulator of G protein signaling that inhibits the amplitude and duration of signals mediated by GQ-coupled GPCRs[25, 26]. Some GQ-coupled GPCRs and their ligands are important drivers of pulmonary fibrosis[27, 28]. Jang HS et al. demonstrated in animal experiments that knockdown of RGS2 leads to renal fibrosis in mice after unilateral ureteral obstruction (UUO), suggesting that endogenous RGS2 has an anti-fibrotic function[29]. Similarly, Xie Y et al. showed that the gene that they screened for rapid upregulation in human lung fibroblasts in response to pirfenidone using gene microarrays was RGS2, and confirmed that endogenous RGS2 exhibited anti-pulmonary fibrosis function and that RGS2 underlies the anti-fibrotic effect of PFD, thus providing some ideas to investigate the mechanism of PFD for the treatment of pulmonary fibrosis[4]. In our in vitro experiments, we observed that hUC-MSCs increased the expression of endogenous RGS2 in MFB, allowing pirfenidone to exert a stronger anti-fibrotic effect, which may be one of the reasons why hUC-MSCs combined with low-dose PFD could achieve better efficacy. However, the mechanism of how hUC-MSCs increase the expression of RGS2 needs to be explored, and the mechanism of how PFD exerts its antifibrotic effect through RGS2 also needs to be further investigated.

The pathogenesis of IPF is still unclear, but the main pathological changes are the proliferation and aggregation of large numbers of myofibroblasts and the deposition of extracellular matrix, of which the main component is collagen [30]. MFB, as the main effector cell of IPF, has a negative correlation with the severity and prognosis of IPF disease [31]. It was found that in addition to their anti-fibrotic effects, hUC-MSCs still have a certain degree in pro-fibrotic effects, especially in the fibrotic environment, which can be converted to MFB through endothelial mesenchymal conversion[32, 33], which may be one of the reasons for the controversial effects of hUC-MSCs in the treatment of pulmonary fibrosis. And PFD, as a multi-cytokine inhibitor, can inhibit the expression of cytokines such as TGF-β1, basic fibroblast growth factor(bFGF), and connective tissue growth factor(CTGF) of target cells (myo)fibroblasts, thus suppressing the proliferation of (myo)fibroblasts and the synthesis of collagen[34, 35]. Therefore, in this study we examined the changes in mRNA levels of endothelial mesenchymal transition markers in hUC-MSCs. The results showed that E-cad mRNA was significantly increased and FN and α-SMA mRNA expression were both significantly decreased after PFD treatment of hUC-MSCs. This result indicated that PFD inhibited the conversion of hUC-MSCs to mesenchymal cells, thereby reducing the conversion of hUC-MSCs to MFB in order to give hUC-MSCs the opportunity to exert maximum anti-fibrotic effects, which may also be one of the mechanisms why hUC-MSCs combined with PFD could achieve better efficacy compared with PFD alone.

Conclusion

In conclusion, the results of the present study provide direct evidence that hUC-MSCs combined with low-dose PFD had a therapeutic effect on a mouse model of pulmonary fibrosis and delayed the progression of IPF, providing a new idea for the clinical treatment of IPF. In addition, this study also found that the possible mechanism of the efficacy of hUC-MSCs in combination with low-dose PFD may be related to a significant increase in the expression of the anti-fibrotic protein RGS2, but the in-depth mechanism of the combined treatment of both needs to be further studied and explored.

Abbreviations

hUC-MSCs, umbilical cord-derived mesenchymal stem cells; PFD, pirfenidone; $P_{10,}$ 10 mg/kg PFD; P_{30}, 30 mg/kg PFD; P_{100}, 100 mg/kg PFD; P_{300}, 300 mg/kg PFD; HE, hematoxylin-eosin ; PCR, polymerase Chain Reaction; RGS2, regulator of G protein signaling 2; MFB, myofibroblasts; IPF, diopathic pulmonary fibrosis; Col1a1, type I collagen a1; Col1a2, type I collagen a2; α-SMA, α-smooth muscle actin; ACTA2, actin alpha 2; E-cad, calcium adhesion protein E; FN, fibronectin; NIH3T3, mouse embryonic fibroblasts; HLF-9, human Embryonic Lung Fibroblasts; DMEM, Dulbecco's Modified Eagle's Medium; F12, Ham's F-12 Nutrient; FBS, fetal bovine serum; P_6, passage 6; h, hours; N, control group; B, bleomycin model group; N+M, hUC-MSCs control group; B+M, hUC-MSCs treatment group; B+M+P_{30}, hUC-MSCs+30 mg/kg PFD group; TGF-β1, transforming growth factor-β; N, NIH3T3 group; H, HLF-9 group; M, hUC-MSCs group, N+M, NIH3T3+hUC-MSCs group; H+M, HLF-9+hUC-MSCs group; M+T, hUC-MSCs+TGF-β1 group; M+T+P, hUC-MSCs+TGF-

β1+PFD group; FDA, Food and Drug Administration; bFGF, basic fibroblast growth factor; CTGF, connective tissue growth factor.

Declarations

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Availability of data and material

All data generated or analyzed during this study are included in this published article and its Additional file 1.

Authors, contributions

XW, and ZF participated in research design. XW, HG, HJQ and OZ conducted experiments. XW, HJQ and OZ contributed materials and reagents. XW performed data analysis. XW, HML, ZF and LNC wrote or contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

No human participants, human data or human tissue involved in studies reported in this manuscript. All animal studies were approved by the Animal Ethics Committee of Chongqing Medical University.

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Figures

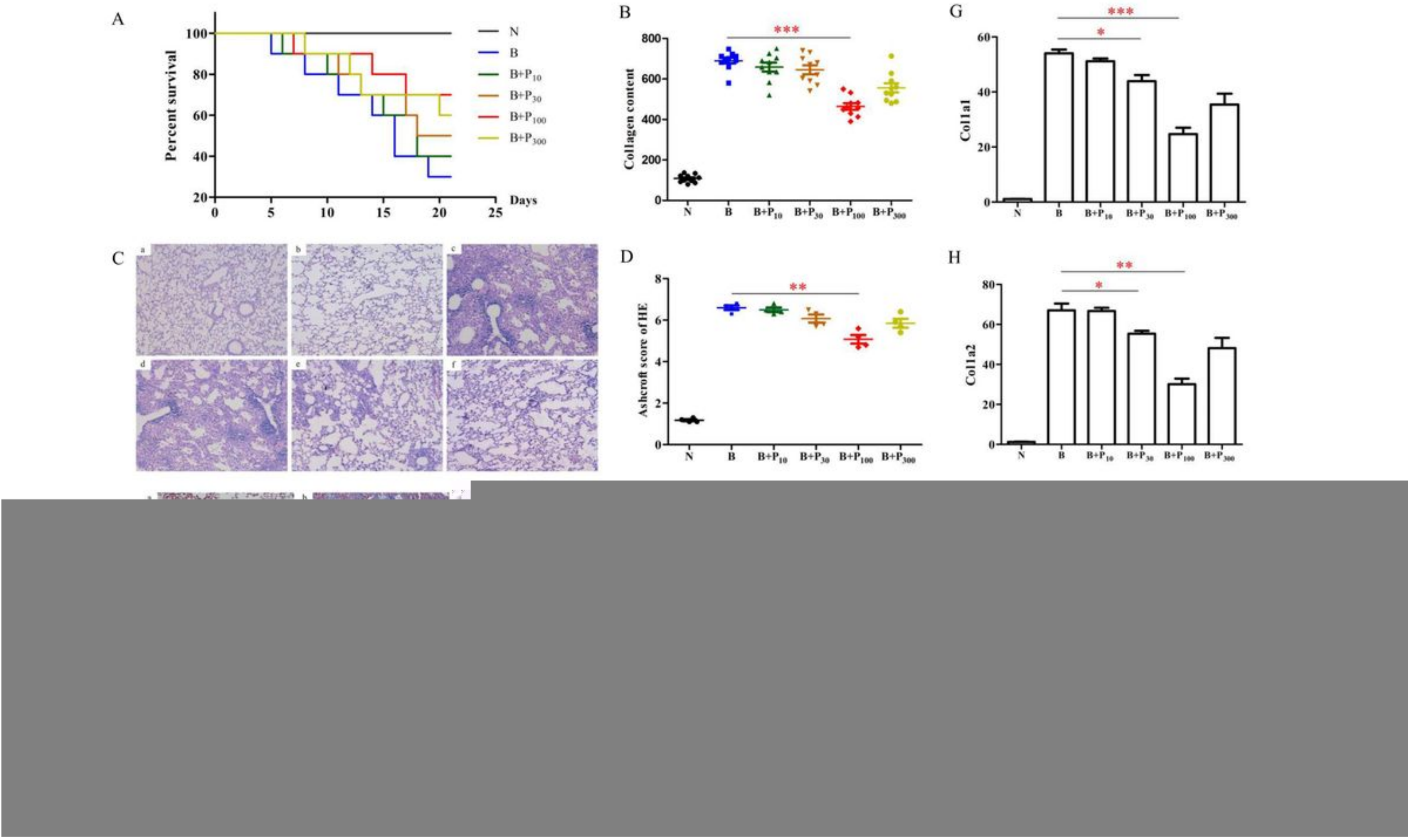

Figure 1

Therapeutic effects of different doses of PFD in BLM induced pulmonary fibrosis mice. **A** The KaplaneMeier survival curves of different doses of PFD groups. **B** The total content of soluble collagen in the right lungs of different groups in mice. **C,E** Representative pictures of HE and Masson staining of lung sections of different groups in pulmonary fibrosis mice. Scale bars: 200 μm. **D,F** The Ashcroft score of HE and Masson staining of different groups in pulmonary fibrosis mice. **G,H,I** The mRNA levels of main pulmonary fibrosis markers(Col1a1, Col1a2 and α-SMA) of different groups in mice.

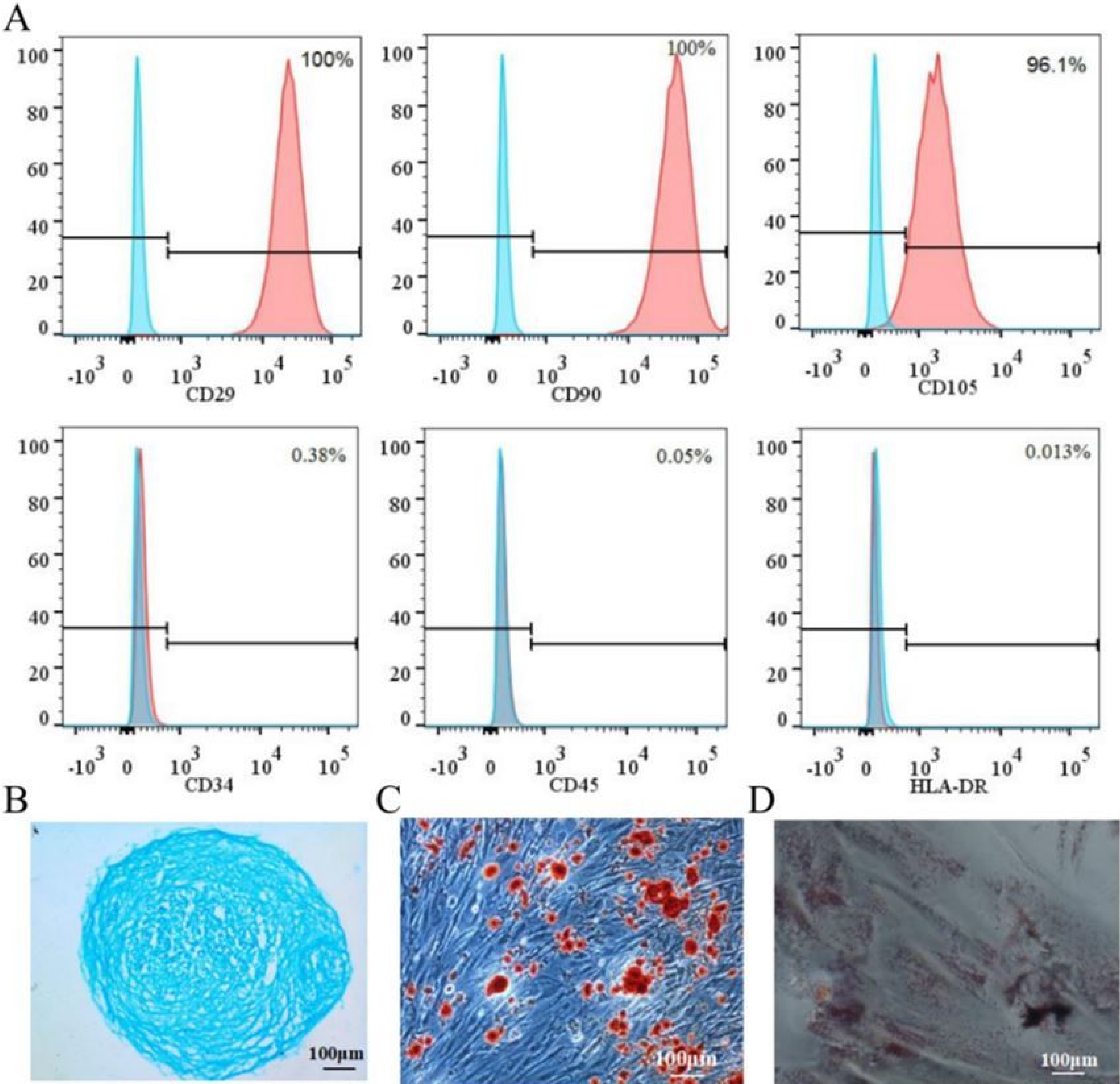

Figure 2

The pluripotency of hUC-MSCs. **A** The expression of the surface markers CD34, CD45, HLA-DR, CD73, CD90, and CD105 on hUC-MSCs detected by flow cytometry. **B, C, D** The results of hUC-MSCs differentiation into chrondrocytes, osteocytes, and adipocytes.

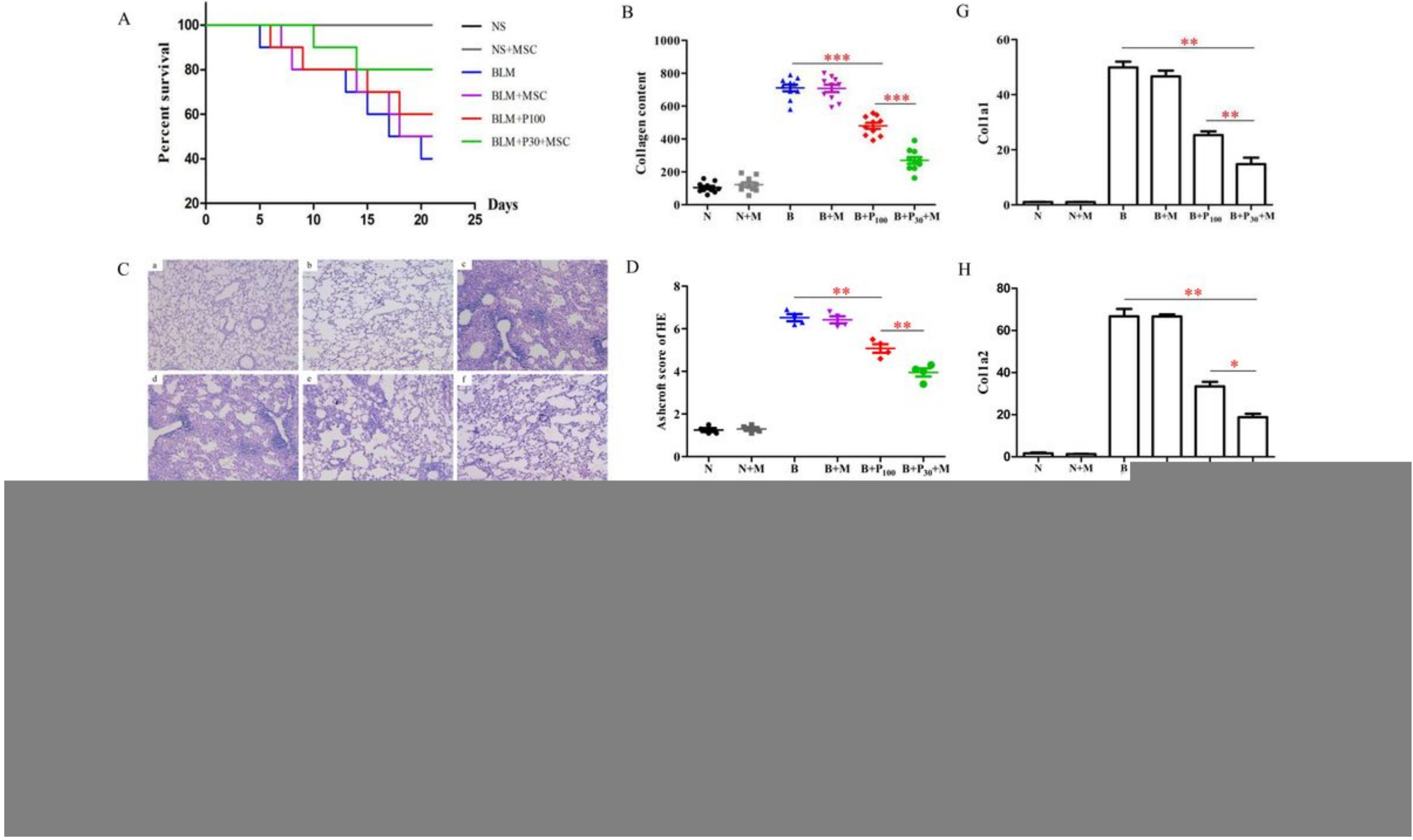

Figure 3

Therapeutic effects of hUC-MSCs combined with P_{30} in BLM induced pulmonary fibrosis mice. **A** The KaplaneMeier survival curves of different groups. **B** The total content of soluble collagen in the right lungs of different groups in mice. **C,E** Representative pictures of HE and Masson staining of lung sections of different groups in pulmonary fibrosis mice. Scale bars: 200 μm. **D,F** The Ashcroft score of HE and Masson staining of different groups. **G,H,I** The mRNA levels of main pulmonary fibrosis markers(Col1a1, Col1a2 and α-SMA) of different groups in pulmonary fibrosis mice.

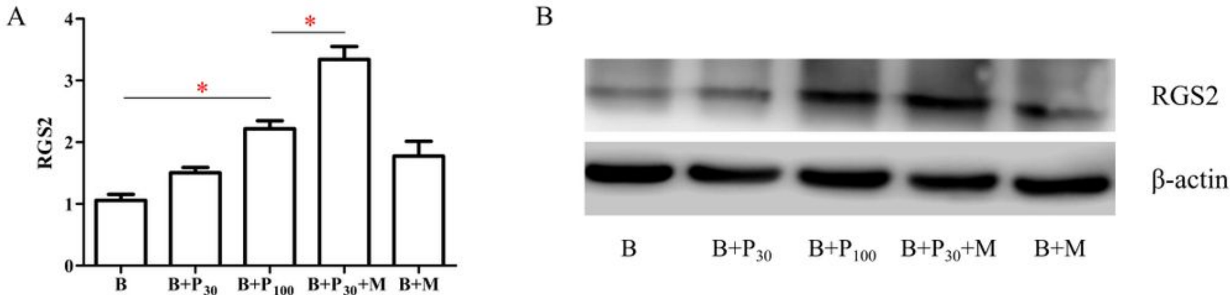

Figure 4

The RGS2 mRNA and protein expression levels in mouse lung tissue of different groups. **A** The RGS2 mRNA levels detected by PCR in mouse lung tissue of different groups. **B** The RGS2 protein expression levels detected by western blot in mouse lung tissue of different groups.

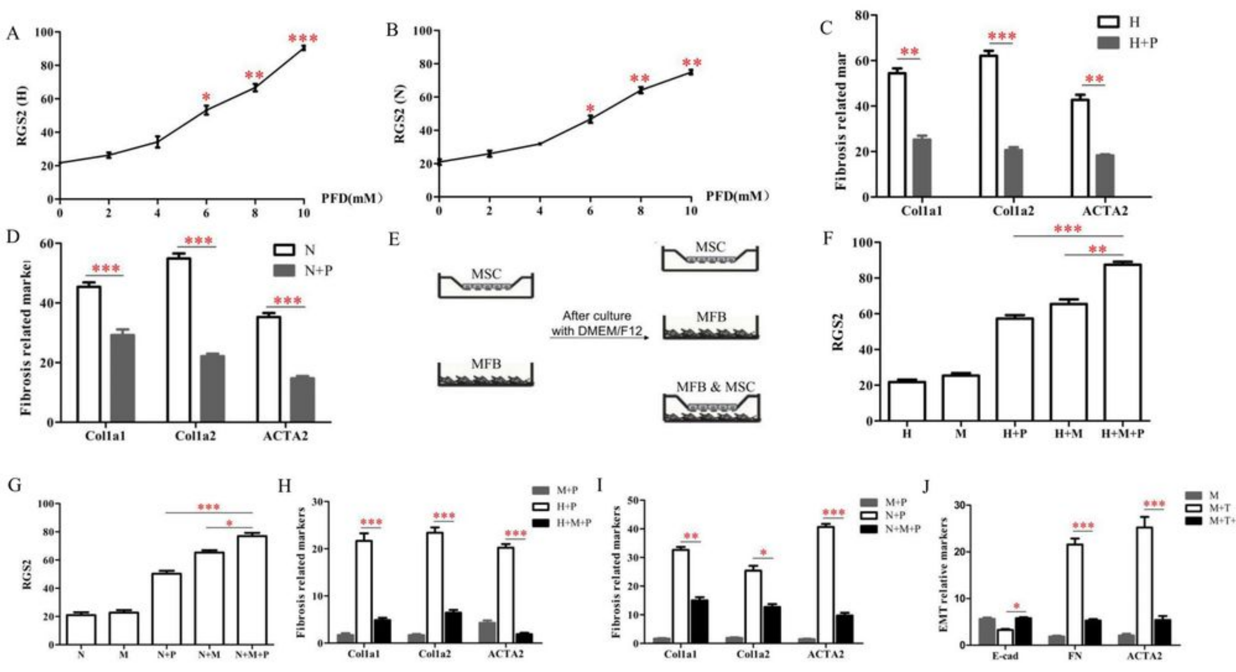

Figure 5

RGS2 suppresses the profibrotic effects in HLF-9 and NIH3T3. **A,B** HLF-9(**A**) and NIH3T3(**B**) were treated with various concentrations of PFD (0-10 mM) for 2 h and then were harvested for RT-PCR analysis of RGS2 and β-actin mRNA levels. **C,D** HLF-9(**C**) and NIH3T3(D) were treated with 8 mM PFD over a time course of 24 h and then were harvested for RT-PCR analysis of fibrosis markers mRNA levels. **E** Schematic diagram of single culture and co-culture of hUC-MSCs and MFB. **F,G** HLF-9(**F**) and NIH3T3(**G**) were single culture or co-culture with hUC-MSCs, then were treated with 8 mM PFD or equal amount of PBS buffer for 2 h and then were harvested for RT-PCR analysis of RGS2 and β-actin mRNA levels. **H,I** HLF-9(**H**) and NIH3T3(**I**) were single culture or co-culture with hUC-MSCs, and were treated with 8 mM PFD for 24 h and then were harvested for RT-PCR analysis of fibrosis markers mRNA levels. **J** hUC-MSCs were treated with 4ng/ml TGF-β1 and cultured for 24h, and treated with 8 mM PFD or equal amount of PBS buffer for 24 h and then were harvested for RT-PCR analysis of E-cad, FN, α-SMA mRNA levels.

Supplementary Files

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