

TGF-beta 1 induced fibroblast proliferation is mediated by the FGF-2/ERK pathway

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
 - 3.1. Type II Cell Culture
 - 3.2. FGF-2 detection
 - 3.3. Immunofluorescence
 - 3.4. Cell proliferation assay
 - 3.5. Cell cycle analysis by fluorescence-activated cells sorting (FACS)
 - 3.6. Statistical analysis
4. Results
 - 4.1. TGF-β1 increased FGF-2 expression in type II alveolar epithelial cells
 - 4.2. TGF-β1 conditioned medium enhanced fibroblast proliferation and differentiation
 - 4.3. FGF-2 mediates the effects of TGF-β1 on fibroblast proliferation
 - 4.4. Fibroblast ERK signaling is required for TGF-β1 CM- induced cell differentiation and proliferation
5. Discussion
6. Acknowledgements
7. References

1. ABSTRACT

Pulmonary fibrosis, defined as the accumulation of connective tissue in the lungs, is a severe and often fatal form of interstitial lung disease. Transforming growth factor-beta (TGF-beta) is a powerful activator of connective tissue synthesis and fibroblast proliferation in the lung, and a critical paracrine signal for the development of pulmonary fibrosis. To investigate signaling pathways downstream of TGF- beta that contribute to lung fibrosis, TGF- beta stimulation of fibroblasts was replicated by treating NIH3T3 fibroblasts with conditioned medium (CM) from TGF- beta -treated type II alveolar epithelial cells (ATII cells). The data showed that fibroblast growth factor 2 (FGF-2) signaling is responsible for TGF-beta 1 CM-induced fibroblast proliferation, while it does not affect TGF-beta 1 CM-induced fibrotic differentiation. Moreover, fibroblast proliferation and differentiation induced by TGF- beta CM was totally abrogated by pretreatment of NIH3T3 cells with the specific ERK1/2 inhibitor, PD98059. These findings indicate that FGF-2 secreted by alveolar epithelial cells in response to TGF- beta 1 induces fibroblast proliferation and fibrotic activation through the ERK kinase pathway.

2. INTRODUCTION

Pulmonary fibrosis results from an irreversible accumulation of connective tissue in the interstitium of the lung, impeding gas exchange and pulmonary mechanics. Possible causes of pulmonary fibrosis include epithelial cell injury, abnormal fibroblast proliferation, inflammation, or deposition of extracellular matrix components (1,2). Standard therapies such as prescription therapies, supplemental oxygen, and pulmonary rehabilitation have shown little benefit, and most patients with fibrosis eventually suffer respiratory failure (3).

The process of fibrosis in the lung is analogous to wound healing in the skin. The fibrotic cells proliferate and release extracellular matrix (ECM) proteins like collagen, while a specialized set of myofibroblasts adhere to the wound edges and pull in the skin by contracting. Research on animal models and studies of human lung disease suggest that the initiating events may include pulmonary injury and the recruitment of inflammatory cells and macrophages, as well as the local release of mitogenic and pro-inflammatory cytokines (4).

Many well-characterized cytokines have been either found in the injured lung or produced by the inflammatory cells removed from the fibrotic lung, indicating their important role in lung fibrosis. Among them, transforming growth factor- β (TGF- β) is probably the most important cytokine for the direct stimulation of lung extracellular matrix (ECM) expression, which typifies fibrosis (5). TGF- β is a member of a larger family of polypeptide transforming growth factor- β (TGF- β) is probably the most important cytokine for the direct stimulation of lung extracellular matrix (ECM) expression, which typifies fibrosis (5). TGF- β is a member of a larger family of polypeptide growth factors that have key functions in growth, development, and tissue remodeling (6). Recent evidence shows that TGF- β 1 is the major TGF- β isoform that induces connective tissue synthesis and promote fibroblast proliferation in an autocrine or paracrine manner across different cell types and disease models (7,8). For instance, the addition of TGF- β 1 to tumor cells leads to tumor fibroblast activation, as indicated by increased ECM protein and cytokine secretion, as well as enhanced contractility mediated by the *de novo* expression of α -smooth muscle actin (α -SMA)-containing stress fibers (9). In addition, TGF- β 1 acts as a paracrine factor in endothelial cell-mediated fibroblast growth and differentiation (10). TGF- β 1 gene expression and protein secretion are also increased in human idiopathic pulmonary fibrosis (IPF) (11). Although much is known about the deleterious effects of chronic TGF- β signaling in cancer, the pathways that mediate the proliferation of lung fibroblasts and synthesis of extracellular matrix proteins (fibrosis) are largely unknown.

In the present study, we tested the paracrine activity of TGF- β by stimulating fibroblasts with conditioned media (CM) obtained from TGF- β -treated type II alveolar epithelial cells. We identified the mechanism underlying CM-induced fibrosis, and we demonstrated that the FGF-2/ERK signaling pathway is essential for CM-induced fibroblast proliferation and fibrotic activation.

3. MATERIALS AND METHODS

3.1. Type II Cell Culture

Rat type II alveolar epithelial cells (ATII cells) were isolated from pathogen-free, 200–250-g Sprague-Dawley rats according to the procedure of Dobbs (12) with some minor modifications (13). Isolated cells were suspended at 100,000 cells/mL in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 50 μ g/mL gentamicin. NIH3T3 fibroblast cells were routinely cultured in 10% FBS/DMEM, as described elsewhere.

3.2. FGF-2 detection

FGF-2 in TGF- β CM was quantified by an enzyme-linked immunosorbent assay (ELISA) as described previously (14). Briefly, ATII cells were seeded directly into 96-well cell culture plates, grown to 80% confluence,

growth-arrested for 48 h, and then treated with different doses of TGF- β 1 for another 48 h. The FGF-2 in the media was quantified using the Quantikine FGF-2 ELISA kit (R&D Systems).

3.3. Immunofluorescence

NIH3T3 cells growing on cover slips were fixed in freshly prepared 4% formaldehyde solution at room temperature. Fixed cells were permeabilized in Solution P containing 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in PBS for 10 min, washed with PBS, and blocked with 10% BSA in PBS. Cells were incubated in primary antibody overnight at 4 °C, and then incubated in a fluorescent-tagged secondary antibody for 1 h at room temperature. Images were captured on a Zeiss ApoTome Axiocam MRc microscope equipped with 10 \times objective lens.

3.4. Cell proliferation assay

NIH3T3 cell viability and proliferation was assessed by MTT assay according to the manufacturer's instructions (Calbiochem). Briefly, fibroblasts of the NIH3T3 cell line cultured in control or CM were incubated with 1 mL of MTT (5 mg/mL) for 3 h at 37 °C. The precipitated formazan was dissolved by adding three volumes of ethanol, and cellular proliferation was estimated from the optical density of formazan by using a spectrophotometer at 570 nm with background subtraction at 650 nm. All assays were performed in triplicate.

3.5. Cell cycle analysis by fluorescence-activated cell sorting (FACS)

Cell cycle analysis was examined by FACS. Briefly, NIH3T3 cells were harvested by trypsinization, resuspended in 1 mL of nuclear isolation buffer (1 \times PBS, 0.5% BSA, 0.1% NP-40) with 10 μ L of propidium iodide (0.5 mg/mL) and 10 μ L of RNase A (10 mg/mL), incubated at 37 °C for 30 min, and then analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

3.6. Statistical analysis

All experiments were performed at least three times. Data are presented as the mean \pm SD or SEM values. Each experimental group was compared to its control group (cells cultured in fresh growth factor-free medium) by paired t-tests. Mean values were considered significantly different if p value was <0.05. The levels of significance are denoted as *p < 0.05 and **p < 0.01.

4. RESULTS

4.1. TGF- β 1 increased FGF-2 expression in type II alveolar epithelial cells

TGF- β 1 is a key fibrogenic cytokine and activates fibroblast differentiation into myofibroblasts *in vitro* and *in vivo* (2,15,16). To study the paracrine effects of TGF- β 1, ATII cells were treated with different concentrations of TGF- β 1 for 48 h, and the culture medium was assayed for FGF-2 release by ELISA. We found that TGF- β 1 treatment evoked a dose-dependent increase in the concentration of FGF-2 in the medium. A maximum FGF-2 concentration of 300% above control was attained at 3

ng/mL TGF- β 1 (Figure 1).

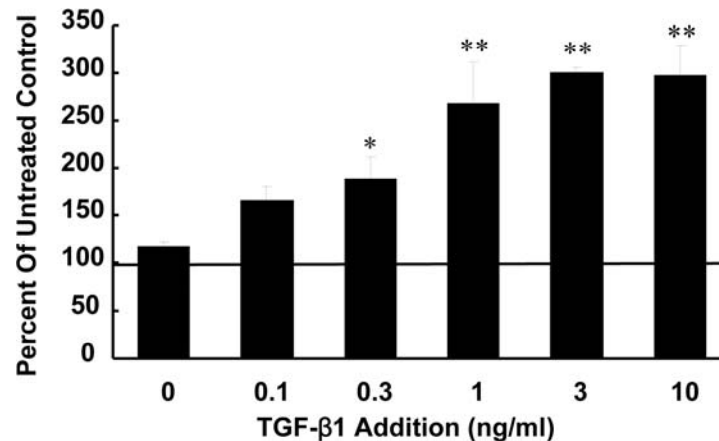


Figure 1. TGF- β 1 stimulation of FGF-2 release from type II alveolar epithelial cells is dose-dependent. Type II cells were cultured with different doses of TGF- β 1 and FGF-2 protein in the medium was measured after 48 h by ELISA. Results are presented as mean \pm SEM relative to FGF-2 release induced by control media without TGF- β 1. * P <0.05, ** P <0.01, compared to untreated cultures (Student's t-test).

4.2. TGF- β 1 CM enhanced fibroblast proliferation and differentiation

Interstitial accumulation of ECM proteins such as collagen and α -SMA is a hallmark of lung fibrosis. Collagen I is the predominant collagen type produced by lung fibroblasts (17,18). To examine whether TGF- β CM can induce fibrosis *in vitro*, we measured the production of total collagen I and α -SMA by NIH3T3 fibroblasts in the culture medium. Medium retrieved from ATII cultures after 48-h treatment with 3 ng/mL TGF- β 1 or vehicle was added to NIH3T3 culture for 24 h. As shown in Figures 2A and B, the collagen I and α -SMA levels in the fibroblast medium were greatly elevated when cells were cultured in the CM from TGF- β 1-treated ATII cells (TGF- β 1 CM) rather than in the medium from vehicle-treated ATII cells (normal CM). Thus, only CM from TGF- β 1-treated ATII cells can induce fibroblast activation.

To analyze the effect of TGF- β 1 CM on fibroblast proliferation, we examined the level of phosphorylated histone 3 (Phos-H3), a standard cell cycle marker of the G2/M transition, in NIH3T3 cells by immunofluorescence with Hoechst 33342 as a nuclear counterstain. Expression of Phos-H3 was enhanced in NIH3T3 cells incubated in TGF- β 1 CM (Figure 2C). An MTT cell viability assay was also performed to estimate the rate of NIH3T3 cell proliferation following exposure to TGF- β 1 CM for 24, 48, and 72 h. Incubation in TGF- β 1 CM greatly increased the relative number of viable cells (Figure 2D). In addition, we also tested fibroblast proliferation by FACS. As shown in Figure 2E, the proportion of cells in the G2/M phase was significantly increased after treatment with TGF- β 1 CM (Figure 2E). We concluded that TGF- β 1 CM can promote both fibrotic differentiation and fibroblast proliferation.

4.3. FGF-2 mediates the effects of TGF- β 1 on fibroblast proliferation

Fibroblast growth factor (FGF) is another critical cytokine implicated in the pathogenesis of lung fibrosis (19-21). Many FGF members function in the pathogenesis of organ scarring. FGF-1 and FGF-2 are the most representative isoforms of the response to fibrotic lung disease (14, 22-25). Since FGF-2 content was increased in the media from TGF- β 1-treated ATII cells (TGF- β 1 CM), we investigated whether an FGF-2 signaling pathway is involved in TGF- β 1-induced fibroblast proliferation and fibrotic differentiation. Specific antibodies against FGF-2 were added to the TGF- β 1 CM to neutralize FGF-2, and the CM was then used to culture NIH3T3 fibroblasts. For comparison, antibodies against TGF- β 1 and FGF-1 were also tested.

Fibroblasts incubated in TGF- β CM exhibited a marked increase in collagen I and α -SMA expression (Figure 3A and B). When the TGF- β 1 in TGF- β 1 CM was neutralized by TGF- β 1 antibody, fibroblast release of collagen I and α -SMA was not significantly reduced, indicating that fibrotic activation was mediated by a factor released by ATII cells in response to TGF- β 1, but not directly by TGF- β 1. Meanwhile, neutralization of FGF in CM by anti-FGF-1 or anti-FGF-2 had no effect on collagen I and α -SMA expression (Figure 3A and B), suggesting that FGF-1 and FGF-2 are not involved in TGF- β 1 CM-induced fibrosis.

We then examined whether FGF-1 or FGF-2 mediates the proliferative response of fibroblasts induced by TGF- β 1 CM. Immunofluorescence staining of phos-H3 revealed that anti-TGF- β 1 and anti-FGF1 neutralization did not change the proliferative response to TGF- β 1 CM, while FGF-2 antibody neutralization significantly attenuated phos-H3 expression (Figure 3C). The results of an MTT assay and cell cycle analysis confirmed that FGF-2 antibody neutralization reduced the mitogenic effect of TGF- β 1 CM (Figure 3D and E). Therefore, FGF-2 is necessary for the fibroblast proliferation induced by TGF-

Role of FGF-2/ERK pathway in Fibroblast proliferation

$\beta 1$ CM, but not for fibrotic differentiation characterized by collagen I and α -SMA expression.

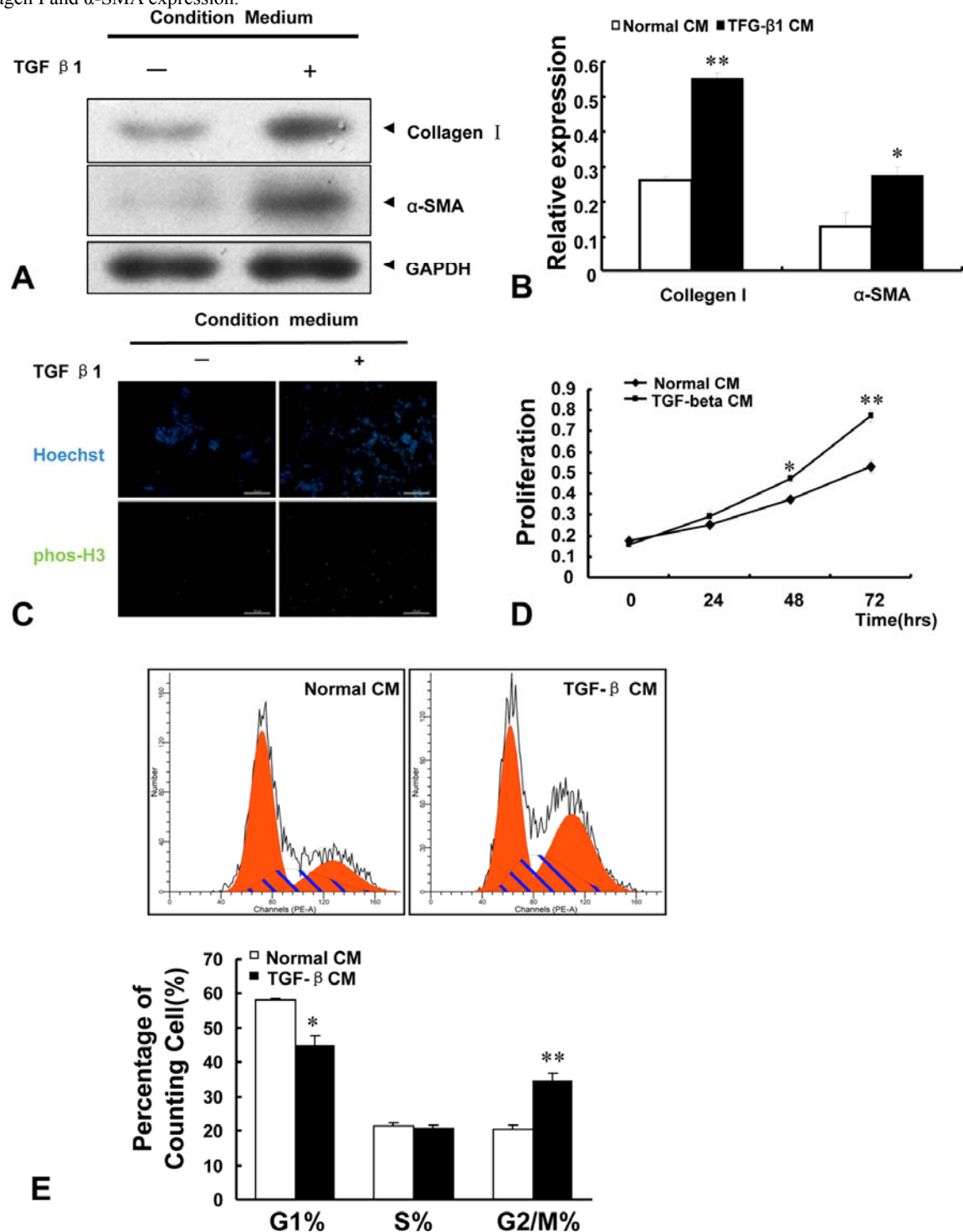


Figure 2. TGF- $\beta 1$ conditioned media promotes fibrotic differentiation and fibroblast proliferation. (A) Western blot analysis of NIH3T3 cells revealed increased expression of the fibrosis markers collagen I and α -SMA after treatment by TGF- $\beta 1$ conditioned medium. (B) Statistical analysis of collagen I and α -SMA expression in the presence or absence of TGF- $\beta 1$ conditioned medium. (C) Immunostaining of phos-Histone H3 (phos-H3) in NIH3T3 fibroblasts showing that TGF- $\beta 1$ conditioned medium induces fibroblast proliferation. (D and E) Proliferation of NIH3T3 cells in response to TGF- $\beta 1$ conditioned medium as measured by MTT assay at 24 h intervals for 72 h (D) and by FACS analysis (E). Data are presented as mean \pm SEM from at least three

Role of FGF-2/ERK pathway in Fibroblast proliferation

experiments. * $P < 0.05$, ** $P < 0.01$, Student's *t* test.

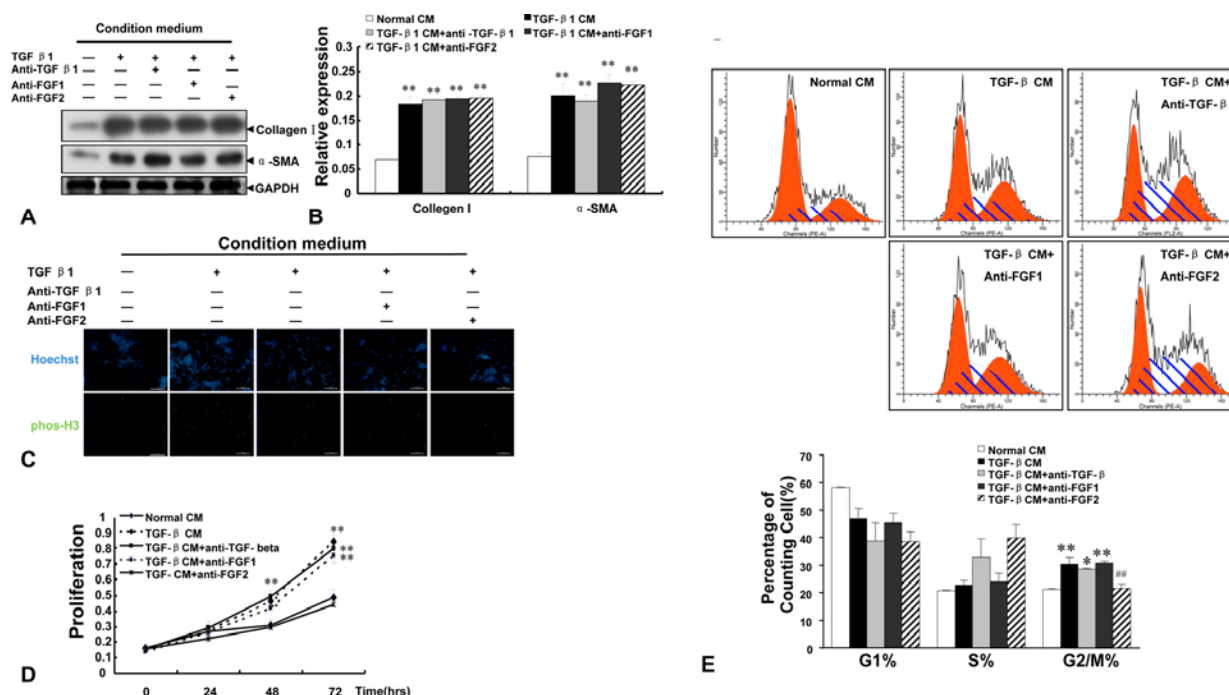


Figure 3. Fibrotic proliferation in response to TGF-β1 conditioned medium was dependent on FGF-2. (A and B) NIH3T3 cells cultured in conditioned medium neutralized by specific antibodies against TGF-β1, FGF1, or FGF2 were assayed for collagen I and α-SMA expression by Western blot. None of these antibodies affected fibrotic differentiation. (C, D and E) NIH3T3 cells cultured in conditioned medium neutralized by specific antibodies against TGF-β1, FGF1, or FGF2 were subjected to phospho-Histone H3 immunostaining or MTT and FACS. FGF-2 antibody neutralization greatly decreased fibroblast proliferation. Data are presented as mean±SEM from at least three experiments. *P<0.05, **P<0.01, compared with normal conditioned medium; ##P<0.01, compared with TGF-β1 conditioned medium. (Student's t test).

4.4. Fibroblast ERK signaling is required for TGF-β1 CM-induced cell differentiation and proliferation

FGF-2 is a basic growth factor that signals to the nucleus by binding to FGF receptor and activating multiple signal transduction pathways, including those involving Ras, mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERKs), Src, p38 MAPKs, phospholipase-Cγ (PLCγ), Crk, jun N-terminal kinase (JNK), and protein kinase C (PKC) (26). The ERKs are the main downstream FGF signaling molecules implicated in fibrotic lung disease (27). As shown in Figure 4A, ERK1/2 was also phosphor-activated by TGF-β1 CM in NIH3T3 cells, indicating that ERK1/2 signaling accompanied the upregulation of collagen I and α-SMA. To further elucidate the role of ERK signaling in TGF-β1 CM-induced fibroblast proliferation, we blocked ERK1/2 signaling by treating fibroblasts with a specific pharmacological inhibitor, PD98059. As shown in Figure 4A and B, ERK activity induced by TGF-β1 CM was significantly reduced by PD98059, and both collagen I and α-SMA protein levels were dramatically downregulated (Figure 4A and B), indicating that TGF-β1 CM-induced fibrotic differentiation depended on the activation of the ERK1/2 signaling pathway.

We then examined the influence of FGF/ERK signaling on TGF-β1 CM-induced fibroblast proliferation. The inhibitor PD98059 also suppressed phospho-H3

expression, indicating that fibroblast proliferation was blocked in the absence of intact FGF-2/ERK1/2 signaling. To confirm this observation, the proliferation rate was estimated by MTT and FACS in the fibroblasts cultured in normal CM or TGF-β1 CM in the presence or absence of PD98059. As shown in Figures 4D and E, the cells remained responsive to TGF-β1 supplementation. However, when FGF/ERK signaling was blocked by the specific inhibitor, fibroblast proliferation was adversely affected.

Taken together, these results show that FGF/ERK activity is required for TGF-β1 CM-induced fibrotic differentiation and fibroblast proliferation.

5. DISCUSSION

Identification of the critical regulatory pathways that control lung fibrosis is of profound importance to the fields of lung disease control and drug discovery. By identifying a signaling pathway that acts to induce fibroblast differentiation and proliferation, the work presented here extends our understanding of the role of cytokines like TGF-β1 and FGF-2 in controlling pulmonary progress and provides insight into the management of lung disease. Controlling fibrotic progress is crucial to medical applications that hope to repair lung fibrosis. Moreover, the identification of pathways that regulate lung fibrotic proliferation and differentiation will be essential to the

Role of FGF-2/ERK pathway in Fibroblast proliferation

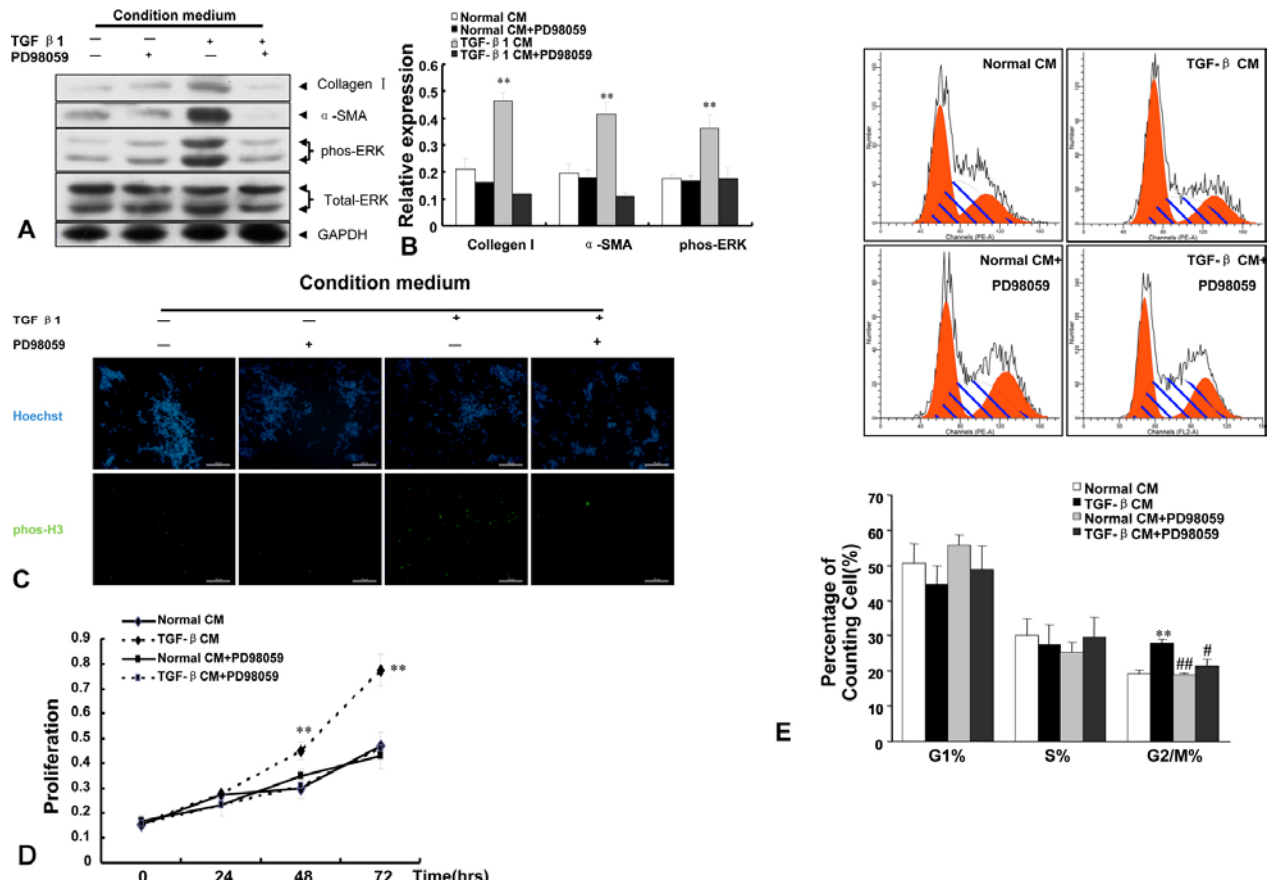


Figure 4. The ERK1/2 inhibitor PD98059 abrogated both fibrotic differentiation and proliferation induced by TGF-β1 conditioned medium. (A and B) NIH3T3 cells were cultured in serum-free DMEM for 24 h prior to TGF-β1 conditioned medium treatment in the absence or presence of the ERK1/2 inhibitor PD98059. The expression of collagen I and α-SMA, as well as ERK activation were examined by Western blotting. PD98059 attenuated the fibrotic differentiation. (C, D and E) NIH3T3 cells cultured in conditioned medium were treated by PD98059. NIH3T3 cells cultured in normal condition medium from ATII cells treated with vehicle (DMSO) were used as the controls. Cell proliferation was examined by phos-Histone H3 staining (C), MTT (D), and FACS analysis (E). All measures indicated that PD98059 abrogated fibrotic proliferation induced by TGF-β1 conditioned medium. Data are presented as mean±SEM from at least three experiments. *P<0.05, **P<0.01, compared with normal conditioned medium; #P<0.05, ###P<0.01, compared with TGF-β1 conditioned medium. (Student's t test)

development of improved therapeutic strategies for combating fibrotic lesions in the lung, as well as other tissues such as damaged heart valves, which are known to be affected by similar cytokines (28-30).

TGF-β1 is a very important cytokine that has been shown to be involved in many aspects of the lung fibrotic response and has long been believed to be a central mediator of this response (31). TGF-β1 may induce epithelial-mesenchymal transition (EMT) of alveolar epithelial type II cells (32), and thereafter function in both autocrine and paracrine manners to promote the aggression of fibrosis (33). However, the underlying mechanism that regulates this process is still unclear. In our study, incubating NIH3T3 cells in the media from TGF-β1-treated type II alveolar cells (Figure 2) was used to mimic the paracrine action of TGF-β1 *in vitro*, and indeed, such treatment enhanced both fibroblast proliferation and the activation of collagen and α-SMA secretion. We also found that FGF-2 was abundant in TGF-β1 CM (Figure 1),

indicating that TGF-β1 can induce FGF-2 expression and release from type II alveolar epithelial cells. This upregulation of FGF-2 is possibly due to the stimulated release of a previously synthesized and stored pool of FGF-2, which has been manifested in alveolar epithelial cell (AEC) fibroblasts (8). Importantly, we demonstrated that FGF-2 is specifically responsible for TGF-β1-stimulated fibroblast proliferation by antibody neutralization (Figure 3). However, we were surprised to find that FGF-2 is not involved in TGF-β1-induced fibrotic differentiation (as illustrated by the expression of collagen I and α-SMA), which illuminates a selective role of FGF-2 in TGF-β1 CM. Interestingly, although FGF-1 is increased in TGF-β1 transgenic mice (34) as well as observed in our study (data not shown), FGF-1 does not appear to be a functional constituent in TGF-β1 CM. The possible functions of FGF-1 in TGF-β1 CM remain to be determined.

In an in-depth study of FGF-2 function in the TGF-β1-induced fibrotic response, we selectively blocked

the FGF/ERK signaling pathway by using PD98059. The addition of this ERK1/2 inhibitor to the CM dramatically reduced both fibroblast proliferation and differentiation, suggesting that ERK activity might be essential for the paracrine action of TGF- β 1. It is worth noting that the inhibitor not only suppressed TGF- β 1-induced fibroblast proliferation, but also downregulated the expression of collagen I and α -SMA. Given that FGF-2 only plays a role in TGF- β 1-induced fibroblast proliferation, it is possible that a non-FGF dependent pathway is also present that responds to TGF- β 1. In a previous study, Khalil *et al.* concluded that expression of proliferating genes such as c-Myc, p21^{CIP1}, p27^{KIP}, and p15^{INK46} was not associated with TGF- β 1/FGF-2-mediated proliferation of primary lung fibroblasts (8). However, in Khalil's model, ERK1/2 is not a decisive factor in TGF- β 1-induced AECs interstitial pulmonary fibroblasts proliferation (8). This difference may reflect the distinct actions of paracrine versus autocrine TGF- β 1 signaling, but further work is necessary to differentiate these modes of TGF- β 1 action.

TGF and FGF are two very important signaling cytokines that regulate growth and differentiation in the lung, especially after lung injury. Our study provides a functional interaction link between these two signaling pathways and suggests possible therapeutic targets for clinical intervention. However, we still do not know the mechanisms by which TGF- β 1 induces FGF-2 expression or release. A more detailed investigation of the precise signals linking TGF- β 1 and FGF is anticipated to lead to novel therapeutic protocols for the treatment of fibrosis in the lung and other organs.

6. ACKNOWLEDGMENTS

This study was financially supported by the Post-Doctoral Research Startup Found of Liaoning Province (#20061036).

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Abbreviations: TGF- β : Transforming growth factor- β ; ATII cells: type II alveolar epithelial cells; CM: conditioned media; FGF-2: fibroblast growth factor 2; ECM: release extracellular matrix; α -SMA: α -smooth muscle actin; IPF: idiopathic pulmonary fibrosis; ELISA: enzyme-linked immunosorbent assay; BSA: bovine serum albumin; FACS: fluorescence-activated cells sorting; MAPKs: mitogen-activated protein kinases; ERKs: extracellular signal-regulated kinases; PKC: protein kinase C; PLC γ : phospholipase-C γ ; EMT: Epithelial-mesenchymal transition; AECs: alveolar epithelial cells

Key Words: TGF- β ; ATII cells; ERK, EMT; AECs

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