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FGF-1 Activates several Signaling Pathways as an Antifibrogenic Mediator in Human Lung Fibroblasts

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Abstract

Background: In idiopathic pulmonary fibrosis (IPF), fibroblasts characteristically perform abnormal turnover of the extracellular matrix by downregulating matrix metalloproteinase-1 (MMP-1) and upregulating collagen I and alpha-smooth muscle actin (α-SMA), mostly through the stimulation of transforming growth factor beta-1 (TGF-β1). Alternatively, fibroblast growth factor-1 combined with heparin (FGF-1/H) inhibits these effects. In view of this, the aim of this research was to determine which signaling pathways stimulated by FGF1/H in fibroblasts upregulate MMP-1 and downregulate collagen I and TGF-β1-induced α-SMA expression in human lung fibroblasts (HLF).

Methods: Cells were stimulated with FGF-1/H in the presence or absence of selective pharmacological inhibitors of different signaling pathways to block MMP-1, collagen I, and TGF-β1-induced α-SMA expression. Expression levels of the aforementioned proteins and genes were evaluated by RT-qPCR and Western blot.

Results: Data showed that FGF-1/H upregulates MMP-1 chiefly through the JNK, MEK, and TGF-β/Smad signaling pathways; meanwhile, it downregulates collagen I expression mostly through the MEK, p38, and PI3K signaling pathways; and downregulation of TGF-β1-induced α-SMA expression happens predominantly through the JNK, MEK, and TGF-β/Smad signaling pathways. On the other hand, pharmacological inhibitors alone had no effect on the expression of MMP-1 or collagen I. Likewise, FGF-1/H activates ERK1, AKT1, JNK1, and p38 proteins and upregulates transcription factor ETS-1. These findings might provide useful insights for future pharmacological treatments targeting fibrosing lung diseases such as IPF.

Conclusion: FGF-1/H stimulates different signaling pathways to upregulate MMP-1, downregulate collagen I and to inhibit TGF-β1-induced α-SMA expression.

Keywords: α-smooth muscle actin; FGF-1; Human lung fibroblasts; MEK signaling pathway; MMP-1; Type I collagen; P38 signaling pathway; PI3K signaling pathway; TGF-β/Smad signaling pathway

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Introduction

Fibroblasts are the main mesenchymal cells found in connective tissues and synthetize, secrete and turnover several molecules of the extracellular Matrix (ECM), including structural proteins, adhesive proteins, glycosaminoglycans and proteoglycans [1]. Fibroblasts are involved in all ECM physiological and pathophysiological procedures and participate actively in

idiopathic pulmonary fibrosis (IPF), a frequent interstitial lung disease (ILD). IPF is a progressive, irreversible, and lethal lung disease characterized by the expansion and prevalence of activated fibroblasts and myofibroblast subpopulations, which accumulate focally. These cells develop an active and abnormal ECM remodeling and participate in their increase, finishing eventually with an exaggerated accumulation of ECM and irreversibly altering the lung histo-architecture [2].

Abnormal ECM turnover in IPF is dependent on fibroblast and myofibroblast subpopulations, including the downregulation of matrix metalloproteinase(MMP-1), and the upregulation of collagen and alpha-smooth muscle actin (α-SMA), a hallmark of myofibroblast differentiation; all these processes are mediated predominantly by TGF-β1 stimulation, recognized as the foremost profibrogenic cytokine in different organs [3]. Therefore, fibroblast and myofibroblast subpopulations play a pivotal role in fibrotic diseases, such as IPF, as these cells are involved in the turnover of the ECM and may exhibit fibrogenic activity [4]. Myofibroblasts can derive from classic interstitial fibroblasts and from type II alveolar epithelial cells by epithelial-mesenchymal transition and possess an ECM secretory phenotype. In lungs, they mainly synthetize I and III type collagens in a 3:1 proportion and other molecules [5]. On the other hand, we have found that the acidic fibroblast growth factor (FGF-1) combined with heparin (FGF-1/H) chains several antifibrogenic effects, antagonizing the pro-fibrogenic activity of TGF-β1 in fibroblasts obtained from human lung fibroblasts (HLF). Some of these effects observed in HLF *in vitro* include the upregulation of MMP-1, the downregulation of collagen I and TGF-β1-induced α-SMA [6,7]. Similar results have been demonstrated in a pulmonary fibrosis model in female Sprague-Dawley rats *in vivo,* where lung fibrosis was attenuated when prolonged transient overexpression of FGF-1 (AdFGF-1) and TGF-β1 (AdTGF-β1) using adenoviral vectors was used [8]. In this model, the FGF-1 had preventive and therapeutic effects on TGF-β1-induced lung fibrosis by inhibiting myofibroblasts differentiation and the proliferation of alveolar epithelial cells, by downregulating TGF-β1, and degrading transforming growth factor-β receptor type 1 (TBRI), through the inhibition of Smad2/3 phosphorylation [8]. Furthermore, signaling pathways activated by FGF-1/H to upregulate MMP-1 and to downregulate collagen I and α-SMA are only partly understood. Consequently, we studied the signaling pathways activated by FGF-1/H in HLF that upregulate MMP-1 and downregulate collagen I and TGF-β1-induced α-SMA expression. We hypothesized that FGF-1/H activates different signaling pathways that regulate the expression of MMP-1 and collagen I or inhibit the upregulation of α-SMA induced by TGF-β1 in HLF.

Different aspects were taken into account for the development of the present work. Mainly, the fact that physiopathogenetic mechanisms involved in IPF are complex and involve different physiological mediators such as cytokines, chemokines, interleukins and growth factors that, in turn, activate several signaling pathways, including MEK, JNK, p38, pI3K and TGF-β/Smad. The latter stimulate fibroblasts, myofibroblasts and type II cells subpopulations characteristic of IPF, a non-inflammatory disease [1-3]. Besides, FGF-1 can activate all these pathways independently of the cellular type, interacting with all the receptors of the FGF family (FGFR1-4), thus efficiently participating in the modulation of the metabolic turnover of the ECM [9]. Also, FGF-1/H anti-fibrous potential, as evidenced both *in vitro* and *in vivo* [6-8], can lower the expression of collagen, especially type I, the most abundant protein in fibrous lung tissue. It also increases the expression of MMP-1, that constitutes the initial degrading enzyme of type I fibrillar collagen in the native state [2,3], and decreases the expression of α-SMA, essential in the trans-differentiation of fibroblast into myofibroblasts [1,5]. Consequently, our interest was to find out which of these signaling pathways were activated in fibroblasts to promote the expression of these three proteins. To achieve this goal, we used a cellular model that can broaden our knowledge of the role of FGF-1 in the pathophysiology of IPF. Patients affected by this illness generally die between 3 to 5 years after the appearance of the first respiratory symptoms [2,3,10], a fact that clearly points out the relevance of this research. We expect that, by integrating the results obtained herein with the previous information of the anti-fiber potential of FGF-1, novel research will be developed to finally find an effective therapy for this illness.

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Research Methodology

The aim of this research was to determine the signaling pathways stimulated by FGF1/H in fibroblasts derived from human lung that upregulate MMP-1 and downregulate collagen I and TGF-β1-induced α-SMA expression. To achieve this goal, the following experimental approaches were designed.

Fibroblasts obtention from human lung

Human lung fibroblasts (HLF) in passage 4-6 (n=3), were obtained as described elsewhere [6,7]. In brief, the fibroblasts were obtained from samples of lung tissue via the open lung biopsy from normal areas from patients with lung cancer and cultured in Ham's F12 (F-12 Nutrient Medium), supplemented with 10% FBS. Experiments were performed when cells reached 70% confluence while cultured in serum-free media (SFM). In all experiments the cells were then treated with 20 ng/ml recombinant human FGF-1 (R&D, Minneapolis, MN) plus 100 μg/ml heparin (FGF-1/H) or TGF-β1 (5 ng/ml). Control cells were incubated in SFM only.

FGF-1, heparin, and TGF-β1 concentration was based in previous studies [6,7]. Heparin was used because it plays an essential role in FGF signaling as it has a direct association with it and its receptor [11-13]. FGF and heparin were used in combination, because it has been confirmed that when incubated alone, they have a negligible effect [6,7].

FGF-1/H-activated signaling pathway pharmacological inhibition

HLF were kept for 24 h in serum-free media. On the next day, they were pre-incubated for 30 min with selective pharmacological inhibitors of signaling pathways (see below), followed by the addition of FGF-1/H; incubating the cells finally for a period of up to 24 h, depending on the experiment: for MMP-1 and type I collagen proteins the last period was 24 h, for MMP-1 and α1(I) pro-collagen mRNA expression analysis for 6h, for evaluation of ERK1, p38, AKT1, JNK1 up to 8h, and finally to ETS-1 expression for 30 min.

The analysis of α-SMA was performed as follows: cells were stimulated for 24 h in serum-free medium (SFM), followed by incubation with TGF-β1 (5 ng/ml) during 24 h to stimulate protein expression. On the next day, cells were pre-incubated for 30 min with the respective pharmacological inhibitor, and finally FGF-1/H was added to the same medium and incubated during 24 h (without eliminating the inhibitors). In parallel experiments, HLF were incubated in the presence of the corresponding pharmacological inhibitor in the absence of FGF-1/H or TGF-β1 for 24 h (negative controls).

The selective pharmacological inhibitors of specific signaling pathways were: PD098059 (40μM; Calbiochem, San Diego, CA, USA), an inhibitor of ERK1/2/3 of the MEK-ERK MAPK signaling pathway; SB203580 (20μM), a p38-MAPK signaling pathway inhibitor Calbiochem, San Diego, CA); SB600125 (50μM; Cell Signaling Technology, Beverly, MA) the JNK1/2/3 MAPK pathway inhibitor; LY294002 (50μM; Cell Signaling Technology, Beverly, MA), a phosphatidylinositol 3-kinase (PI3K) inhibitor; and SB431542 (10μM; Calbiochem, San Diego, CA), an inhibitor of TBRI kinase activity and consequently, an indirect inhibitor of Smad2/3 phosphorylation (an integrant of the TGF-β/Smad signaling pathway).The dosage of the inhibitors was based on previous studies of our study group [14].

Western blot analysis

Proteins drawn from whole-cell extracts and serum-free conditioned media were obtained independently from fibroblasts stimulated with 20 ng/ml FGF-1 (R&D, Minneapolis, MN) plus 100μg/ml heparin (FGF-1/H) or TGF-β1 (5 ng/ml) 24 h. We separated 20μg of protein from whole-cell extracts or 12μg from conditioned media by electrophoresis on SDS-PAGE gels, and these simples were then transferred to nitrocellulose membranes and incubated with primary antibodies: anti-human α1(I) collagen (Col 1α1 (L-19) Goat polyclonal (sc-8783; Santa Cruz Biotechnology, INC; dilution 1:200); anti-MMP-1 (Ab-1) Mouse Mab (41-1E5; Calbiochem; dilution 1:200); monoclonal anti-α-smooth muscle actin (1A4; Sigma-Aldrich, St. Louis MO; dilution 1:300); rabbit polyclonal anti-Erk1 (K-23) ab (sc-94; Santa Cruz Biotechnology; dilution 1:500); anti-phospho-Erk1 (12d4), Mouse mab (sc-81492; Santa Cruz Biotechnology; dilution 1:200); anti-JNK1 (D-2), Mouse mab (sc-7345 Santa Cruz Biotechnology; dilution 1:500); anti-phospho-JNK1 (9H8), Mouse mab (sc-81502; Santa Cruz Biotechnology; dilution 1:500); anti-AKT1 (H-136), Rabbit polyclonal antibody (sc-8312 Santa Cruz Biotechnology; dilution 1:500); antiphospho-AKT1 (Ser 473), Rabbit mab (Santa Cruz Biotechnology; dilution 1:500); anti-p-38 Map Kinase, Rabbit pab (#9212; Cell Signaling; dilution 1:200); anti phospho-p-38 MAPK (Thr180/Tyr182) (D3F9), Rabbit mAb (#4511; Cell Signaling; dilution 1:1000); and anti-β-tubulin (Santa Cruz Biotechnology; dilution 1:200). A Western blot quantitative analysis was conducted with Image Lab 5.2.1 software with results expressed in mean±SD values of relative intensity units (RIU) as a ratio of the band density to total β-tubulin. Changes observed in phosphorylated protein levels were measured with reference to respective nonphosphorylated proteins. The measure of MMP-1 in conditioned media has not a currently control intern; therefore, the concentration of protein charged in the gel is accurately quantitated.

RNA extraction, cDNA synthesis and RT-qPCR

Total RNA was isolated from treated or control HLF (incubated 6h), using the TRIzol reagent (Invitrogen Life Technologies, Grand Island NY) and reverse-transcribed into cDNA using High-Capacity cDNA reverse transcription kit (Applied Biosystems™, Foster city USA) according to the manufacturer´s instructions. Quantitative real-time PCR (RT-qPCR) was performed with Quant Studio 12K Flex (Applied Biosystems™) using the following specific TaqMan probes (Applied Biosystems™). Collagen 1 COL 1A1 (Hs00164004_m1) and MMP1 (Hs00899568_m1). The cycling conditions were 95° for 20sec, then 40 cycles at 95° for 15s and 60° for 1min. The levels of mRNA expression were assessed after normalization using the HPRT (hypoxanthine-guanine phosphoribosyltransferase) TaqMan probe (Hs00399225_m1) as endogenous control. All amplifications reactions were performed in triplicate, and the relative quantification of mRNA expression was calculated using the comparative threshold cycle method (2^-ΔΔCT) [15]. RT-qPCR analysis was performed for three independent experiments. Data are presented as the mean ± standard deviation of three independent experiments.

Collagen content measurement

Concentrations of newly synthesized collagen were measured in the conditioned media via a quantitative Sircol Soluble Collagen Assay (Biocolor Ltd, Carrickfergus, Northern Ireland), according to the manufacturer's specifications [16]. Results are expressed as the mean ± standard deviation values of μg collagen/mg protein.

Expression of the ETS-1 transcription factor

To determine whether some of the transcription factors that regulate different genes of the metabolism of extracellular matrix in fibroblasts is activated by FGF-1, and if heparin potentiates this action, ETS-1 was chosen [17]. For this, cells were incubated for 30 min with FGF-1, heparin alone or the combination FGF-1/H. We examined the expression of transcription factor ETS-1 by Western blot; ETS-1 was purified from the nucleus after stimulating the HLF with FGF-1/H, FGF-1, or heparin using a commercial kit (NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit, Thermo Scientific 7883) according to the manufacturer's specifications [18]. Western blot was performed on the nuclear fraction with an anti-ETS-1 rabbit polyclonal antibody (N276) Rabbit pab (sc-

111; Santa Cruz; dilution 1:200); Anti-Lamin A + C Rabbit pab (GTX101127; Genetex; dilution 1:500) was used as a load control charge. A Western blot quantitative analysis was conducted using Image Lab 5.2.1 software and results are expressed as mean ± SD of relative intensity units (RIU) as a ratio of the band density to nuclear lamin A/C.

Statistical analysis

Results are presented graphically based on the means ± SD of at least three independent experiments. Statistically significant changes between samples were assessed by one-way ANOVA and with Tukey´s post hoc tests for multiple comparisons. The analyses were performed using the statistical package GraphPad version 6.01 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was deemed statistically significant.

Results

FGF-1/H induces the overexpression of MMP-1 by the activation of JNK, MEK, and TGF-β/Smad signaling pathways

The effect of FGF-1/H on MMP-1 expression was evaluated by Western blot and RT-qPCR. Our Western blot analysis of MMP-1 secreted into the culture media revealed a significant overexpression when compared to the control **(Figures 1A-1B; Lane 2 vs. 1; p<0.0**1).When cells were incubated with FGF-1/H in the presence of selective inhibitors of the signaling pathways of interest, the overexpression of MMP-1 was significantly blocked by PD098059, SB600125, and SB431542, inhibitors of JNK, MEK, and TGF-β/Smad signaling pathway, respectively **(Figures 1A-1B; Lanes 3, 5, and 7;** p<0.01). On the other hand, inhibitors of the p38 and PI3K pathways, LY294002 and SB203580 respectively, did not block FGF-1/H-induced MMP-1 expression (**Figures 1A-1B; Lanes 4 and 6**). HLF incubated with the pharmacologic inhibitors in the absence of FGF-1/H show that these molecules alone did not have any effect on the expression of MMP-1 (**Figure 1C; Lanes 3-7 vs. 2**); showing similar levels of expression to as those of control cells (**Figure 1C; Lane 1**).

RT-qPCR experiments using mRNA extracted from the HLF (**Figure 2**) confirm the results of the Western blot: MEK, JNK, and TGF-β/Smad signaling pathways inhibitors diminished the upregulation of MMP-1 gene induced by FGF-1/H (**Figure 2; Lanes 3, 5 and 7 vs. 2; p<0.001**).

FGF-1/H downregulates collagen I through the MEK, P38, and PI3K signaling pathways

The effect of FGF-1/H on collagen I protein expression was analyzed via Western blot, to pro-collagen α1(I) chain collagen with RT-qPCR, and also the amount of total collagen secreted into the culture media using the Sircol reagent was measured. The Western blot analysis shows that HLF express high quantities of pro α1(I) collagen (**Figures 3A-3B; Lane 1**), which is significantly downregulated by FGF-1/H (**Figures 3A-3B; Lane 2 vs. 1; p<0.01**). When FGF-1/H was incubated in the presence of selective

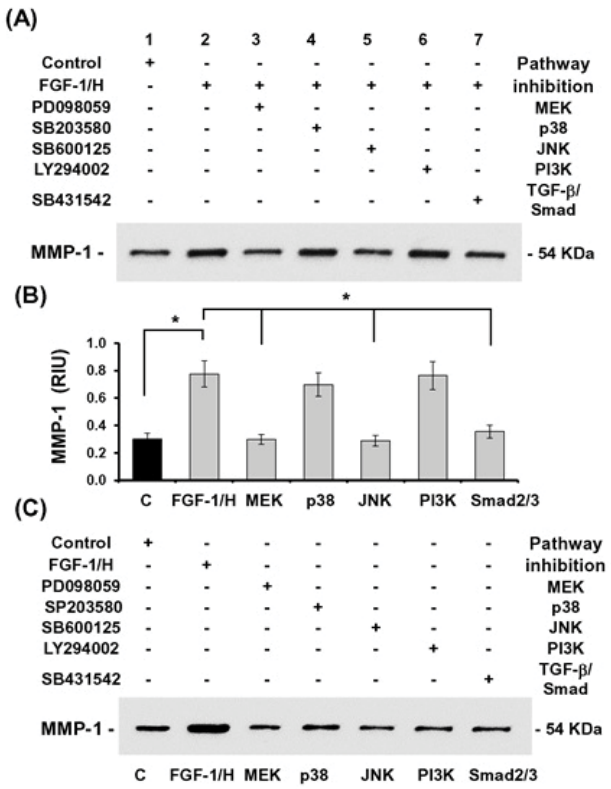

Figure 1 The JNK, MEK, and TGF-β/Smad signaling pathways mediate the upregulation of FGF-1/H-induced MMP-1 at the translational level. **Panel A**: Representative Western blot of conditioned media of HLF showing the effect of several pharmacological signaling pathway inhibitors on the expression of MMP-1 induced by FGF-1/H. **Panel B**: Densitometric analysis of the Western blot shown in panel a. **Panel C**: Representative Western blot of the conditioned media showing the effect of signaling pathway pharmacological inhibitors incubated in the absence of FGF-1/H. **Lane 1:** Control cells. **Lane 2:** Cells treated with FGF-1/H. **Lanes 3-8:** Cells treated with FGF-1/H 24 h after pre-incubation with specific pharmacologic signaling pathway inhibitors. **Lane 3:** PD098059. **Lane 4:** SB203580. **Lane 5:** SB600125. **Lane 6:** LY294002. **Lane 7:** SB431542. Each bar illustrated in B represents the mean ± SD of 3 independent Western blot experiments. *p<0.01.

pharmacologic inhibitors, the MEK, P38, and PI3K inhibitors blocked the effect of FGF-1/H (**Figures 3A-3B; Lanes 3, 4, and 6 vs. 2; p<0.01**). JNK and TGF-β/Smad pathways inhibition did not restore collagen downregulation induced by FGF-1/H (**Figures 3A-3B; Lanes 5 and 7 vs. 2; NS**). A control of this analysis was performed through the incubation of HLF cells in the presence of the pharmacologic inhibitors only and in the absence of FGF-1/H, showing that these molecules alone did not have effect on the expression of pro α1(I) collagen (**Figure 3C; Lanes 3-7 vs. 2**). Only FGF-1/H induced the downregulation of pro α1 (I) collagen (**Figure 3C; Lane 2 vs. 1; p<0.01**).

The collagen expression gene measured by RT-qPCR confirms the results evaluated by Western-blot. The MEK, p38, and PI3K

signaling pathways are involved in the activity of FGF-1/H on the collagen expression gene in HLF (**Figure 4; Lanes 3, 4, and 6 vs. 2; p<0.001**).

The inhibitors of MEK, p38, and PI3K signaling pathways blocked the decrement of total collagen protein secretion induced by FGF-1/H

Newly total collagen synthesized and secreted into the culture medium by HLF was measured by Sircol reagent, confirming the Western blot results. Thus, while FGF-1/H decremented collagen protein secreted into culture media (**Figure 5; Lane 2 vs. 1; p<0.01**), pharmacological inhibitors of MEK, p38, and PI3K signaling pathways reversed this effect (**Figure 5; Lanes 3, 4, and 6 vs. 2; p<0.01**).

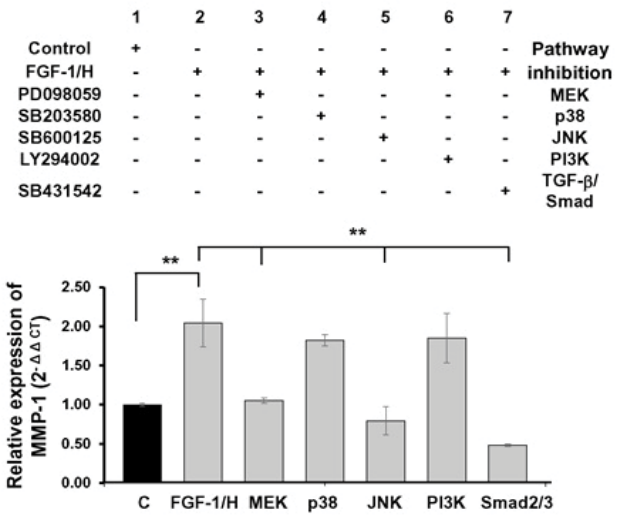

Figure 2 The JNK, MEK, and TGF-β/Smad signaling pathways mediate the upregulation of FGF-1/H-induced MMP-1 at the transcriptional level in HLF. Quantification of the expression levels of MMP1 mRNA induced by FGF-1H by RT-qPCR in HLF and the effect of several signaling pathway pharmacological inhibitors evaluated at 6 h of incubation**. Lane 1:** control cells. **Lane 2:** Cells treated with FGF-1/H. **Lanes 3-8:** Cells treated with FGF-1/H 24h after pre-incubation with specific pharmacologic signaling pathway inhibitors. **Lane 3:** PD098059. **Lane 4:** SB203580. **Lane 5:** SB600125. **Lane 6:** LY294002. **Lane 7:** SB431542. RT-qPCR results are illustrated in the bar graph; Bar graph depicting RT-qPCR of MMP-1 mRNA extracted from cells incubated with and without FGF-1/H and selective signaling pathway pharmacological inhibitors. Bar graph showing the RT-qPCR results of mRNA extracted from HLF cells showing the effect of several signaling pathway pharmacological inhibitors on the expression of MMP-1 induced by FGF-1/H. Bars represent the mean ± SD of 3 experiments performed in triplicate and data are expressed as relative expression values of the MMP-1 mRNA normalized to HPRT mRNA (2^-ΔΔCT). *p<0.001.

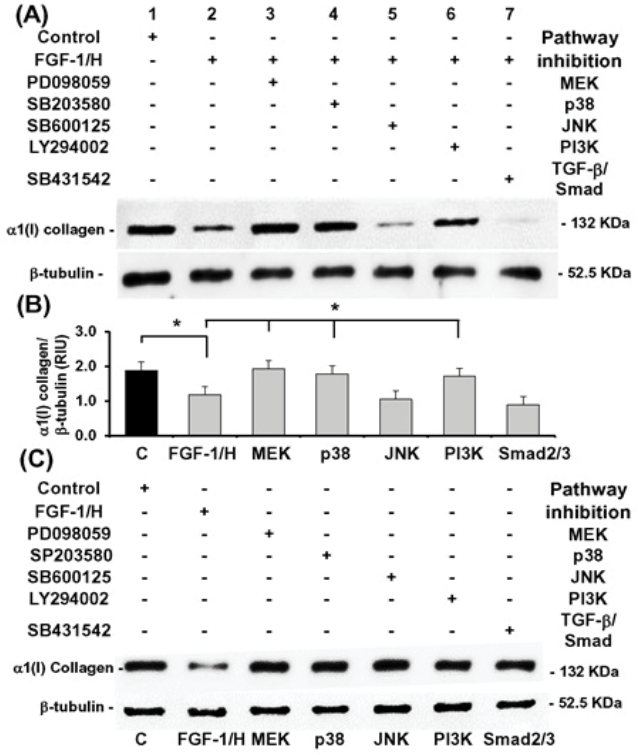

Figure 3 FGF-1/H-induced downregulation of α1(I) collagen at the translational level is mediated by the MEK, p38, and PI3K pathways in HLF. **Panel A**: Representative Western blot of HLF showing the effect of several signaling pathway pharmacological inhibitors on the expression of α1(I) collagen and -tubulin induced by FGF-1/H. **Panel B**: densitometric analysis of the Western blots illustrated in (**a**) showing the normalization of α1(I) collagen with-tubulin as a loading control. Statistical analysis showed that cells incubated with FGF-1/H had significantly less α1(I) collagen (2 vs. 1; p <0.01) and that selective pharmacological inhibitors of the MEK, P38, and PI3K blocked the effect of FGF-1/H (**Lanes 3, 4 and 6 vs. 2**; p<0.01). **Panel C**: Representative Western blot of conditioned media of HLF showing the effect of pathway inhibitors incubated in the absence of FGF-1/H. **Lane 1:** control cells. **Lane 2:** Cells treated with FGF-1/H only. **Lanes 3-8:** Cells treated with FGF-1/H 24h after preincubation with specific pharmacologic signaling pathway inhibitors. **Lane 3:** PD098059. **Lane 4:** SB203580. **Lane 5:** SB600125. **Lane 6:** LY294002. Lane 7:SB431542. Each bar of the densitometry represents the mean ± SD of 3 independent experiments. *p <0.01.

At the translational level, FGF-1/H induces downregulation of TGF-β1-induced α-SMA expression through the JNK, MEK, and TGF-β/ Smad signaling pathways

HLF extracted from human lung are composed of about 14.8 ± 11.6 % of myofibroblasts that constitutively express α-SMA [4]. To confirm that TGF-β1 induces the expression of α-SMA, cells were incubated with TGF-β1 for 48 h (**Figures 6A-6B; Lane 3**

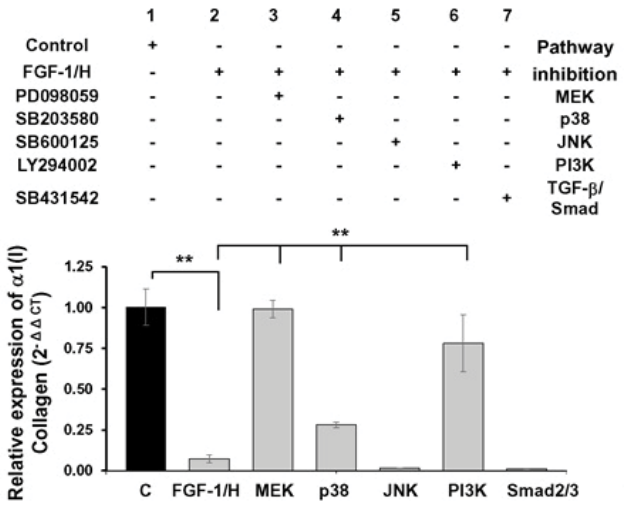

Figure 4 The downregulation of α1(I) pro-collagen at the transcriptional level occurs through the MEK, p38, and PI3K pathways in HLF. FGF-1/H significantly diminishes type I collagen gene expression (lane 2 vs.1). The MEK, p38, and PI3K signaling pathways are involved in the activity of FGF-1/H on the collagen expression gene in HLF (**Lanes 3, 4 and 6 vs. 2).** Quantification of the expression levels of α1(I) collagen mRNA induced by FGF-1H by RT-qPCR in HLF and the effect of several signaling pathway pharmacological inhibitors. **Lane 1:** control cells. **Lane 2:** Cells treated with FGF-1/H. **Lanes 3-8:** Cells treated with FGF-1/H 24h after pre-incubation with specific pharmacologic signaling pathway inhibitors. **Lane 3:** PD098059. **Lane 4:** SB203580. **Lane 5:** SB600125. **Lane 6:** LY294002. **Lane 7:** SB431542. Bar graph illustrating the RT-qPCR results of mRNA extracted from HLF cells showing the effect of several signaling pathway pharmacological inhibitors on the expression of α1(I) pro-collagen induced by FGF-1/H. Bars represent the mean ± SD of 3 experiments performed in triplicate and results are expressed as 2^-ΔΔCT values of α1(I) pro-collagen mRNA normalized to HPRT mRNA. **p<0.001.

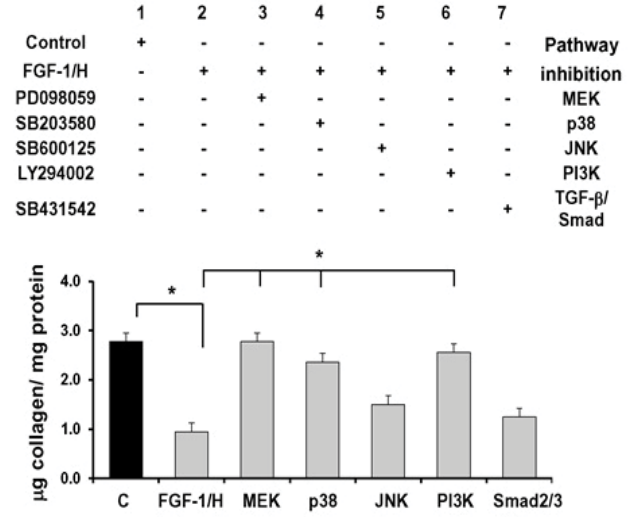

Figure 5 Inhibitors of MEK, p38, and PI3K block the decrement of newly total collagen synthesis induced by FGF-1/H in HLF. While FGF-1/H decremented amount of soluble collagen secreted in the media (lane 2 vs. 1), pharmacological inhibitors of MEK, p38, and PI3K signaling pathways reversed this effect (**Lanes 3, 4 and 6 vs. 2**). **Lane 1:** control cells. **Lane 2:** Cells treated with FGF-1/H. **Lanes 3-8:** Cells treated with FGF-1/H 24 after pre-incubation with selective pharmacologic signaling pathway inhibitors. **Lane 3:** PD098059. **Lane 4:** SB203580. **Lane 5:** SB600125. **Lane 6:** LY294002. **Lane 7:** SB431542. Each bar represents the mean ± SD of 3 independent experiments. *p<0.01.

vs.1; p<0.01). Furthermore, the α-SMA upregulation induced by TGF-β1 was significantly diminished by FGF-1/H **(Figures 6A-6B; Lane 3 vs. 2;** p<0.01). For the other samples, cells were incubated first with TGF-β1 for 24 h followed by incubation with selective pharmacologic inhibitors of the signaling pathways of interest for 30 min, and they were finally stimulated with FGF-1/H for 24 h. We were thus able to evaluate which signaling pathway is activated by FGF-1/H to inhibit TGF-β1-mediated α-SMA expression (**Figures 6A-6B; Lane 4, 6 and 8 vs. 2; p<0.01**). The pharmacological inhibitors of the JNK, MEK, and TGF-β/Smad signaling pathways restored the expression of α-SMA to levels observed in cells stimulated only with TGF-β1 during 48 h (**Figures 6A-6B; Lane 3**). Thus, FGF-1/H inhibits effects of TGF-β1-mediated α-SMA expression through these pathways. The p38 and PI3K inhibitors did not affect the diminution induced by FGF-1/H on the TGF-β1-induced α-SMA expression (**Figures 6A-6B; Lanes 5 and 7**).

FGF-1/H stimulates the activation of AKT1, ERK1, JNK1, and p38 Proteins

To confirm that the downregulation in α-SMA expression induced by FGF-1/H observed in our experiments corresponded with the activation of the above-mentioned signaling pathways, kinetics of the phosphorylation of specific proteins in pathways were determined. Thus, by Western blot, the FGF-1/H-induced phosphorylation of ERK1, JNK1, AKT1, and p38 was assayed. Accordingly, ERK1, which forms part of the MEK MAPK signaling pathway, was phosphorylated (p-ERK1) by the exposure of HLF to FGF-1/H for 15-60 min (**Figures 7A-7B; Lanes 2 and 3 vs. 1; p<0.01**). JNK1, which forms part of the JNK signaling pathway, showed an increase in phosphorylation (p-JNK1) after 1-30 min exposure to FGF-1/H **(Figures 7C-D; Lanes 2-6 vs. 1; p<0.01**). AKT1, a protein that forms part of the PI3K signaling pathway, showed an increase in phosphorylation (p-AKT1) after 3-30 min exposure to FGF-1/H **(Figures 8A-8B; Lanes 3-6 vs. 2; p<0.01**), while p38 exhibited significant phosphorylation (p-p38) after 15 and 30 min exposure (**Figures 8C-8D; Lanes 2 and 3 vs. 1;** p<0.01).

FGF-1/H stimulates the upregulation of transcription factor ETS-1

With the aim of evaluate one of the transcription factors involving in the regulation of genes of the metabolism of the ECM in fibroblasts, that might be activated by FGF-1/H in HLF, ETS-1

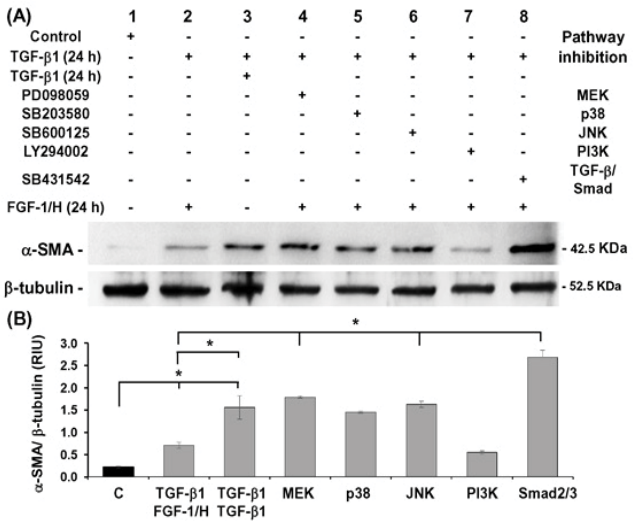

Figure 6 The upregulation of TGF-β1-induced α-SMA expression is inhibited by FGF-1/H through the JNK, MEK, and TGF-β/Smad signaling pathways of HLF. The pharmacological inhibitors of the JNK, MEK, and TGF-β/Smad signaling pathways restored the expression of α-SMA to levels observed in cells stimulated only with TGF-β1 during 48h (lane 3). **Panel A**: Representative Western blot of an HLF cell protein extract showing inhibitory effect of FGF-1/H on the TGF-β1-induced α-SMA expression. **Panel B**: densitometric analysis of Western blots illustrated in Panel a, showing the normalization of α-SMA with β-tubulin as a loading control. **Lane 1:** control cells. **Lane 2:** Cells treated with TGF-β1/FGF-1/H. **Lane 3:** Cells treated with TGF-β1 for 48 h. **Lanes 4-8:** Cells treated with TGF-β1/FGF-1/H and specific selective pharmacological inhibitors: **Lane 4:** PD098059. **Lane 5:** SB203580. **Lane 6:** SB600125. **Lane 7:** LY294002. **Lane 8:** SB431542. TGF-β1 induces the expression of α-SMA in HLF (lane 3 vs. 1) and FGF-1/H diminishes this effect (lane 3 vs. 2). Each bar represents the mean ± SD of 3 independent experiments. *p <0.01.

was chosen [17,19]; we examined the expression of transcription factor ETS-1 by Western blot using nuclear extracts. We found that FGF-1/H upregulates the expression of this factor after 30 min of stimulation relative to the control **(Figure 9; Lane 3 vs. 1; p<0.01**) while FGF-1 and heparin alone have an incipient effect (**Figure 9; Lanes 2 and 4; NS**), showing that ETS-1 is efficiently upregulated by FGF-1/H. Results about the different signaling pathways activated by FGF-1/H are summarized in **Figure 10.**

Discussion

FGF-1/H has been studied in our lab, and it exhibits potent anti-fibrosing characteristics, antagonizing several effects mediated by TGF-β1 [6-8], through mechanisms involving signaling pathways that have not been widely studied. Therefore, for this study we analyzed signaling pathways stimulated by FGF-1/H to downregulate type I collagen and α-SMA, and to upregulate MMP-1 in HLF. Heparin is a polyanionic, linear, and highly sulfated glycosaminoglycan that retards the degradation of FGF-1 and that favors stabilization with the FGFR1 and subsequent signaling cascades [11-13], integrated into a complex network of signaling pathways activated by FGF-1, such as JNK, MEK, p38, and MEK [9,13]. FGF-1 and heparin were not used alone in the experiments because they have an incipient effect only, as we have demonstrated previously [5,6], showing an effect on ETS-1 expression similar to the one observed in this work. The results are summarized in **Figure 10**.

The activation of the JNK, MEK, and TGF-β/Smad signaling pathways by FGF-1/H in HLF to downregulate MMP-1, observed in Western blot and confirmed by RT-qPCR examination, has not been demonstrated previously. However, these features have been documented in other fibroblast types. For instance, the activation of the JNK and MEK pathways has been documented in fibroblasts derived from explant cultures of normal skin and hypertrophic scars, where FGF-2 induces the expression of MMP-1 in both types of cells [20]. The MEK pathway activation was also demonstrated in four invasive melanoma cells lines constitutively overexpressing MMP-1 via Ras/Raf/MEK/ERK, which, when blocked, allows cells to proliferate, prevent collagenolysis, and diminish their survival and metastatic potential [21].The TGF-β/Smad signaling pathway participation in the overexpression of

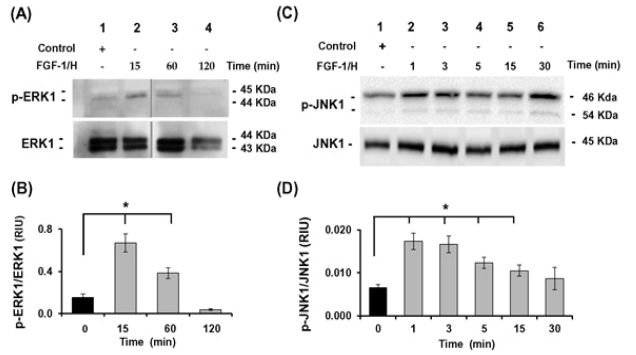

Figure 7 FGF-1/H induces the phosphorylation of ERK1 and JNK1 in HLF. **Panel A**: A representative Western blot of cell extracts of HLF showing the temporal effect of FGF-1/H on the phosphorylation of ERK1. This image was cut and assembled to match similar **Lanes** to those shown in other figures. **Panel B**: densitometric analysis of the Western blots from panel a, comparing p-ERK1/ ERK1. **Lane 1:** control cells. **Lanes 2-4** cells treated with FGF-1/H at different incubation periods. p-ERK was significantly increased in cells treated with FGF-1/H for 15 and 60 min (**Lanes 2 and 3 vs. 1**). **Panel C**: Representative Western blot of a cell protein extract of HLF showing the temporal FGF-1/H effect on the phosphorylation of JNK1. **Panel D**: Densitometric analysis of Western blots from panel c comparing p-JNK1/JNK1. **Lane 1:** control cells. **Lanes 2-5:** Cells treated with FGF-1/H at different incubation periods. p-JNK1 was significantly increased in cells treated with FGF-1/H during 1, 3, 5 and 15 min (**Lanes 2-5 vs. 1**). Each bar represents the mean ± SD of 3 independent experiments. *p<0.01.

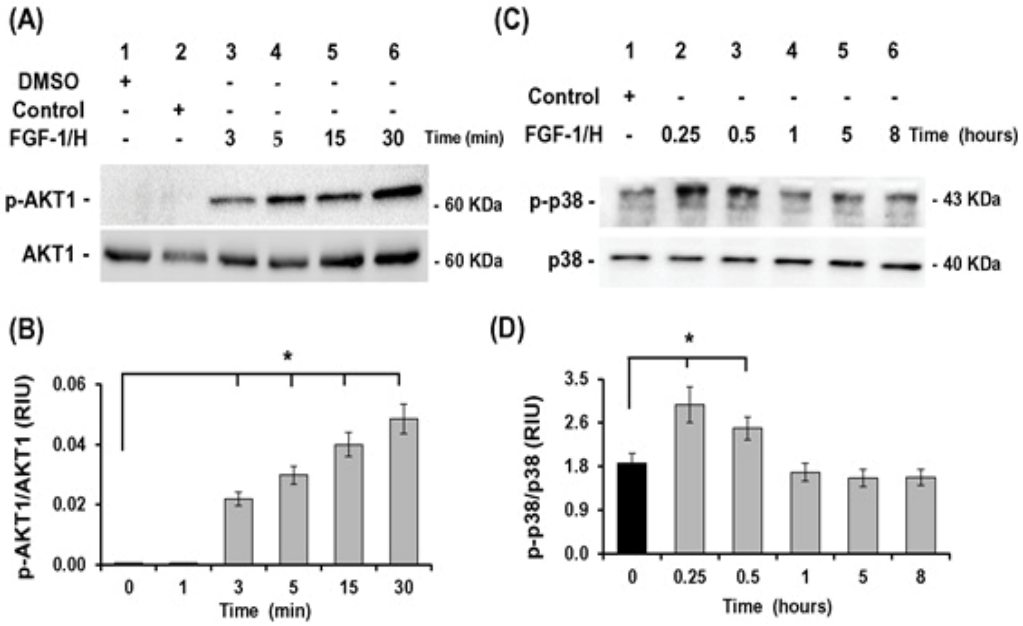

Figure 8 FGF-1/H induces the phosphorylation of AKT1 and p38 in HLF. **Panel A**: representative Western blot of a cell protein extract from HLF showing the temporal effect of FGF-1/H on the phosphorylation of AKT1. **Panel B:** densitometric analysis of Western blots from panel a comparing p-AKT1/AKT1. **Lane 1:** control cells. **Lanes** 2-6: Cells treated with FGF-1/H at different incubation periods. p-AKT1 was significantly increased in cells treated with FGF-1/H for 3, 5, 15 and 30 min (**Lanes 3-6 vs. 1**). **Panel C**: representative Western blot of a cell protein extract from HLF, showing the temporal effect of FGF-1/H on the phosphorylation of p38. **Panel D**: densitometric analysis of the Western blot from panel c comparing p-p38/p38. **Lane 1:** control cells. **Lanes 2-6:** Cells treated with FGF-1/H at different incubation periods. p-p38 was significantly increased in cells treated with FGF-1/H for 15 and 30min (**Lanes 2 and 3 vs. 1**). Each bar represents the mean ± SD of 3 independent experiments. *p<0.01.

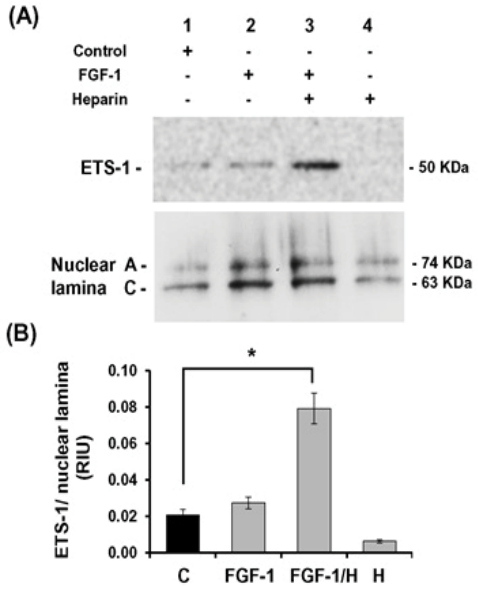

Figure 9 FGF-1/H stimulates the upregulation of transcription factor ETS-1 in HLF after 30 min incubation. **Panel A**: Representative Western blot of a cell protein extract from HLF showing the time-dependent effect of FGF-1 and heparin combined or alone on the ETS-1 activation. **Panel B**: Densitometric analysis of the Western blots from panel a, comparing nuclear ETS-1 and lamin A/C as a loading control. FGF-1/H significantly induced ETS-1 production (lane 3 vs 1). **Lane 1:** control cells. **Lane 2:** FGF-1. **Lane 3:** FGF-1/H. **Lane 4:** heparin. Each bar represents the mean ± SD of 3 independent experiments. *p<0.01.

MMP-1, has only been confirmed by FGF-2, that inhibits this effect as part of an anti-scar mechanism in wound repair [22]; a feature like the one observed in HLF with FGF-1/H.

We found that MEK, p38, and PI3K signaling pathways are activated by FGF-1/H in HLF to decrease the type I collagen synthesis, which was observed in Western blot and corroborated in the RT-qPCR exploration. This effect has also not been seen before in HLF. However, another member of the FGF family, FGF-2, inhibits collagen I expression in human dental pulp cells, invasive melanoma cells lines, and mouse osteoblastic cells (MC3T3-E1) through MEK signaling pathway activation [20,23,24]. Regarding the activation of the p38 and PI3K pathways by FGF-1/H and diminishing type I collagen synthesis, there are no previous reports on this issue and we show for the first time that this biological role of FGF-1/H might be related to the antifibrogenic role of this factor.

To determine which pathway is inhibited by FGF-1/H to lower the expression of α-SMA, as spontaneous inhibition in basal cells was inconclusive (data not shown), and to confirm that α-SMA is expressed, we performed an assay stimulating α-SMA-expression with TGF-β1 addition. Afterwards, these cells were incubated with selective pharmacological signaling pathway inhibitors and FGF-1/H. Our results demonstrated that the JNK, MEK, and TGF-β, Smad signaling pathways mediate the FGF-1/H-induced inhibition of TGF-β1-induced α-SMA expression, because their inhibition restored the α-SMA expression to the levels of cells incubated with TGF-β1 only.

The direct activation of the MEK pathway by FGF-1/H to inhibit the TGF-β1-induced α-SMA expression has been observed with the soluble ectodomain of fibroblast growth factor receptor-IIIc (sFGFR2c), which binds to fibroblast growth factor (FGF) ligands to block the activation of the FGF-signaling pathway, dramatically diminishing lung fibrosis *in vitro* and *in vivo* through the upregulation of α-SMA induced by TGF-β1 in fibroblasts by means of the activation of the MEK/ERK pathway [25].

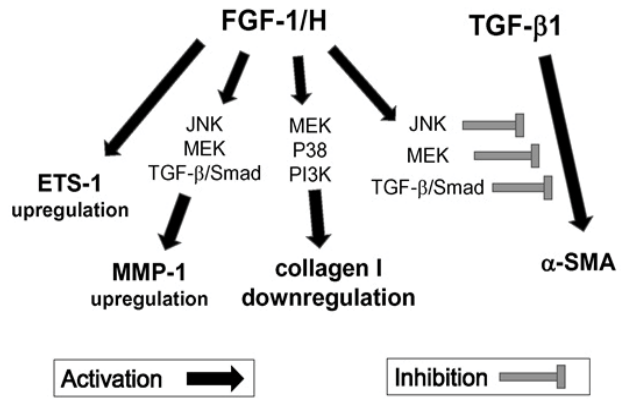

Figure 10 Schema summarizing the signaling pathways activated by FGF-1/H in HLF to upregulate ETS-1, MMP-1, and downregulate collagen type I, and inhibit the TGF-β1-induced α-SMA upregulation. JNK, MEK, and TGF-β/Smad signaling pathways mediate the upregulation of MMP-1 induced by FGF-1/H, and the inhibition of TGF-β1-induced α-SMA expression. Collagen type I downregulation is induced by FGF-1/H activation of MEK, p38, and PI3K signaling pathways. Additionally, the ETS-1 transcription factor expression is upregulated by FGF-1/H.

We previously showed that FGF-1/H blocks TGF-β1-induced α-SMA expression and that this is accompanied by the inhibition of Smad2 phosphorylation in HLF (7), and also, through experiments conducted *in vivo* via the prolonged transient overexpression of FGF-1 (AdFGF-1) in HLF, it was found that α-SMA is inhibited through the reduction of Smad2 phosphorylation, and also that AdFGF-1 attenuates an increase in TBRI protein and mRNA levels in HLF and alveolar epithelial cells *in vitro* [8]. Additionally, in the TGF-β/Smad pathway, an inhibitory effect of FGF-1/H on α-SMA down regulation has been reported with the FGF-2 inhibition of TGF-β1-induced α-SMA in airway smooth muscle cells *in vitro* through the inhibition of TBRI [26].

The inhibitory effects of FGF-1/H on α-SMA (downregulation) through the JNK pathway has not been reported in HLF, which suggests that the activation of these signaling pathways by TGF-β1-induced α-SMA must be analyzed further using an experimental approach.

The direct activation of the TGF-β1/Smad pathway by FGF-1/H in HLF has not been demonstrated. However, some cross talk between signaling pathways has been described in the TGF-β induction of FGF-2 expression in stromal cells that needs integrated Smad3 and MAPK pathways signaling cascades to operate an antifibrogenic mechanism in a prostate fibroblast cell line [27]. Probably, this might occur in our HLF also, although it must be studied further to be better understood.

Experiments on kinetic activation through the phosphorylation of ERK1, JNK1, AKT1, and p38 proteins confirm that FGF-1/H effectively activates corresponding pathways in HLF. It is important to notice that some of these molecules may be activated by canonical or crosstalk mechanisms occurring between signaling pathways [28,29]. However, poorly understood features regarding crosstalk dependent on signaling pathways that can activate or inhibit some other pathways and their specific target genes, some of which have been documented in the family of fibroblast growth factors (FGFs) relative to receptors (FGFR), but also in relation to growth factors like TGF-β1 and Wnt [30-32], must be considered.

On the other hand, looking for one of the major transcription factors involved in the regulation of genes of the ECM metabolism, ETS-1 was chosen [17]. It was upregulated by FGF-1/H, and their expression was significantly higher than that observed for FGF-1 or heparin alone, confirming the synergy between FGF-1 and heparin on the physiological effects stimulated by FGF-1. This phenomenon has been well documented [6,7,11-13]. ETS-1 was upregulated efficiently by FGF-1/H; this is consistent with the fact that this transcription factor may be activated by several members of the FGF family to regulate genes involved in ECM metabolism as proteinases wall as proteins [17], playing a critical role in cell

migration and tumor invasion; importantly, ETS-1 mediates the upregulation of MMP-1 expression in cultured endothelial cells [19]. Additionally, ETS-1 stimulates angiogenesis in chorioallantoic membrane in an *in vivo* model in chick through a system involving the regulation of the PI3K/AKT/MMP-1 pathway, suggesting that Ets-1 activation is a requisite for FGF-1-mediated angiogenesis *in vivo;* thus Ets-1 might be a potential target for the generation of inhibitor drugs for the treatment of FGF-dependent pathological angiogenesis such as metastatic tumors, rheumatoid arthritis and diabetic retinopathy [33,34], but also to fibrosing diseases, were it down regulates several genes, including collagen type 1 and other ECM molecules in stromal fibroblasts [17]; however, its FGF-1/H-induced upregulation in HLF requires further studies.

That FGF-1/H signaling pathways can be used as a therapeutic means to target fibrosis diseases was demonstrated by Shimbori et al. who analyzed anti-fibrogenic effects of FGF-1 on pulmonary fibrosis *in vitro* and *in vivo* through the prolonged transient overexpression of FGF-1 from adenoviral vectors (AdFGF-1) and TGF-β1 AdTGF-β1. Their results showed also that FGF-1 induces decrements in α-SMA accompanied with TBR1 downregulation, increments collagen degradation and MMP-1 expression in TGF-β1-treated cells and alveolar epithelial cells as part of the inhibition of TGF-β1 activation of the signaling pathway Smad2/3, and FGFR1 upregulation induced by FGF-1. Furthermore, these authors were able to establish an *in vivo* model of pulmonary fibrosis that was reduced by FGF-1. Moreover, it has been recognized that FGF-1 levels are increased in the serum of IPF patients relative to control subjects´ serum [8]. FGF-1 was also incremented in lungs of a rat lung fibrosis model induced by paraquat plus hyperoxia, and in human lung tissue with IPF FGF-1 colocalized with the receptor FGFR1; interestingly, FGF-1 concentration was especially higher in tissue areas with no inflammation or damage associated with fibrogenic lesions [35]. These facts may reflect FGF-1-related systemic protective antifibrogenic responses in IPF patients, though this assumption requires further examination.

At this point, it is pertinent to mention that HLF cells were used instead of fibroblasts derived from human lungs with IPF because they constitute a reliable surrogate model for the study of this ailment. In this regard, we have shown that lung fibroblasts with IPF contain 62.8 ± 25.2 (n=5) α-SMA positive cells, while those derived from HLF 14.8 ± 11.6 (n=5) [4]. We consider that both fibroblasts and myofibroblasts contained in these HLF cells responded to the stimulation of FGF-1, as can be seen in the various experiments shown. However, the effect seems to be most noticeable when cells were previously stimulated with recombinant TGF-β1 *in vitro*, or with the viral transfection vector AdTGF-β1, followed by AdFGF-1 *in vivo* [8]. In this regard, the assessment the expression of α-SMA *in vivo* seems to show better the fact that both cell populations actually responded to the stimulation by FGF-1 when pulmonary fibrosis was induced with bleomycin in female Sprague-Dawley rats by AdTGF-β1, and reversed with AdFGF-1. This evidence confirms that both cell populations respond *in vitro* and *in vivo,* further corroborating the antagonism between FGF-1 and TGF-β1.

Regarding the use of other anti-fibrotic agents against IPF, another member of the FGF family, FGF-2 must be mentioned. This factor has shown a therapeutic potential very similar to that evidenced for FGF-1 in HLF, as well as in bleomycin-induced pulmonary fibrosis in mice [26,36,37]. Both factors have been reported to be increased in lungs of patients with IPF in comparison to those of normal subjects [8,38].

The therapeutic potential of FGF-1 in IPF seems promising, given the fact that the drugs currently used are nintedanib and pirfenidone, that do not stop fibrogenic progress or reverse the disease, but improve decreased lung function (FEV_1) and patient survival [39,40]. Further investigation to define the precise therapy for IPF through FGF-1 alone or in combination with other drugs that could show satisfactory therapeutic efficacy is evidently needed.

In relation to other molecules with antifibrotic potential, two inorganic salts were used at very low doses both *in vitro* in HLF cells, and *in vivo* through a bleomycin model in mice. Arsenic trioxide (ATO) and sodium arsenite (SA) showed effects similar to those of FGF-1 and some of FGF-2; they inhibit the TGF-β1-induced expression of α-SMA, of collagen type I protein and mRNA, as well as the phosphorylation of ERK and Smad2/3 [41,42]. Seemingly, these salts possess interesting therapeutic potential that could include a combination of FGF1 and/or FGF-2, together with ATO and/or SA.

Our analysis shows that the results obtained are consistent with our hypothesis, thus offering new insights on signaling pathways inactivated by FGF-1/H to upregulate MMP-1, to downregulate type I collagen and to inhibit TGF-β1-induced α-SMA expression in HLF. Characterization of the intracellular signaling pathways that participate in FGF-1-induced decrement in fibrosis may certainly prove useful for novel therapeutic targeting of fibrosing diseases such as IPF.

Conclusion

This paper describes that FGF-1/H upregulates MMP-1 principally through the JNK, MEK, and TGF-β/Smad signaling pathways and downregulates type I collagen chiefly through expression by JNK, MEK, and TGF-β/Smad pathways. In addition, FGF-1/H induces downregulation in TGF-β1-induced α-SMA expression chiefly through the JNK, MEK, and TGF-β/Smad signaling pathways, phosphorylating ERK1, AKT1, JNK1, and p-38, and upregulating transcription factor ETS-1 in fibroblasts derived from human lung.

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