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Fibrocyte CXCR4 regulation as a therapeutic target in pulmonary fibrosis

Borna Mehrad, **Marie D. Burdick**, and **Robert M. Strieter**
Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Virginia, P.O. Box 800466, Charlottesville, Virginia 22908

Abstract

Fibrotic interstitial lung diseases are characterized by progressive decline in lung function and premature death from respiratory failure. Fibrocytes are circulating bone marrow-derived progenitor cell that traffic to the lungs and contribute to fibrosis and may represent novel therapeutic targets in these diseases. We have previously found the recruitment of fibrocytes to the lung to be dependent on the chemokine ligand CXCL12. Given that the expression of the CXCL12 receptor, CXCR4, can be modulated pharmacologically in other cell types, we tested the hypotheses that the regulation of CXCR4 expression on fibrocytes mediates their influx to the lung in the context of pulmonary fibrosis and that pharmacologic inhibition of this process results in attenuated disease severity. CXCR4 was the predominant chemokine receptor on human fibrocytes, and its expression on fibrocytes was enhanced by hypoxia and by growth factors including platelet-derived growth factor. Both hypoxia-induced and growth factor-induced CXCR4 expressions were attenuated by specific inhibition of PI3 kinase and mTOR. Finally, in the mouse model of bleomycin-induced pulmonary fibrosis, treatment with the mTOR inhibitor rapamycin resulted in reduced numbers of CXCR4-expressing fibrocytes in the peripheral blood and lung as well as reduced lung collagen deposition. Taken together, these experiments support the notion that pharmacologic inhibition of the CXCR4/CXCL12 biological axis is achievable in human fibrocytes and reduces the magnitude of pulmonary fibrosis in an animal model. This approach may hold promise in human fibrotic lung diseases.

Keywords

chemokines; stem cells; lung; cell traffic; signal transduction

Introduction

Fibrotic interstitial lung diseases are illnesses characterized by tissue remodeling, fibroproliferation, and deposition of extra-cellular matrix in the lung parenchyma. Some of these illnesses occur in response to known insults – such as inorganic dusts, inhaled antigens, or autoantigens – whereas others are of unknown cause. The prototypical fibrotic lung disease is idiopathic pulmonary fibrosis (IPF), a progressive disorder that culminates in premature death from respiratory failure and in which no treatment intervention has been effective to date. There is thus an urgent clinical need for new therapeutic approaches these diseases.

Corresponding author: Robert M. Strieter, M.D., P.O. Box 800466, Charlottesville, VA 22908, Tel (434) 243 7363, Fax (434) 924 2824, E-mail: E-mail: Strieter@Virginia.edu.

A fundamental question in the pathogenesis of fibrotic interstitial lung diseases is the cellular precursors of the lung fibroblasts and myofibroblasts. In this context, we have previously examined the contribution of a population of bone marrow-derived circulating progenitor cells, the fibrocytes (Phillips et al., 2004). Specifically, we found fibrocytes to accumulate in the lungs of mice with bleomycin-induced pulmonary fibrosis in associated with deposition of collagen and that human fibrocytes given to SCID mice challenged with bleomycin specifically trafficked to the lungs (Phillips et al., 2004). Consistent with this, we also found an expanded pool of fibrocytes in the peripheral blood of patients with fibrotic interstitial lung diseases (Mehrad et al., 2007) findings that have been subsequently reproduced by another group (Andersson-Sjoland et al., 2008). We have recently found that the expanded pool of circulating fibrocytes in patients with IPF is an independent predictor of mortality (Moeller et al., 2009), lending further support to the relevance of these cells in the pathogenesis of pulmonary fibrosis.

Identification of the chemokine ligand-receptor pair that mediates the mobilization of human fibrocytes from the bone marrow to the lung in the context of pulmonary fibrosis has the potential to open new avenues for therapy for these illnesses, but the issue remains unsettled in the literature. This is, at least in part, due to differences between human and mouse fibrocyte chemokine receptor repertoire: human fibrocytes express CXCR4, CCR3, CCR5 and CCR7 (Phillips et al., 2004, Quan et al., 2004) whereas mouse fibrocytes express CXCR4, CCR2 and CCR7 (Phillips et al., 2004, Abe et al., 2001, Moore et al., 2005, Quan et al., 2004). CXCR4 is a critical chemokine receptor in haematopoietic and non-haematopoietic stem cell homing in both mice and humans, and the differential expression of CXCL12 in tissue creates a gradient essential for traffic of CXCR4-expressing cells (Murdoch, 2000). Consistent with their progenitor cell phenotype, human fibrocytes express CXCR4 and migrate in response to CXCL12 in vitro (Phillips et al., 2004). In the context of the mouse model, there was a marked induction in lung CXCL12 after bleomycin challenge and the neutralization of this ligand resulted in reduced accumulation of fibrocytes in the lungs and attenuated lung fibrosis (Phillips et al., 2004). In human interstitial lung disease, we found elevated plasma and lung levels of CXCL12 protein associated with ~90% CXCR4 expression in circulating fibrocytes (Mehrad et al., 2007, Phillips et al., 2004). Other groups have shown mouse fibrocytes to traffic to tissue via the CCR2/CCL12 axis in the FITC-induced model of pulmonary fibrosis (Moore et al., 2005, Moore et al., 2006), via the CCL3-CCR5 axis in bleomycin-induced pulmonary fibrosis (Ishida et al., 2007), and via CCR7 in models of renal fibrosis and wound healing (Abe et al., 2001, Sakai et al., 2006).

Prior work has demonstrated the expression of CXCR4 on fibrocytes in the context of mouse and human pulmonary fibrosis and implicated CXCL12-mediated traffic of fibrocytes to the lungs in the pathogenesis of pulmonary fibrosis (Phillips et al., 2004, Mehrad et al., 2007, Andersson-Sjoland et al., 2008). The purpose of the current work was to provide proof-of-principle that this mechanism can be manipulated pharmacologically as a therapeutic strategy in fibrotic lung disease. We therefore tested the hypotheses that the regulation of CXCR4 expression on fibrocytes mediates their influx to the lung in the context of pulmonary fibrosis and that pharmacologic inhibition of this process results in attenuated disease severity.

Materials and methods

Human fibrocyte isolation

Human fibrocytes were quantified in fresh blood samples collected in EDTA tubes or were isolated from leukophoresis packs (HemaCare, Woodland Hills, CA) as previously described (Phillips et al., 2004). Briefly, peripheral blood mononuclear cells were plated on fibronectin-coated flasks and cultured in complete media [Dulbecco's Modified Eagle Media (DMEM, Invitrogen, Carlsbad, CA) supplemented with 20% FCS, 25 mM HEPES, 100 units/mL penicillin, and 100 ng/mL streptomycin]. Non-adherent cells were discarded after 5 days and

remaining cells were incubated for an additional 7-10 days, then negatively selected with anti-CD2, anti-CD14, and anti-CD19 (Dynal, Lake Success, NY), and cultured in complete media for 24 hours. The resulting cells were >90% fibrocytes, as defined by simultaneous expression of CD45 and collagen-1 (Col1). To further establish their phenotype, fibrocytes (prepared as above), peripheral blood monocytes, or normal human lung fibroblasts (Lonza, Allendale, NJ) were cultured in complete media at 3×10^4 cells/ml with or without TGF-β and the concentration of soluble collagen in the supernatant was quantified (Table). These data showed that human fibrocytes, like fibroblasts, produce substantial amounts of collagen in vitro.

In some experiments, fibrocytes were pre-treated with 20 μM LY294002 or 10 ng/mL rapamycin (Cell Signaling Technology, Beverly, MA) for 1 h prior to stimulation with 30 ng/mL PDGF. Cells were then cultured in normoxic or hypoxic conditions (94% nitrogen, 5% carbon dioxide, 1% oxygen) in modular incubator chambers (Billups-Rothenberg, Del Mar, CA), and whole-cell or nuclear and cytoplasmic extracts were prepared, as previously described (Phillips et al., 2005).

RNA isolation and quantitative PCR

Total lung RNA was extracted from mouse lungs using Trizol reagent (Gibco-BRL, Grand Island, NY) according to manufacturer's instructions. One microgram of total RNA was reversed transcribed into cDNA and amplified using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Real-time quantitative polymerase chain reaction (PCR) was performed using the ABI Prism 7700 Sequence Detector and SDS analysis software (Applied Biosystems). For both CXCR4 and 18S, TaqMan Pre Developed Assay Reagents (PDAR) (Applied Biosystems) was used for amplification. Reactions were assembled in 96-well reaction plates using TaqMan Universal PCR Master Mix (2x); the appropriate PDAR; and template. PCR was performed under the following conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles at 95°C for 15 s; and 60°C for 1 min. Negative controls (no template cDNA) were performed on each PCR plate. Quantitative analysis of gene expression was done using 2^{-ddCT} method.

Flow cytometry

Analysis of mouse fibrocytes was performed as previously described on freshly collected peripheral blood and lung single cell suspensions (Phillips et al., 2004). Human cells were stained with PE-Cy7 anti-CCR7, PerCP anti-CD45 (BD Biosciences, San Jose, CA), PE anti-CCR2, APC anti-CXCR4 (R&D systems, Minneapolis, MN) or respective isotype controls. To stain for intracellular collagen-1, cells were permeabilized with a commercial kit (Cytofix/Cytoperm, BD Biosciences) and stained with biotinylated polyclonal rabbit anti-human collagen-1 (Rockland, Inc., Gilbertsville, PA), goat anti-mouse procollagen I C-terminus, procollagen I N-terminus, or procollagen III (Santa Cruz Biotechnology, Santa Cruz, CA) and detected with streptavidin-FITC or anti-goat PE (BD Biosciences). Samples were analyzed a FACS Calibur instrument using Cellquest 3.2.1f1 software (BD Biosciences).

Chemotaxis assay

Fibrocytes were tested for chemotaxis to CXCL12 after exposure to various conditions, using a modification of a previously described protocol (Phillips et al., 2003). Briefly, the bottom well of blind-well modified Boyden 12-well chemotaxis chambers (Neuroprobe, Gaithersburg, MD) was filled with DMEM with 20% FCS, 5 μg/mL fibronectin and various concentrations of CXCL12. Polycarbonate filters with 5 μm pores were and the top wells filled with fibrocytes suspended in DMEM with 20% FCS (10^5 cells/100 μl). Chambers were incubated for 6 hrs at 37°C in 5% CO_2. Filters were then removed, fixed in methanol, stained with 2% toluidine blue, and the number of cells per high power field that had migrated through the filters was counted in 5 high-power fields per well. Each condition was run in triplicate.

Western blot analysis

Proteins were analysed by immunoblotting using 40 μg of total protein from nuclear/cytoplasmic extracts (Phillips et al., 2005). After SDS-PAGE and transfer to PVDF, membranes were blocked in 5% non-fat dry milk for 30 min and incubated with primary antibody at 4°C overnight. Primary antibodies used were mouse anti-human HIF-1α (1:500; BD Biosciences), mouse anti-β-actin (1:1000; Abcam, Cambridge, MA), mouse anti-β-tubulin (1:1000; Novus Biologicals, Littleton, CO), and rabbit anti-CXCR4 (1:500; Calbiochem, La Jolla, CA). Membranes were then washed in TTBS and incubated with donkey anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA) for 1 h at room temperature. Blots were washed in TTBS and visualized using ECL Plus (Amersham Biosciences, Piscataway, NJ).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously, with slight modifications (Pan et al., 2006). Briefly, formaldehyde-fixed cells were harvested and prepared for immunoprecipitation using a commercial kit (Upstate Biotechnology, Lake Placid, NY). One half of each sample was incubated with either 2 μg anti-human HIF-1α or isotype control antibody at 4°C overnight. Immune complexes were collected with protein A-agarose, washed and eluted, reverse cross-linked and purified by phenol-chloroform extraction. Purified and input DNA was analyzed by conventional PCR using primers specific for the human CXCR4 promoter.

Transient transfections and luciferase assays

Human fibrocytes were transfected using commercial kits according to the manufacturer's instructions (Human MSC Nucleofector and Nucleofector II, Amaxa Biosystems, Gaithersburg, MD). Briefly, cells were resuspended in 100 μL MSC solution, combined with 1 μg pRL-SV40 (Promega) and 4 μg pGL3-CXCR4 WT promoter or pGL3-CXCR4 HRE mut promoter (Schioppa et al., 2003, Phillips et al., 2005), and nucleofected using program U-23. Cells were then immediately plated on fibronectin in DMEM with 20% FCS and incubated at 37°C for 24 h. Subsequently, cells were transferred to DMEM with 0.3% HSA overnight, exposed to either normoxia or hypoxia for 6 h, and harvested using the Dual Luciferase Reporter Assay kit (Promega). Luciferase activity was assessed using a MLX Microplate Luminometer (Dynex Technologies, Chantilly, VA) and then normalized to the Renilla control.

Mice and in vivo studies

C57Bl/6 mice and mice transgenic for green fluorescent protein (GFP) under the control of the human ubiquitin C promoter on a C57Bl/6 background were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained under pathogen-free conditions in the animal care facility at the University of Virginia and in compliance with institutional animal care regulations. Bone marrow chimeras were generated using published protocols (Spangrude, 2008) and preceded bleomycin challenge by 8 weeks. Bleomycin-induced pulmonary fibrosis was induced in 6-8 weeks old female C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) as previously described (Phillips et al., 2004). Briefly, mice were anesthetized with ketamine and given intratracheal injections of either 0.15 U/kg bleomycin in saline (Sigma-Aldrich, St. Louis, MO) or sterile saline. From 1 day before to 15 days after bleomycin administration, animals were given daily i.p. injections of 2 mg/kg rapamycin in saline or saline control. On day 16, animals were euthanized by pentobarbital overdose and whole lungs were removed. Salt- and acid-soluble fractions of lung collagen, which constitute the most recently released collagen and is made up of tropocollagen monomers and non-cross-linked collagen fibrils, were quantified using a commercial kit, according to the manufacturer's instructions (Sircol collagen assay, Biocolor, Belfast, UK).

Statistical analysis

Data were analyzed on a Macintosh computer using Statview 5.0 statistical package (Abacus Concepts Inc., Berkeley, California, USA). All data were expressed as mean ± SEM. Comparisons between 2 groups were performed with unpaired two-tailed Mann-Whitney (non-parametric) test, and comparisons between multiple groups were performed using the Kruskal-Wallis test. Probability values were considered statistically significant if they were less than 0.05.

Results

Fibrocytes traffic to the lungs and differentiate into myofibroblasts

We have previously shown that human fibrocytes transferred to SCID mice challenged with bleomycin traffic to the lungs, and that neutralization of CXCL12 in bleomycin-induced pulmonary fibrosis results in reduced accumulation murine fibrocytes in the lungs that is associated with reduced lung collagen deposition (Phillips et al., 2004). On the other hand, the magnitude of the contribution of bone marrow-derived fibrocytes to total lung fibroblasts in the context of pulmonary fibrosis is not established, and whether host fibrocytes are capable of differentiating into myofibroblasts in the lungs has been questioned (Hashimoto et al., 2004). Since these issues are critical to the premise of our hypothesis, we established stable bone marrow chimera mice between donors with constitutive expression of GFP and wildtype recipients. As expected, in control mice challenged only with saline, all bone marrow $CD45^+$ $Col1^+$ fibrocytes were found to express GFP. In contrast, on day 8 after intrapulmonary challenge with bleomycin, there was a marked expansion of the bone marrow fibrocyte pool, with most but not all cells expressing GFP (Figure 1A). This indicates that, even after lethal irradiation and bone marrow transplantation, a small proportion of fibrocytes are derived from recipient progenitors. At the same time point, we found >60% of the lung $CD45^+$ $Col1^+$ fibrocytes to express GFP, indicating their derivation from donor-derived bone marrow precursors (Figure_1B). To address whether these cells were capable of further differentiation in the lungs, we quantified their expression of the myofibroblast marker, α-smooth muscle actin (αSMA) in the lung fibrocytes. Approximately 10% of lung fibrocytes were found to express αSMA in both bleomycin- and saline-treated mice, of which more than half expressed GFP and were therefore donor-derived (Figure 1C). These data establish that, in the context of this animal model, bone marrow-derived fibrocytes traffic to the lungs and acquire myofibroblast markers.

CXCR4 is the predominant chemokine receptor expressed on human and mouse fibrocytes

We have previously shown that the CXCR4/CXCL12 biological axis is critically important for fibrocyte trafficking to areas of fibrosis in the lung in mouse models (Phillips et al., 2004, Gomperts et al., 2006), but others have shown a role for CCR2 and CCR7 in fibrocyte function (Moore et al., 2005, Abe et al., 2001). To determine the array of constitutively expressed chemokine receptors on normal human and mouse fibrocytes, we examined fresh $CD45^+$ $Col1^+$ cells from buffy coat of normal subjects or naive animals for expression of these chemokine receptors (Figure 2A-B). We found that most (but not all) freshly isolated human fibrocytes expressed CXCR4, whereas 46% expressed CCR2 and 9% expressed CCR7. Approximately 30% of the CCR2-expressing cells were also $CXCR4^+$ and most CCR7-expressing cells also expressed CCR2, but there was no overlap between $CXCR4^+$ and $CCR7^+$ fibrocytes. As we have noted previously, the peripheral blood fibrocyte concentration was lower in mice as compared to humans (Mehrad et al., 2007, Phillips et al., 2004), but, similar to humans, most mouse fibrocytes expressed CXCR4 (Figure 2B). We have previously reported that ~90% of fibrocytes in both the circulation and lungs of patients with pulmonary fibrosis express $CXCR4^+$ (Mehrad et al., 2007), and we have found a similar expression profile as normal human for fibrocytes for CCR2 and CCR7 from these patients (data not shown).

Given these data and our previous work linking fibrocyte traffic to the pathogenesis of pulmonary fibrosis, we next examine factors that might regulate the expression of CXCR4 in fibrocytes.

Hypoxia increases CXCR4 expression and function in human fibrocytes

CXCR4 expression is known to be regulated by HIF-1α and to be enhanced in hypoxic conditions in cancer cells (Phillips et al., 2005, Staller et al., 2003), but the relevance of this condition in fibrocytes has not been determined. Since the bone marrow microenvironment is known to be hypoxic relative to arterial blood (Harrison et al., 2002, Parmar et al., 2007, Schwartz and Stats, 1949, Skouby, 1976), examination of hypoxic regulation of CXCR4 expression in fibrocytes is likely relevant to their function in vivo. We therefore tested the expression of CXCR4 in human fibrocytes cultured in hypoxic as compared to normoxic conditions (Figure 3A). There was a marked induction of CXCR4 mRNA in fibrocytes cultured in hypoxia as compared to cells cultured in normoxic conditions. This effect was observed in fibrocytes from every donor tested, although the magnitude of the effect varied between donors. To determine whether this increased mRNA expression resulted in increased cell surface expression of CXCR4 protein, we performed flow cytometry on fibrocytes cultured in normoxic or hypoxic conditions for 12 hours (Figure 3B). Cell-surface expression of CXCR4 was augmented in cells cultured under hypoxic conditions, as compared to normoxic controls. In order to demonstrate the functional consequence of this enhanced expression, we next tested the chemotaxis of fibrocytes that had been cultured under hypoxia or normoxia towards CXCL12, the natural ligand of CXCR4 (Figure 3C). Hypoxic preconditioning of fibrocytes exhibited markedly enhanced chemotaxis of these cells to all concentrations of CXCL12 tested, providing evidence that hypoxia enhances the transcription and cell surface expression of CXCR4 in fibrocytes, and results in their greater chemotaxis to CXCL12.

Hypoxia increases HIF-1α expression and interaction with the CXCR4 promoter in human fibrocytes

We next investigated the mechanism by which hypoxia results in CXCR4 expression in fibrocytes. HIF-1α, a critical component of the HIF-1 transcription factor, is an essential component of cellular detection of hypoxia (Semenza, 1999, Semenza, 2000) and is known to regulate CXCR4 expression in cancer cells (Phillips et al., 2005). We found that fibrocytes from healthy donors had low or undetectable HIF-1α protein in the cytosol and nucleus when cultured in normoxic conditions, but that HIF-1α was abundantly present in both the cytoplasm and nucleus of cells incubated in hypoxia for 3 and 6 h (Figure 4A).

To assess whether the increase in HIF-1α protein levels correlated with an increase in CXCR4 transcription, a construct containing either a wild-type 2.6 kilobase fragment of the CXCR4 promoter or an HRE mutant CXCR4 promoter upstream of a luciferase reporter was transfected into fibrocytes (Phillips et al., 2004, Gomperts et al., 2006). Incubation of the cells transfected with the wild-type promoter in hypoxia resulted in a 2-fold increase in transcription, whereas hypoxia had no effect on the transcription in cells transfected with the mutant promoter (Figure 4B). To confirm that the effect of hypoxia on CXCR4 transcription was mediated by HIF-1α, the interaction of HIF-1α with the endogenous CXCR4 promoter was determined by ChIP assay. Chromatin isolated from fibrocytes incubated in hypoxia was immunoprecipitated with anti-HIF-1α or isotype control mAb, and the presence of the CXCR4 promoter was determined by conventional PCR. CXCR4 promoter DNA was present in input chromatin of all samples but was markedly detectable in anti-HIF-1α immunoprecipitates, and was greatly increased by hypoxia (Figure 4C). These data indicate that in human fibrocytes, hypoxia increases HIF-1α protein levels and CXCR4 transcription, and that HIF-1α directly interacts with the CXCR4 promoter.

Growth factors control CXCR4 expression in human fibrocytes

A substantial body of literature supports a role for growth factors in the pathogenesis of pulmonary fibrosis (Abdollahi et al., 2005, Antoniades et al., 1990, Aston et al., 1995, Ishii et al., 2006, Martinet et al., 1987, Pilewski et al., 2005, Rice et al., 1999, Uh et al., 1998, Vanhee et al., 1994, Yasuoka et al., 2006), and we have previously shown that growth factors that activate tyrosine kinase receptors increase CXCR4 expression in cancer cells (Phillips et al., 2005, Richard et al., 2000). We reasoned that fibrocytes that have left the bone marrow may encounter normoxic microenvironments (for example in arterial blood) but are likely to be exposed to high levels of growth factors in the context of the fibrotic lung. We therefore tested whether hypoxia-independent mechanisms can mediate CXCR4 up-regulation in human fibrocytes. As compared to fibrocytes grown without growth factors, we found fibrocyte CXCR4 mRNA to increase by 4.3-fold in cells cultured with PDGF, 4.1-fold with insulin-like growth factor (IGF), and 1.8-fold with EGF under normoxic conditions (Figure 5). Thus, these data indicated that human fibrocytes up-regulate CXCR4 transcription in response to PDGF, EGF and IGF under normoxic conditions.

The PI3-kinase pathway and mTOR activation mediate up-regulation of CXCR4 expression in human fibrocytes

The PI3-kinase pathway is a major signal transduction pathways downstream of PDGF receptor (Klinghoffer et al., 1996) and is also implicated in hypoxia-induced HIF-1α activation in several cancer cell lines (Zundel et al., 2000, Zhong et al., 2000). Given the data showing that exposure to PDGF resulted in the greatest induction of CXCR4 transcription in human fibrocytes (Figure 5), we tested the hypothesis that the PI3 kinase pathway mediates CXCR4 induction in fibrocytes.

Under normoxic conditions, we found the PI3 kinase inhibitor, LY294002, had little effect on basal CXCR4 mRNA levels, suggesting that this basal transcription is independent of the PI3 kinase pathway (Figure 6A). Also under normoxic conditions, PDGF-induced CXCR4 induction was markedly inhibited, but not entirely abolished, by LY294002, suggesting that the PI3 kinase pathway is the major mechanism in operation in PDGF-induced expression of CXCR4. Similar to our experience with cancer cells (Phillips et al., 2003), we have made similar observations when fibrocyte PI3 kinase inhibition was achieved with wortmannin (our unpublished data). PDGF and hypoxia demonstrated an additive effect in inducing CXCR4 induction in fibrocytes and, remarkably, LY294002 was found to abolish hypoxia-induced CXCR4 induction and markedly diminish the combined effect of PDGF and hypoxia on CXCR4 expression (Figure 6A). These data implicate the PI3 kinase pathway in CXCR4 induction in response to both hypoxia and to growth factors. We next assessed the effect of PDGF and PI3 kinase signaling pathway in HIF-1α nuclear accumulation. Fibrocytes were incubated with PDGF in the presence or absence of LY294002 under normoxic or hypoxic conditions (Figure 6B). The results of these experiments paralleled CXCR4 induction: PDGF mediated an increase in nuclear HIF-1α that was more prominent in hypoxic conditions, and this effect was abrogated by inhibition of the PI3 kinase pathway. Thus, the effect of PDGF and hypoxia on nuclear localization of HIF-1α correlates with their effect on up-regulation of CXCR4 transcription, suggesting that this pathway may represent a means of targeting fibrocyte CXCR4 expression.

Since AKT mediates phosphorylation of mTOR and mTOR can be targeted pharmacologically (Schlessinger, 2000, Lawrence et al., 2004), we examined the specific mTOR inhibitor, rapamycin, for its ability to inhibit up-regulation of CXCR4 mRNA. Under normoxic conditions, rapamycin did not dramatically affect basal CXCR4 mRNA levels, but the CXCR4 induction by hypoxia, PDGF, or both were all dramatically inhibited by rapamycin by >75%

(Figure 7A). In addition, nuclear localization of HIF-1α in response to PDGF was inhibited under both normoxic and hypoxic conditions (Figure 7B).

Rapamycin inhibits extravasation of fibrocytes in the lungs of bleomycin-treated animals

We have previously shown that, in the context of bleomycin-induced pulmonary fibrosis, $CXCR4^+$ fibrocytes accumulate in the lung and contribute to lung collagen deposition, which could be inhibited by in vitro CXCL12 neutralization (Phillips et al., 2004). CXCL12 levels cannot be targeted pharmaceutically at present, however. Given our in vitro data indicating that inhibition of the PI3 kinase/mTOR pathways abrogates growth factor- and hypoxia-induced CXCR4 up-regulation, we sought to determine whether inhibition of this pathway in vivo, using a currently available pharmaceutical agent, can be used to block influx of fibrocytes into the lung in this model. To assess this, mice challenged with intratracheal bleomycin or saline were treated with daily injections of rapamycin (2mg/kg) or vehicle for 16 days. In this context, rapamycin treatment inhibited the bleomycin-induced increase in circulating fibrocytes in the peripheral blood and abolished bleomycin-induced fibrocyte infiltration into the lung (Figure 8A-B). Interestingly, rapamycin had no detectable effect on basal numbers of fibrocytes in the peripheral blood or lung in saline treated animals. To ascertain that the lung $CD45^+$ $Col1^+$ cells in this context were in fact collagen-producing cells, we also quantified the lung CD45-expressing cells that stained for C- and N-terminus of procollagen I and procollagen III (Figure 8C). The $CD45^+$ pro-$collagen^+$ cells accumulated in the lungs of mice challenged with bleomycin, similar to our previous observations with $CD45^+$ $Col1^+$ cells (Figure 8B and reference (Phillips et al., 2004)).

We next assessed the effect of rapamycin treatment on lung collagen content after bleomycin challenge. As expected, rapamycin treatment had no effect on basal lung collagen content, but resulted in a notable 58% decrease in lung collagen deposition in response to bleomycin (Figure 9), consistent with the hypothesis that inhibition of the PI3-kinase/AKT/mTOR signal transduction pathway with rapamycin results in attenuated expression of CXCR4 in fibrocytes and thus reduces their homing to the lung. We noted, however, that the inhibition of fibrocyte homing to the lung with rapamycin treatment was more marked than the previous observation with neutralization of the CXCR4 ligand CXCL12 (Phillips et al., 2004). Given prior reports of HIF-1α regulation of CXCL12 expression (Ceradini et al., 2004), we also assessed the effect of rapamycin treatment on lung expression of CXCL12 in the bleomycin model. Interestingly, rapamycin treatment resulted in a 50% in decrease in lung CXCL12, suggesting that interruption of the PI3-kinase/AKT/mTOR pathway inhibits the expression of both the ligand, CXCL12, and the receptor, CXCR4, mediating fibrocyte influx to the fibrotic lung.

Discussion

We have previously shown that $CD45^+$ $Col1^+$ $CXCR4^+$ circulating fibrocytes accumulate in the lung in response to CXCL12 and mediate fibrosis (Phillips et al., 2004). In the context of human pulmonary fibrosis, there is a correlation between lung and plasma levels of CXCL12 and circulating and lung fibrocyte numbers (Mehrad et al., 2007, Andersson-Sjoland et al., 2008). To provide the proof-of-principle that the CXCL12-CXCR4 axis can be targeted therapeutically in pulmonary fibrosis, the present work demonstrates that fibrocytes specifically traffic from the bone marrow to the lungs in the in vivo model of bleomycin-induced pulmonary fibrosis, that CXCR4 is the major chemokine receptor on fibrocytes and that its expression was induced by hypoxia and growth factors via the PI3 kinase/Akt/mTOR pathway. In addition, we found that in the bleomycin-induced pulmonary fibrosis, the pharmaceutical interruption of this pathway using a currently available medication resulted in reduced expression of CXCR4, reduced recruitment of fibrocytes to the lung, and attenuated pulmonary fibrosis.

While multiple reports in the literature indicate that bone marrow-derived fibrocytes are capable of contributing to the lung fibroblast population in fibrotic lung diseases (Andersson-Sjoland et al., 2008, Hashimoto et al., 2004, Ishida et al., 2007, Mehrad et al., 2007, Moore et al., 2005, Moore et al., 2006, Phillips et al., 2004), the magnitude of this effect has not been clearly established. Specifically, epithelial to mesenchymal transformation (EMT) has been shown to contribute to lung fibroblast and myofibroblast populations in the bleomycin model of pulmonary fibrosis (Kim et al., 2006, Kim et al., 2009, Wu et al., 2007), although EMT has not been found to contribute to lung myofibroblasts in human pulmonary fibrosis (Yamada et al., 2008). In addition, studies using ex vivo culture of lung fibroblasts from mice with bleomycin-induced pulmonary fibrosis have questioned whether fibrocytes can differentiate into lung myofibroblasts in this model (Hashimoto et al., 2004) while αSMA^+ fibrocytes are detectable in the peripheral blood and lungs in human pulmonary fibrosis (Mehrad et al., 2007, Andersson-Sjoland et al., 2008). In this context, the data presented in the current work show that bone marrow-derived cells contribute to more than half of the lung $CD45^+$ $Col1^+$ and $CD45^+$ $Col1^+$ αSMA^+ cells in the bleomycin model.

The literature regarding the relevance of specific chemokine receptors to fibrocyte trafficking in various diseases and animal models has been conflicting. This may, in part, be due to differences in chemokine repertoire of human and mouse fibrocytes, in addition to differences in chemokine ligands and receptors relevant in various animal models: the CCL21/CCR7 ligand-receptor pair has been implicated in recruitment of fibrocytes to injured skin and kidney of mice (Abe et al., 2001, Sakai et al., 2006). In the lung, the interaction between CCL12 and CCR2 has been found to mediate fibrocyte influx to the lung in FITC-induced mouse pulmonary fibrosis (Moore et al., 2005, Moore et al., 2006), although the relevance of this finding to human disease is not clear, since CCL12 does not have a human counterpart. More recently, interruption of the CCL3/CCR5 axis has been shown to attenuate fibrosis in the bleomycin model that was associated with reduced lung fibrocytes but also fewer lung neutrophils, macrophages, CXCL12, and TGF-β (Ishida et al., 2007). Since reduced inflammation is known to result in reduced fibrosis in the bleomycin model (Moeller et al., 2008), the effect of CCL3/CCR5 may, in fact, be mediated via the CXCL12/CXCR4 axis. In the present study, we found most circulating fibrocytes to express CXCR4, <10% to express CCR7, with almost no fibrocytes expressing both CXCR4 and CCR7. In addition, less than 50% of circulating fibrocytes also expressed CCR2 as well as CXCR4 or CCR7. Although not specifically examined in the present study, we have found less than half of fresh human fibrocytes to express CCR5 (our unpublished data). We have also found a similar chemokine receptor hierarchy in fibrocytes isolated from patients with pulmonary fibrosis (Mehrad et al., 2007). The chemokine receptor repertoire of fibrocytes might define functionally distinct fibrocyte subsets; for example, in contrast to the role of $CXCR4^+$ fibrocytes in pulmonary fibrosis (Andersson-Sjoland et al., 2008, Mehrad et al., 2007, Phillips et al., 2004), CCR7-expressing fibrocytes have been implicated in mediating renal fibrosis and wound healing (Abe et al., 2001, Sakai et al., 2006). Consistent with their progenitor phenotype, the majority of freshly isolated fibrocytes from both mice and humans express CXCR4, providing the rationale for investigating CXCR4 expression as a potential therapeutic target in pulmonary fibrosis.

Regulation of CXCR4 is well-recognized as critical to interaction of tumor cells within their microenvironment, including promoting cancer growth and mediating metastases (Burger and Kipps, 2006), and we have previously shown that both hypoxia and EGF-induce expression of CXCR4 in non-small cell lung cancer cells (Phillips et al., 2005). Both hypoxia and growth factors are also highly relevant to pulmonary fibrosis; the bone marrows of healthy volunteers are hypoxic relative to arterial blood, and bone marrow of patients with chronic lung disease have long been noted to have exaggerated hypoxia (Harrison et al., 2002, Parmar et al., 2007, Schwartz and Stats, 1949, Skouby, 1976). In steady-state hosts, bone marrow progenitor

cells express CXCR4 (the expression of which is enhanced by the hypoxic milieu of the marrow) and are retained there due to expression of CXCL12 by the bone marrow stromal cells (Sugiyama et al., 2006). In the context of stimulation (for example in pulmonary fibrosis) there is a reduction in bone marrow CXCL12 production (Christopher et al., 2009, Semerad et al., 2005) that occurs in concert with increased blood CXCL12 levels (Mehrad et al., 2007, Phillips et al., 2004). In this setting, the hypoxic environment of the bone marrow promotes the expression of CXCR4, which leads to cell mobilization from the bone marrow to the blood (and subsequently to the lungs, the source of CXCL12). Since hypoxia-induced expression of CXCR4 lasts at least 6 hours according to our data (Figure 3), this is a plausible mechanism for homing of fibrocytes from the bone marrow to the lungs. In addition, a substantial body of literature supports a role for growth factors, including PDGF, EGF and IGF, in the pathogenesis of pulmonary fibrosis (Abdollahi et al., 2005, Antoniades et al., 1990, Aston et al., 1995, Ishii et al., 2006, Martinet et al., 1987, Pilewski et al., 2005, Rice et al., 1999, Uh et al., 1998, Vanhee et al., 1994, Yasuoka et al., 2006). In this context, the current studies provide evidence that both hypoxia and growth factors up-regulate CXCR4 in human fibrocytes via the PI3 kinase/mTOR/HIF-1α pathway. Our studies do not preclude additional post-transcriptional mechanisms by which hypoxia might lead to higher CXCR4 receptor expression, such as enhanced translation and protein stabilization. Since the usual tissue niches of fibrocytes are either the bone marrow or inflamed tissue, it is highly likely that this pathway is operative in vivo. In contrast, we found the basal transcription of CXCR4 in normoxic conditions and in the absence of growth factors, to be independent of this pathway, suggesting the presence of other response elements in the CXCR4 promoter mediating basal transcription. Interestingly, we have previously reported that exposure of human fibrocytes to TGF-β induces them to express α-SMA and to loose expression of CXCR4 (Phillips et al., 2004). We therefore speculate that after arrival in the lung, $CXCR4^+$ $\alpha\text{-SMA}^-$ fibrocytes differentiate into $CXCR4^-$ $\alpha\text{-SMA}^+$ myofibroblasts under the influence of local factors.

Our data also suggest that PI3-kinase is significant for hypoxia-induced CXCR4 up-regulation and largely responsible for PDGF-induced CXCR4 up-regulation, as inhibition of PI3-kinase activity by the small molecule LY294002 can abrogate their effects. Furthermore, both hypoxia- and PDGF-induced induction of CXCR4 expression were inhibited by rapamycin in a pattern similar to the inhibition of PI3-kinase. Interestingly, both LY294002 and rapamycin suppressed the PDGF-induced expression of CXCR4 but did not abolish it entirely; this suggests either that LY294002 and rapamycin are not completely blocking the mTOR pathway, or that other signaling pathways that play a minor role in CXCR4 expression independent of the PI3 kinase/mTOR pathway. In support of the latter, PDGFR has also been shown to activate the src family of tyrosine kinases, PLC-γ, and STAT family members (Heldin and Westermark, 1999), any of which could impinge upon CXCR4 expression. In this context, inhibition of PDGF signaling has recently been shown to be effective in inhibiting radiation-induced pulmonary fibrosis (Abdollahi et al., 2005). PDGF-mediated induction of CXCR4 on fibrocytes therefore provides a potential mechanism for this observation.

The relevance of our in vitro observations with human fibrocytes to the in vivo setting of pulmonary fibrosis was established using the mouse bleomycin model of pulmonary fibrosis. The benefit of a rapamycin analogue in reducing lung fibrosis in bleomycin-mediated lung fibrosis has previously been noted in a rat model (Simler et al., 2002). The notable effectiveness of rapamycin in inhibiting lung fibrocyte accumulation and collagen deposition was consistent with its inhibition of fibrocyte CXCR4 expression, as previously reported in cancer cells (Phillips et al., 2005). In addition, however, we found that rapamycin substantially reduced the lung expression of CXCL12 in response to bleomycin challenge, consistent with prior reports of HIF-1α regulation of CXCL12 expression (Ceradini et al., 2004), and likely contributing to the attenuated accumulation of fibrocytes in the lung. We acknowledge that in the present study, treatment with rapamycin preceded the administration of bleomycin, and that the drug

may be less effective if administered later in the course of fibrosis. In addition, we acknowledge that rapamycin has a number of off-target effects on various aspects of the immune response (Brazelton and Morris, 1996) that could contribute to its effect on bleomycin-induced fibrosis observed in vivo. We note, however, that the basal expression of CXCR4 by fibrocytes was not dependent on the mTOR pathway, and that our observations with rapamycin are very similar to our prior published data with interruption of the CXCL12-CXCR4 axis in the bleomycin-induced model of pulmonary fibrosis (Phillips et al., 2004). It is nevertheless formally possible that some of the observed effects of rapamycin in this model are independent of trafficking of fibrocytes, for example by dampening the recruitment of inflammatory leukocytes to the lungs, reducing proliferation of resident lung fibroblasts or by inhibiting epithelial to mesenchymal transformation.

Taken together with prior work showing the relevance of fibrocytes to mouse models of fibrotic lung disease (Phillips et al., 2004, Ishida et al., 2007, Moore et al., 2005, Moore et al., 2006, Pilling et al., 2007, Vannella et al., 2007) and the relevance of circulating CXCR4-expressing fibrocytes to human pulmonary fibrosis (Mehrad et al., 2007, Andersson-Sjoland et al., 2008, Moeller et al., 2009), the current studies provide a rationale for therapeutic targeting of CXCR4 expression in patients with pulmonary fibrosis in clinical trials. In this context, the potential risk of off-target effects of targeting of mTOR and CXCR4, such as predisposition to infection, may be justifiable given the limited treatment options and poor outcome in this illness. Rapamycin is currently the only approved drug for targeting CXCR4 expression via mTOR inhibition in humans, although 3 other mTOR inhibitors are in human trials (Fasolo and Sessa, 2008). In addition, several small molecule antagonists of CXCR4, developed to block viral entry of X4 variants of HIV and for mobilization of bone marrow progenitor cells, have reached human studies (Cashen et al., 2007, Este and Telenti, 2007). We propose that the available data provide justification for the study of these therapeutic approaches in patients with pulmonary fibrosis.

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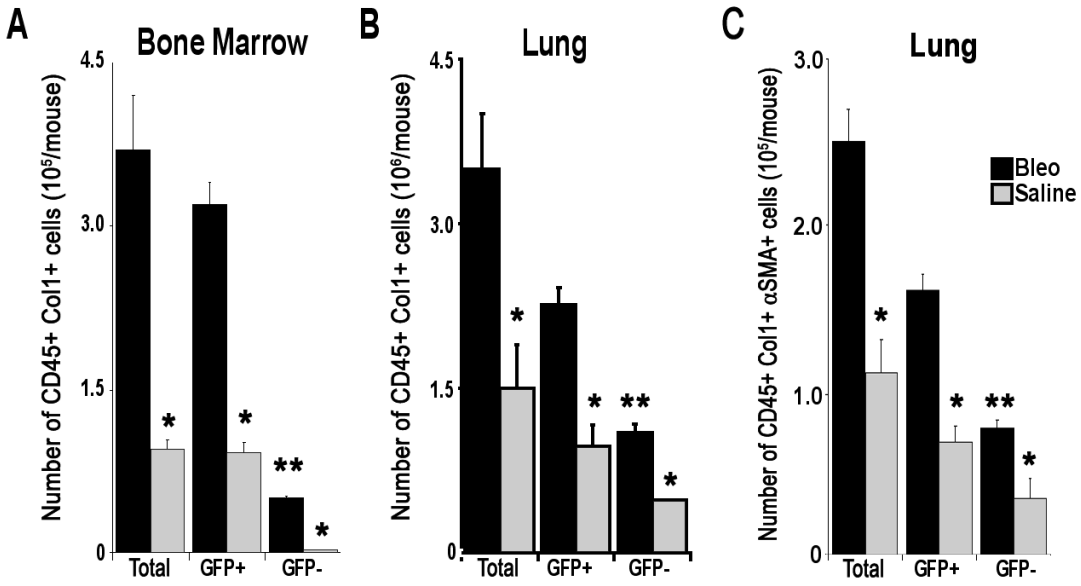

Figure 1.

Bone marrow-derived fibrocytes traffic to the lung in the mouse bleomycin model of pulmonary fibrosis. Stable bone marrow chimeras with GFP-expressing donors and wildtype recipients were established and bone marrow (panel A) and lungs (panels B-C) were examined 8 days after intrapulmonary challenge with bleomycin or saline control. *, $p < 0.05$ compared to respective bleomycin-treated group; **, $p < 0.05$ compared to respective GFP^+ group.

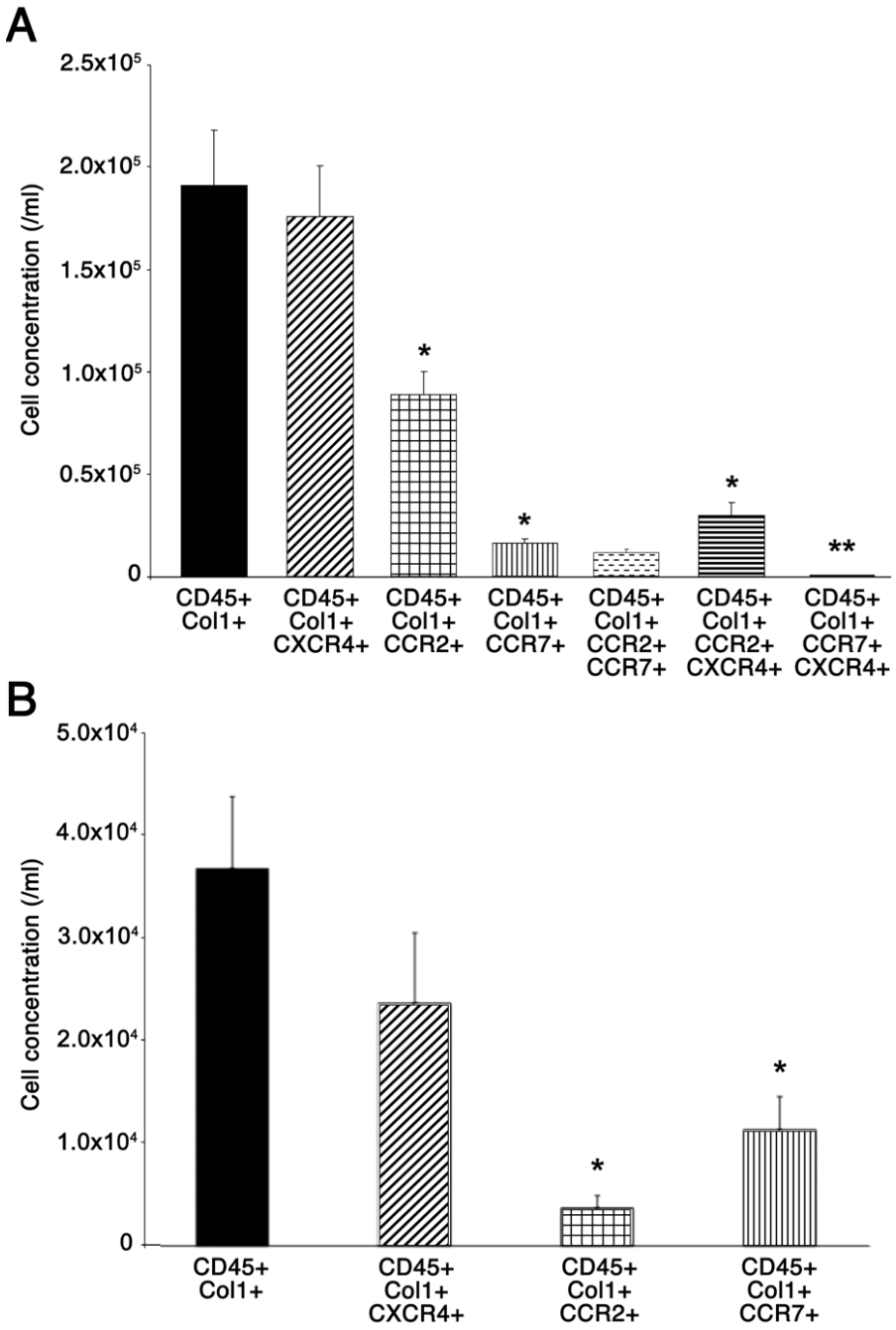

Figure 2.

Chemokine receptor expression on fresh human (panel A) and mouse (panel B) peripheral blood fibrocytes. Buffy coat was isolated from healthy humans or mice and stained for CD45, Col1, and CXCR4, CCR2, or CCR7 alone or in combination and examined by FACS analysis. Data represent the mean ± SEM from 12 normal volunteers for humans (panel A) or 5 mice (panel B). *, $p < 0.05$ compared to $CD45^+$ $Col1^+$ $CXCR4^+$ subset; **, $p < 0.05$ compared to $CD45^+$ $Col1^+$ $CCR2^+$ $CCR7^+$ and $CD45^+$ $Col1^+$ $CCR7^+$ $CXCR4^+$ subsets.

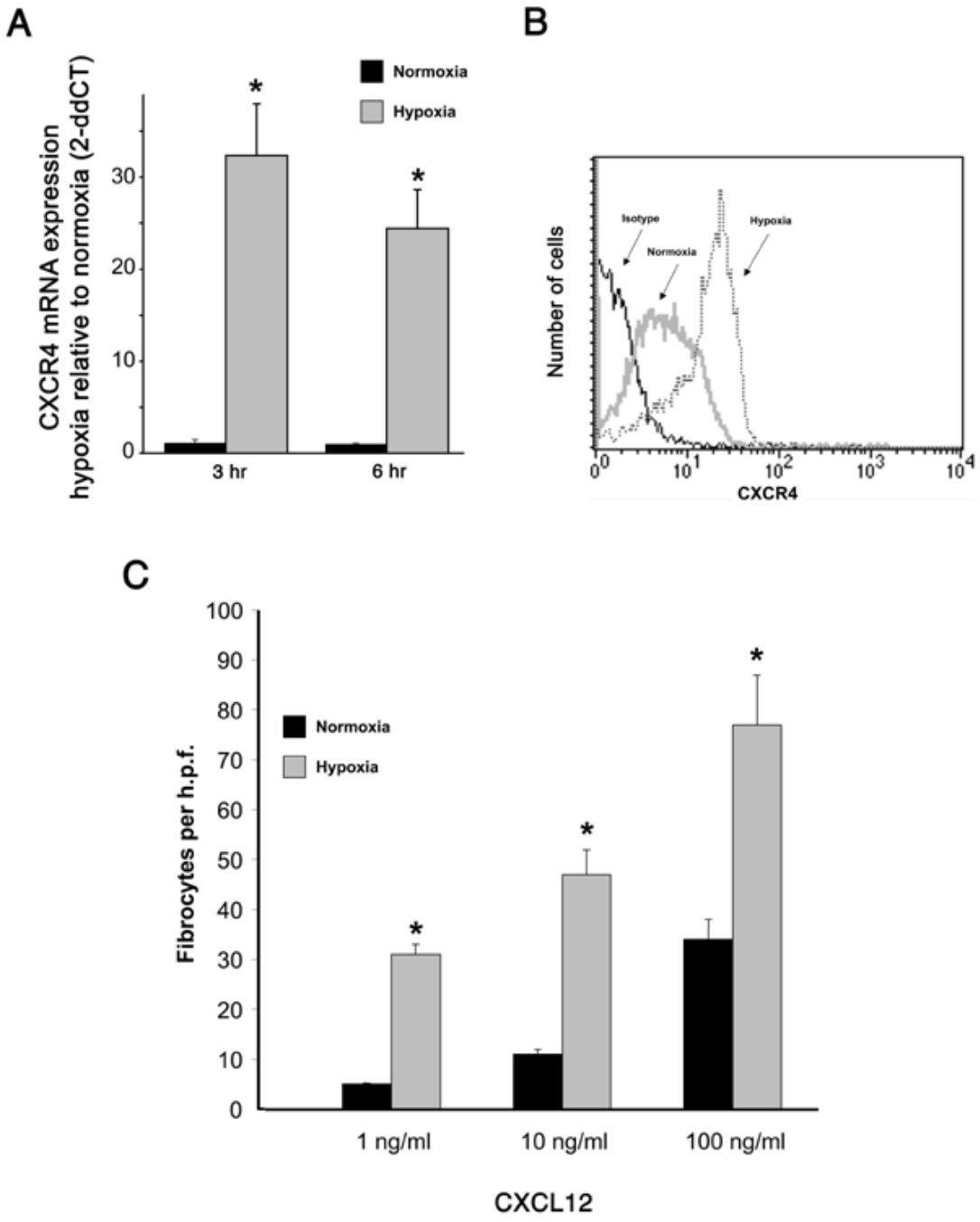

Figure 3.

Fibrocyte CXCR4 expression and function in response to hypoxia. (A) Fibrocytes were exposed to normoxic or hypoxic conditions for the indicated time and changes in gene expression determined by quantitative PCR. Fold inductions represent changes in CXCR4 expression under hypoxic conditions as compared to normoxic controls. Results are representative of three separate experiments. *, $p < 0.05$ for all groups compared to normoxia and for increasing trend. (B) Fibrocytes were exposed to normoxic or hypoxic conditions for 12 h and surface expression of CXCR4 determined by flow cytometry. Representative of three separate experiments. (C) Fibrocytes were prepared as in (B) and subjected to chemotaxis in response to the indicated concentrations of SDF-1/CXCL12 for 6 h. Data represent the mean

± SEM from five high power fields. *, $p < 0.05$ for each group compared to normoxic controls; **, $p < 0.05$ for increasing trend in hypoxic conditions. Representative data from 3 experiments.

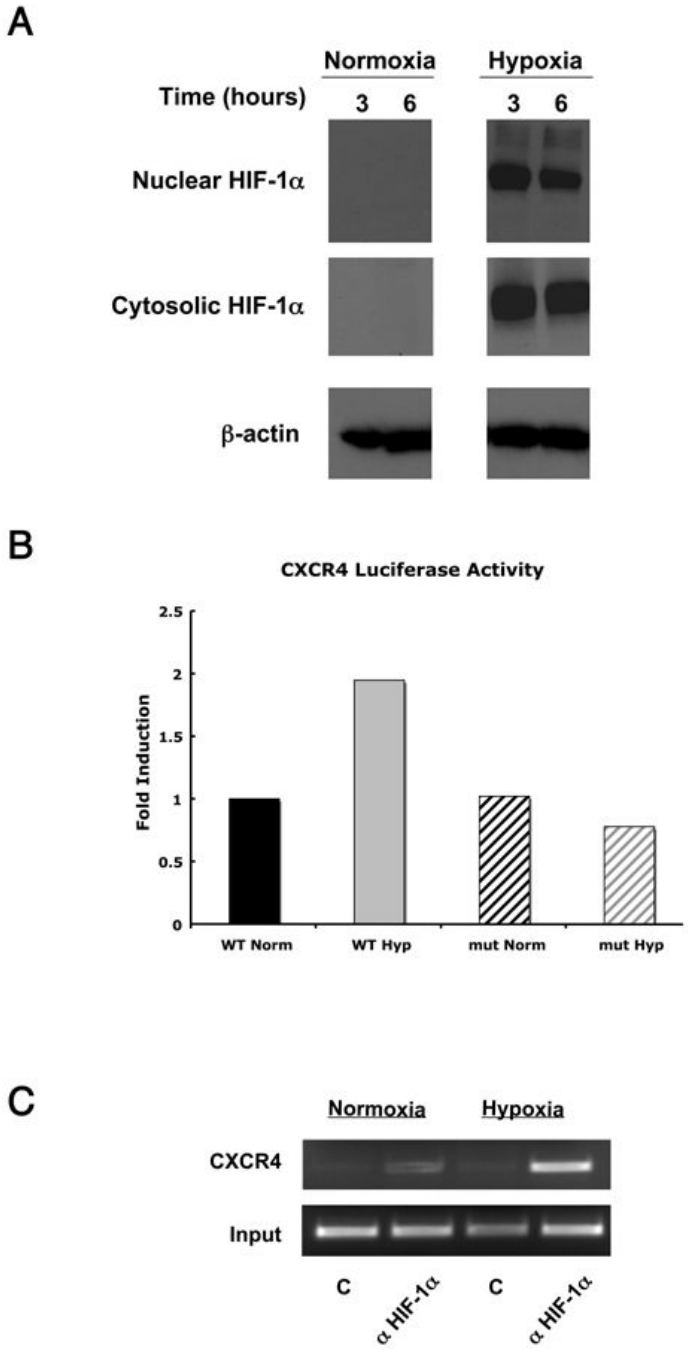

Figure 4.

Hypoxia-induced HIF-1α expression and CXCR4 promoter transactivation in fibrocytes. (A) Fibrocytes were treated as in Fig 3A and western blots performed on cytosolic and nuclear fractions for HIF-1α expression. Results are representative of three different experiments. (B) Fibrocytes were transfected with a reporter construct containing either a wild-type 2.6-kilobase fragment of the CXCR4 promoter (WT) or a fragment containing a mutation in the HIF Responsive Element (mut) upstream of a luciferase gene along with a separate Renilla control reporter. After 48 h, cells were cultured in normoxic or hypoxic conditions for 6 h, and cell extracts were examined for luciferase activity by luminometry and normalized to Renilla control. Data is representative of three separate experiments. (C) Fibrocytes were cultured in

normoxic or hypoxic conditions for 6 h and ChIP assay performed to determine the recruitment of HIF-1α to the CXCR4 promoter. Precleared chromatin solutions were immunoprecipitated with control mouse IgG or mouse anti-human HIF-1α monoclonal antibody. The lower panel represents input genomic DNA before addition of antibody. Representative data from 3 experiments.

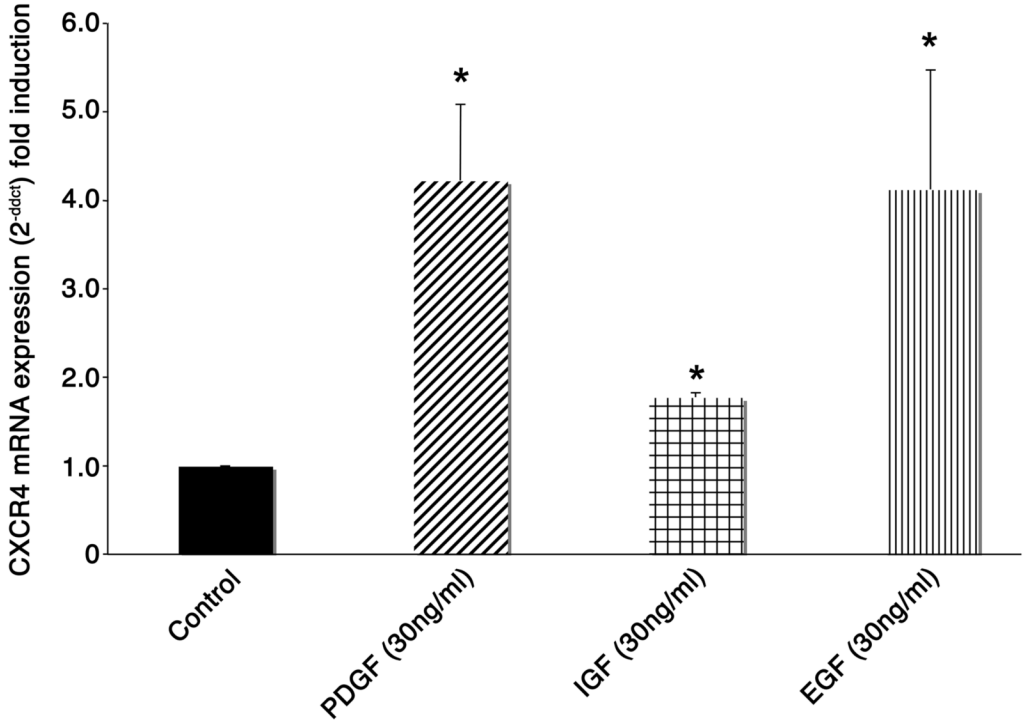

Figure 5.

Growth factors activate CXCR4 transcription. Fibrocytes were exposed to control media, PDGF, IGF, or EGF for 6 h. Changes in gene expression were determined by real-time quantitative PCR and expressed as fold induction over control. Data represent the mean ± SEM from three experiments. *, $p < 0.05$ for all groups as compared to control media.

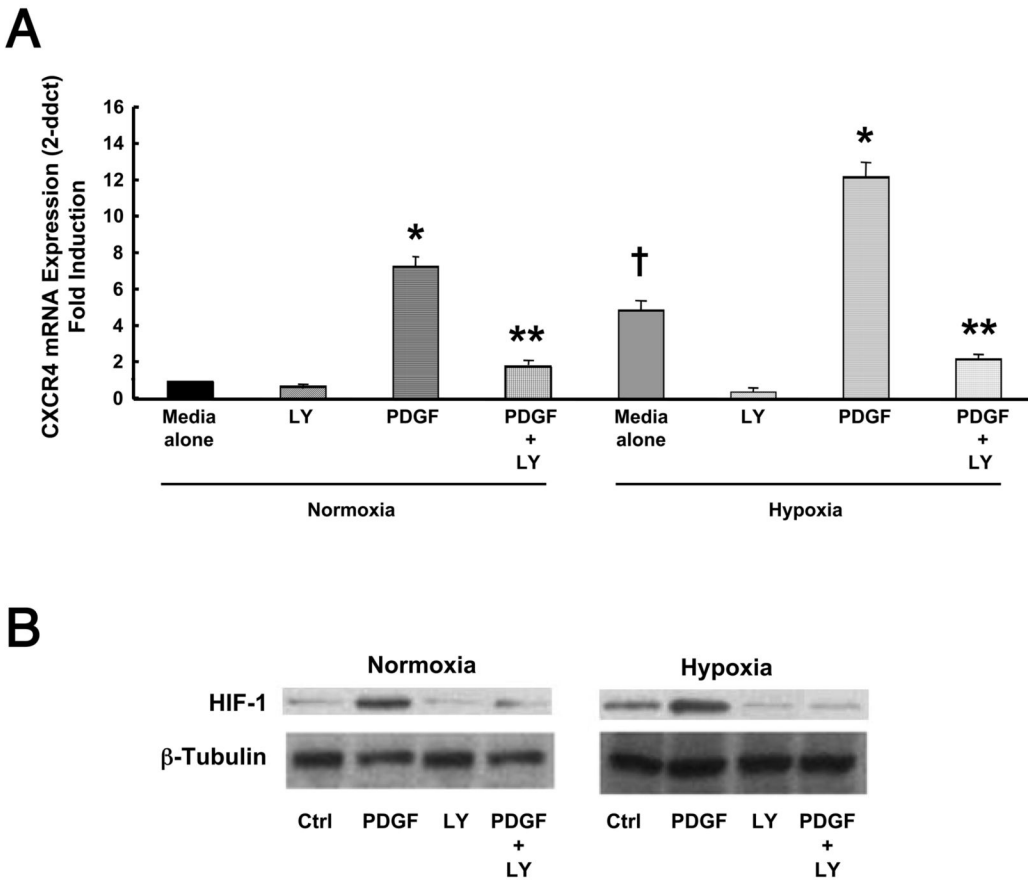

Figure 6.

Effect of PI3-kinase inhibition on CXCR4 transcription and expression. (A) Fibrocytes were pre-incubated with LY294002, PDGF or both, and then incubated in normoxic or hypoxic conditions for 6 h. Changes in gene expression were determined by quantitative PCR and expressed as fold induction as compared to normoxic control. Data shown are representative of three separate experiments. *, $p < 0.05$ as compared to media alone; **, $p < 0.05$ to respective PDGF group; †, $p < 0.05$ compared to media alone under normoxic conditions. (B) Fibrocytes were treated as in (A), nuclear fractions were isolated and examined by western blot for HIF-1α expression. Results are representative of three different experiments.

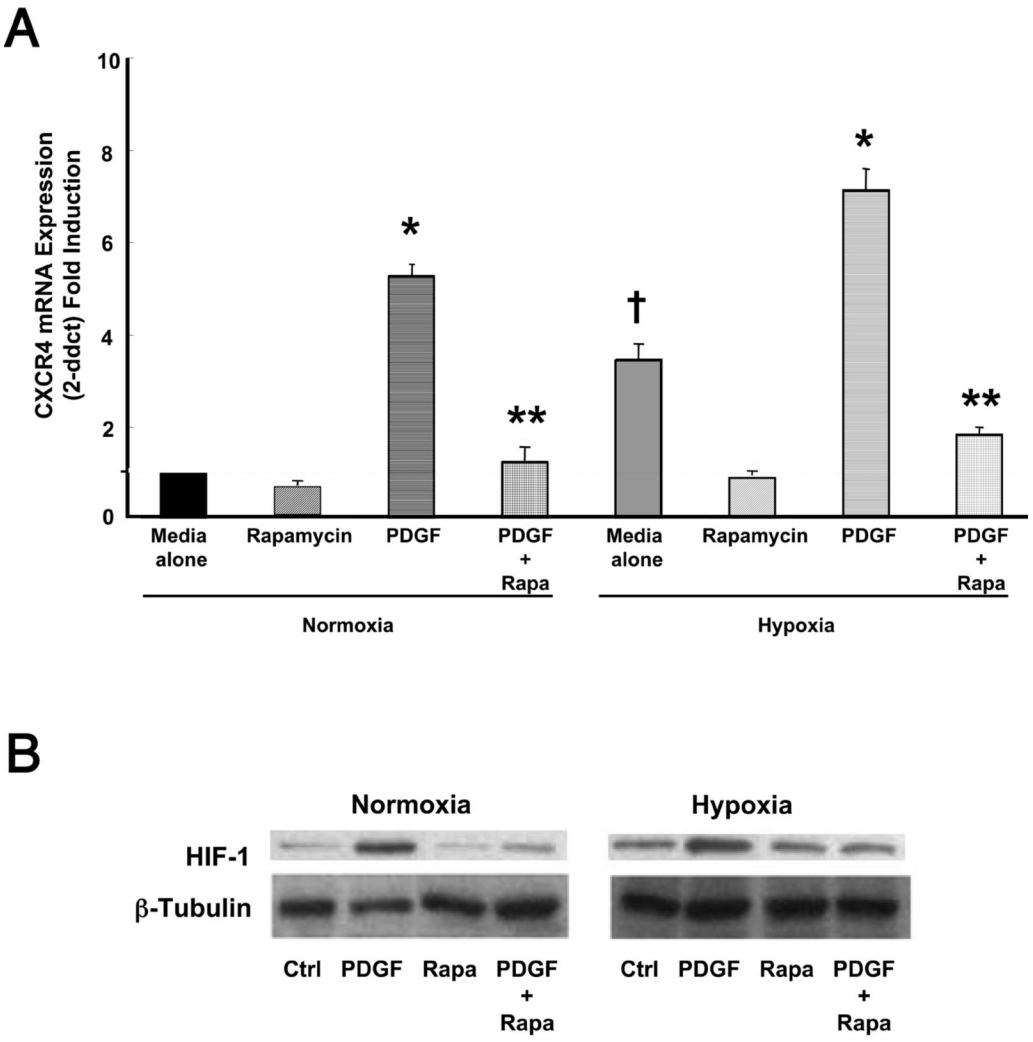

Figure 7.

Effect of mTOR inhibition on CXCR4 transcription and expression. (A) Fibrocytes were pre-incubated with rapamycin where indicated for 1 h, stimulated with PDGF, and then exposed to normoxic or hypoxic conditions for 6 h. Changes in gene expression were then determined by real-time quantitative PCR. Fold induction represents increases in CXCR4 expression under hypoxic conditions as compared to normoxic control. Results are representative of three separate experiments. *, $p < 0.05$ as compared to media alone; **, $p < 0.05$ to respective PDGF group; †, $p < 0.05$ compared to media alone under normoxic conditions. (B) Fibrocytes were treated as in (A). Nuclear fractions were isolated examined for HIF-1α by western blotting. Results are representative of three different experiments.

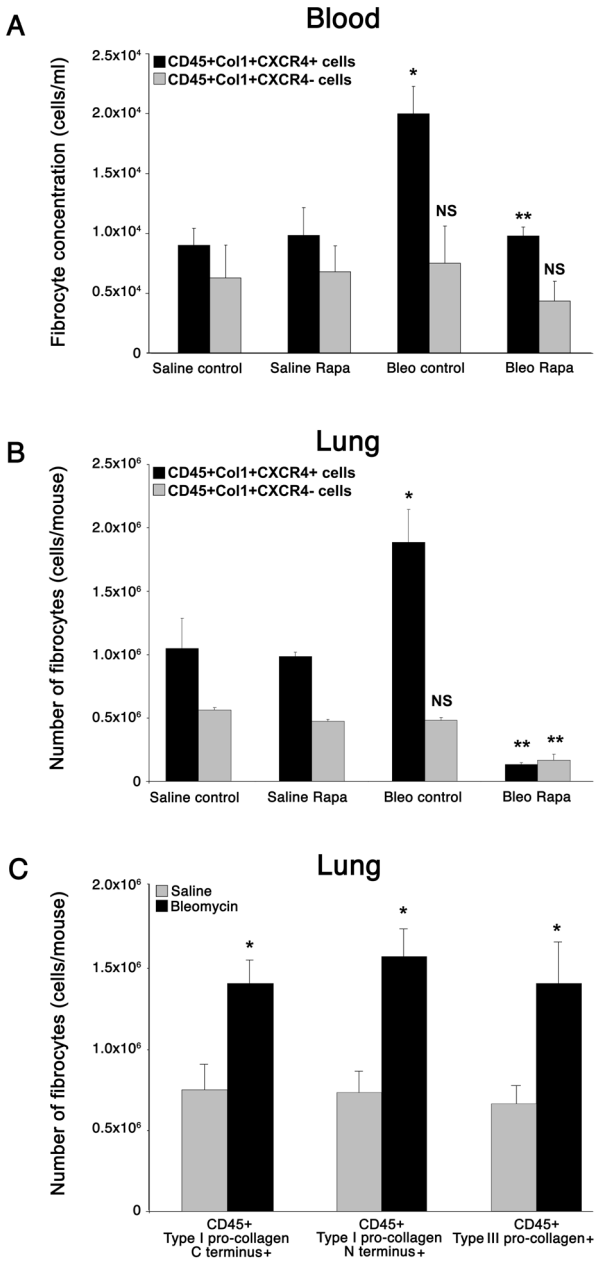

Figure 8.

Effect of rapamycin on fibrocytes in mice with bleomycin-induced pulmonary fibrosis. Fibrocytes were enumerated by flow cytometry in the peripheral blood buffy coat (A) and lung single cell suspensions (B) 16 days after intrapulmonary challenge with bleomycin or vehicle, and daily treatment with rapamycin or saline. *, $p < 0.05$ as compared to $CD45^+$ $Col1^+$ $CXCR4^+$ saline control and saline rapamycin groups; NS, no significant difference as compared to $CD45^+$ $Col1^+$ $CXCR4^-$ saline control or saline rapamycin groups; **, $p < 0.05$ as compared to respective bleo control group. (C) $CD45^+$ cells in lung single cell suspensions from the above experiment, stained for pro-collagens. *, $p < 0.05$ as compared to mice challenged with saline vehicle. $n = 5$ mice per group for all experiments. Data represent the mean ± SEM.

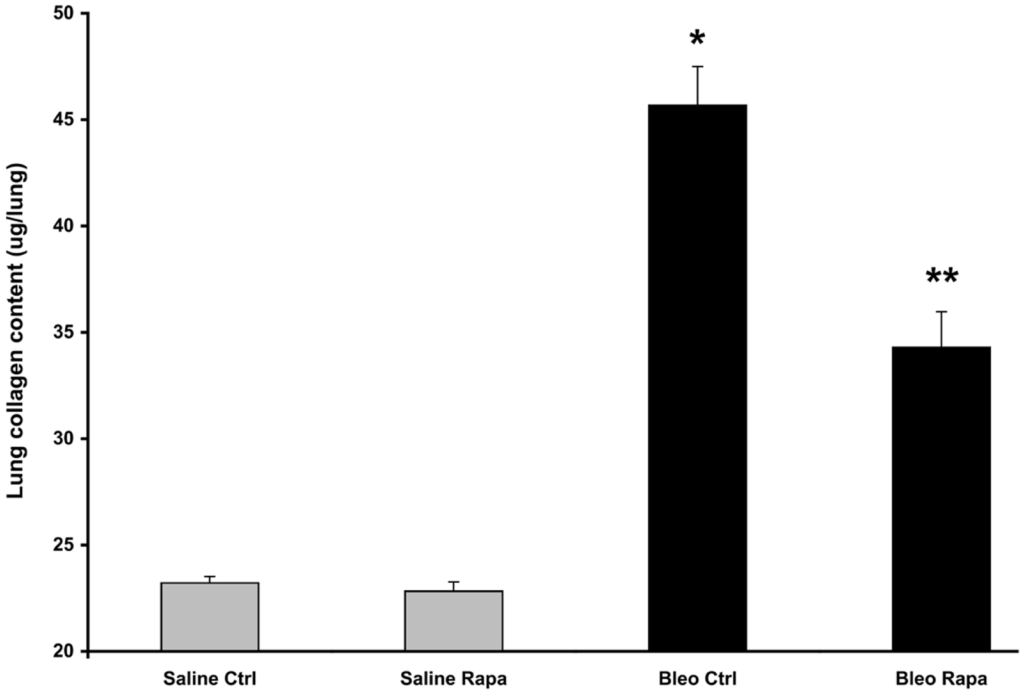

Figure 9.

Effect of rapamycin on lung collagen content in mice with bleomycin-induced pulmonary fibrosis. Whole lung collagen content was quantified using a commercial kit. $n = 5$ mice per group for all experiments. Data represent the mean ± SEM. *, $p < 0.05$ as compared to saline control and saline rapamycin groups; **, $p < 0.05$ as compared all other groups.

Table

Collagen release by cultured human cells in vitro.

Cells	TGF-β in media	Soluble collagen content in supernatant (μg/ml)		
		Week 1	Week 2	Week 3
Fibrocytes	-	81	88	96
Fibrocytes	10 ng/ml	145	183	207
Monocytes	-	ND	ND	ND
Monocytes	10 ng/ml	ND	ND	ND
Fibroblasts	-	78	88	101
Fibroblasts	10 ng/ml	136	190	220

ND, none detected.