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Epithelial and Mesenchymal Cell Biology

The Fibrotic Phenotype Induced by IGFBP-5 Is Regulated by MAPK Activation and Egr-1-Dependent and -Independent Mechanisms

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We have previously shown that insulin-like growth factor (IGF) binding protein- 5 (IGFBP-5) is overexpressed in lung fibrosis and induces the production of extracellular matrix components, such as collagen and fibronectin, both *in vitro* and *in vivo*. The exact mechanism by which IGFBP-5 exerts these novel fibrotic effects is unknown. We thus examined the signaling cascades that mediate IGFBP-5-induced fibrosis. We demonstrate for the first time that IGFBP-5 induction of extracellular matrix occurs independently of IGF-I, and results from IGFBP-5 activation of MAPK signaling, which facilitates the translocation of IGFBP-5 to the nucleus. We examined the effects of IGFBP-5 on early growth response (Egr)-1, a transcription factor that is central to growth factor-mediated fibrosis. Egr-1 was up-regulated by IGFBP-5 in a MAPK-dependent manner and bound to nuclear IGFBP-5. In fibroblasts from Egr-1 knockout mice, induction of fibronectin by IGFBP-5 was abolished. Expression of Egr-1 in these cells rescued the extracellular matrix-promoting effects of IGFBP-5. Moreover, IGFBP-5 induced cell migration in an Egr-1-dependent manner. Notably, Egr-1 levels, similar to IGFBP-5, were increased *in vivo* in lung tissues and *in vitro* in primary fibroblasts of patients with pulmonary idiopathic fibrosis. Taken together, our findings suggest that IGFBP-5 induces a fibrotic phenotype via the activation of MAPK signaling and the induction of nuclear Egr-1 that interacts with IGFBP-5 and promotes fibrotic gene transcription. *(Am J Pathol 2009, 175:605–615; DOI: 10.2353/ajpath.2009.080991)*

Pulmonary fibrosis is characterized by the deposition of extracellular matrix (ECM) components in the lung.[1] Histopathological features of pulmonary fibrosis include cell proliferation, production of ECM by resident fibroblasts, and formation of fibroblastic foci.[2] Recent reports suggest that cellular infiltration is another important component in the disease process.[3] Pulmonary fibrosis results in significant morbidity and mortality due to functional loss of gas exchange.[4–5] Therapeutic options in pulmonary fibrosis are limited and suboptimal.

Induction of ECM production by exogenous stimuli such as growth factors or cytokines is mediated by signal transduction cascades. The transforming growth factor (TGF)-β-Smad pathway is one of the important pathways responsible for regulating ECM production and fibrosis.[6] Non-canonical TGF-β signaling pathways, such as the p44/p42 mitogen-activated protein kinase (MAPK) pathway, also play a role in the development of fibrosis.[6–7] Since the p44/p42 MAPK pathway is activated by various triggers of ECM production,[8–12] it is not surprising that it plays an important role in the regulation of fibrosis.

ECM production is regulated, at least in part, at the level of gene transcription. Early growth response (Egr)-1 is a zinc-finger transcription factor that is up-regulated in response to TGF-β and regulates collagen gene expression.[13] Egr-1 orchestrates the cellular response to several growth factors and other exogenous stimuli[14–15] and is likely to act downstream of multiple pro-fibrotic agents to regulate transcription of fibrosis-associated genes.

Our group previously reported increased expression of insulin-like growth factor binding protein (IGFBP)-5 in primary dermal fibroblasts from patients with systemic sclerosis.[16] We also reported that IGFBP-5 mRNA and

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protein levels are increased *in vivo* in lung tissues of patients with idiopathic pulmonary fibrosis (IPF) and *in vitro* in primary fibroblasts cultured from fibrotic skin and lung tissues.[17] Interestingly, IGFBP-5 induces the production of ECM components *in vitro*,[17] and triggers a fibrotic phenotype *in vivo* that includes induction of ECM production, myofibroblastic transformation, and infiltration of mononuclear cells.[18–19] However the mechanism regulating IGFBP-5-induced fibrosis has not been delineated.

IGFBPs bind insulin growth factor (IGF)-I and modulate its function.[20] IGFBPs can also exert IGF-I-independent effects. The IGF-I-independent effects of IGFBP-5 are significant and include migration of vascular smooth muscle cells and stimulation of growth and IGF-I secretion by human intestinal muscle cells.[21–22] There is evidence that IGFBP-5 binds to a receptor on the surface of fibroblasts that likely mediates its IGF-I-independent effects. However, this putative receptor has not yet been identified.[23]

Our goal was to identify downstream mediators of IGFBP-5's fibrotic and chemoattractant activities. Our findings identify an important role for the MAPK-signaling cascade and Egr-1 in the regulation of IGFBP-5 action.

Materials and Methods

Reagents

Wild-type C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Egr-1 knockout (KO) mice were from Taconic (Hudson, NY). Dulbeccos' Modified Eagle Medium was from Mediatech (Herndon, VA). Transwell culture dishes and filters were from Corning Incorporated (Corning, NY), and 35-mm well cell culture dishes were from Costar (Cambridge, MA). Anti-IGF-I antibody was from R&D Systems (Minneapolis, MN). MAPK/ERK kinase (MEK) inhibitor U0126, anti-phospho Raf, anti-phospho MEK1/2, anti-phospho p44/p42 MAPK, anti-phospho Elk1, anti-phospho p90rsk, anti-phospho STAT3, anti-phospho Akt, anti-histone H3, and anti-total Akt antibodies were from Cell Signaling (Beverly, MA). Anti-fibronectin, anti-type I collagen α1 chain, anti-total MEK1/2, anti-total p44/p42 MAPK, anti-IGFBP-3, anti-Egr-1, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-vitronectin antibody was from AbD Serotec (Oxford, UK). Fetal bovine serum, anti-α-smooth muscle actin (SMA), and anti-β-actin antibodies were from Sigma-Aldrich (St. Louis, MO). Recombinant human IGFBP-3 and IGFBP-5 and anti-IGFBP-5 antibody were from Gropep Ltd. (Adelaide, Australia). Type I collagen was from BD Biosciences (Bedford, MA). Chemiluminescence reagents were from Perkin Elmer Life Sciences, Inc. (Boston, MA). The Aminoethyl Carbazole Substrate kit was from Zymed (San Francisco, CA). Fluorescence-conjugated or biotinylated secondary antibodies, 4,6-diamidino-2-phenylindole (DAPI), and Hematoxylin QS were from Vector laboratories (Burligame, CA). Penicillin, streptomycin, and anti-mycotic agent, TRIzol, oligo $(dT)_{12-15}$ primer, Superscript II reverse transcriptase, Protein A, and Protein G agarose were from Invitrogen Life Technologies (Carlsbad, CA).

Adenovirus Construct Preparation

Adenovirus constructs for IGFBP–5 (Ad-IGFBP-5), IGFBP-3 (Ad-IGFBP-3), and control (cAd) were obtained as previously reported[17] and used at a multiplicity of infection (MOI) of 50. Adenovirus expressing mouse Egr-1 was generously provided by Drs. Swati Bhattacharyya and John Varga at Northwestern University and used at an MOI of 25 in combination with Ad-IGFBP-5.

Primary Fibroblast Culture

Human primary lung fibroblasts were cultured under a protocol approved by the University of Pittsburgh Institutional Review Board from the explanted lungs of normal organ donors and patients with IPF undergoing lung transplant surgery, following informed consent. Mouse primary lung fibroblasts were cultured from lung tissues of C57BL/6J wild-type or Egr-1 KO mice. Fibroblasts were cultured as previously described[16] and used between passages 4 to 6. Fibroblasts were stimulated with 500 ng/ml recombinant IGFBP-5 unless indicated otherwise.

Western Blot Analysis

Western blot analysis was done using culture supernatants and cellular lysates harvested as previously described.[18] Nuclear, cytoplasmic, and ECM fractions were collected as previously described.[17,24]

Immunoprecipitation

Fibroblasts were cultured in 10-cm dishes, infected with cAd or AdIGFBP-5 for 72 hours, and scraped in 350 μl of radio-immunoprecipitation assay buffer (50 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate) containing protease inhibitor cocktail (Sigma, St. Louis, MO). Lysates were incubated with 2 μg Egr-1 antibody or control IgG at 4°C. A mixture of agarose beads conjugated with Protein A and Protein G was added for an additional 4 hours. Bound complexes were resuspended in 50 μl 2 $\times$ SDS sample buffer (125 mmol/L Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 715 mmol/L mercaptoethanol, 0.003% bromophenol blue) for Western blot analysis.

Mice and Adenoviral Administration

Administration of adenovirus to mice was done as previously described.[19] Briefly, 8-week old wild-type C57BL/6J mice were intratracheally injected with PBS, or 10^9 Pfu cAd or Ad-IGFBP-5 in a 55-μl volume. Mice were sacrificed 3 days after adenoviral administration. Lung tissues were fixed with 10% formalin and embedded in paraffin.

Immunohistochemistry

Six-micron sections of paraffin-embedded lung tissues were de-paraffinized and antigens retrieved using 10 mmol/L sodium citrate, pH6.0. Endogenous peroxidase was quenched using 3% H_2O_2, and endogenous biotin was blocked using the biotin blocking kit (Dakocytomation, Carpinteria, CA). Sections were blocked with 5% serum and incubated with anti-Egr-1 antibodies followed by secondary antibody. Bound secondary antibody was detected using the Aminoethyl Carbazole Substrate kit. A light hematoxylin counterstain was used to identify nuclei. Images were taken on a Nikon Eclipse 800 microscope (Nikon Instruments, Inc., Huntley, IL) using identical camera settings.

Immunocytostaining

Fibroblasts were cultured on coverslips coated with type I collagen. Cover glasses were blocked with 5% serum and incubated with anti-IGFBP-5 or anti-Egr-1 antibodies followed by secondary antibody. Bound secondary antibody was detected using the Fluorescence Avidin kit (Vector, Burlingame, CA). DAPI was used to identify nuclei. Images were taken on an Olympus Fluoview 1000 microscope (Olympus America Inc., Melville, NY) using identical camera settings. Some of the images were reconstructed using MetaMorph software (Molecular Devices Corporation, Sunnyvale, CA).

Detection of Egr-1 mRNA

Egr-1 and β-actin mRNA expression in cultured fibroblasts was examined using RT-PCR. Total RNA from cultured lung fibroblasts was extracted using TRIzol. First-strand cDNA was synthesized using oligo $(dT)_{12-15}$ primer and Superscript II reverse transcriptase. Egr-1 and β-actin mRNAs were detected by PCR using cDNA (50 ng total RNA equivalent) as a template. Primer sets were forward: 5′-TGACCGCAGAGTCTTTTCCT-3′, reverse: 5′-GATGAGCTGGGACTGGTAGC-3′ to amplify Egr-1 (650 bp), and forward: 5′-ATGTTTGAGACCTTCAACAC-3′, reverse: 5′-CACGTCACACTTCATGATGG-3′ to amplify β-actin (494 bp). PCR products were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

Migration Assay

The chemoattractant activity of IGFBP-5 was assessed using splenocytes[19] and primary lung fibroblasts from wild-type and Egr-1 KO mice. Chemoattractant activity was assessed in a 24-well Trans-well cell culture dish with 5-μm pore-size polycarbonate filters for splenocytes and 8-μm pore size filters for fibroblasts. Splenocytes or fibroblasts were resuspended in RPMI1640 or Dulbeccos' Modified Eagle Medium, respectively, supplemented with 1% bovine serum albumin, and 2×10^5 splenocytes or 5×10^4 fibroblasts were applied to the upper compartment of each chamber. Recombinant IGFBP-5, or vehicle (10 mmol/L HCl) were diluted in medium supplemented with 1% bovine serum albumin and added to the lower compartment. For splenocytes, after a 4-hour-incubation at 37°C, cells were harvested from both upper and lower chambers and manually counted under a phase contrast microscope. The percentage of migrated cells was calculated as a ratio of the cell count of the lower chamber to the total cell count of the upper and lower chambers. For fibroblasts, after a 6-hour-incubation at 37°C, cells penetrating through the filter pores and adhering to the bottom side of the membrane were defined as migrated cells, stained with hematoxylin, and counted.

Statistical Analysis

Statistical comparisons were performed using the Mann-Whitney U-test as appropriate.

Results

IGFBP-5 Activates the MAPK Pathway in Primary Human Lung Fibroblasts

IGFBP-5 has been shown to activate p44/p42 MAPK in mouse and human osteoblasts and smooth muscle cells.[22,25] To examine the effect of IGFBP-5 on intracellular signaling in primary human lung fibroblasts, we examined MAPK activation. As shown in Figure 1A, IGFBP-5 stimulates MAPK signaling as early as 5 minutes, peaks at 10 minutes, and then gradually decreases. We also examined upstream and downstream components of MAPK signaling. V-raf-1 murine leukemia viral oncogene homolog 1, member of ETS oncogene family, ribosomal protein S6 Kinase (p90rsk), and STAT3 were also phosphorylated in response to IGFBP-5 with kinetics similar to those of p44/p42 MAPK activation. We then compared the effects of IGF-I and IGFBP-5 (Figure 1B). IGF-I stimulated both the MAPK and PI3K pathways, whereas IGFBP-5 only induced activation of the MAPK pathway, suggesting that IGFBP-5 may exert effects independent of IGF-I. To further examine IGF-I-independent activities of IGFBP-5 signaling, we blocked paracrine/autocrine IGF-I using IGF-I neutralizing antibody. The neutralizing activity of the anti IGF-I antibody was first confirmed in primary fibroblasts (Figure 1C) and was consistent with the manufacturer reported neutralizing dose ND_{50} of 2 to 5 μg/ml. IGFBP-5-induced MAPK pathway activation was not blocked by IGF-I neutralization, showing that IGFBP-5-induced MAPK activation is IGF-I-independent (Figure 1D).

IGFBP-5-Induced ECM Expression is Regulated by the MAPK Pathway

We previously reported that IGFBP-5 induces ECM production and myofibroblastic transformation *in vitro* and *in vivo*.[18–19] To identify the signaling cascade mediating the effects of IGFBP-5 on ECM production, we blocked

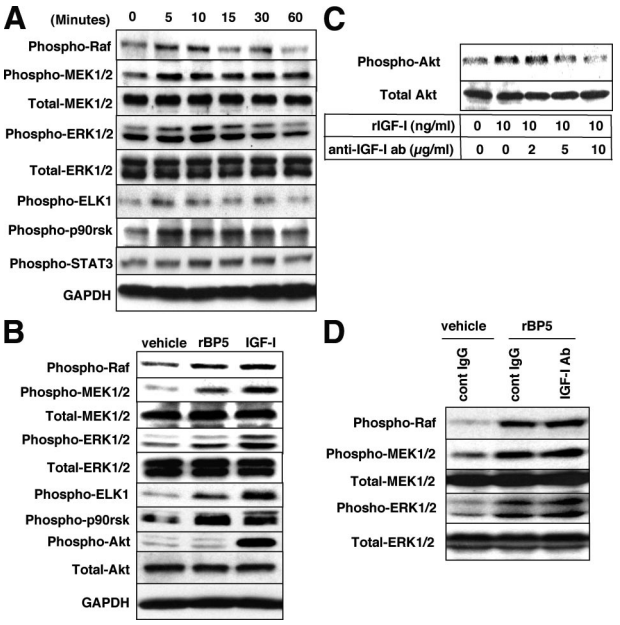

Figure 1. Activation of intracellular signaling cascades induced by IGFBP-5. **A:** Fibroblasts were stimulated with 500 ng/ml recombinant IGFBP-5. Lysates were analyzed using Western blot. **B:** Fibroblasts were treated with 500 ng/ml IGFBP-5 or 50 ng/ml IGF-I for 10 minutes and protein phosphorylation was examined by Western blot. **C:** Fibroblasts were treated with 10 ng/ml IGF-I and increasing amounts of anti-IGF-I antibody. Lysates were harvested 10 minutes post-treatment. **D:** Fibroblasts were stimulated with 500 ng/ml IGFBP-5 for 10 minutes following a 1-hour pretreatment with anti-IGF-I antibody or rabbit IgG. MAPK activation was assessed using Western blot.

MAPK signaling using the MEK inhibitor U0126. As shown in Figure 2A, IGFBP-5-mediated induction of fibronectin, type I collagen α1 chain, and SMA expression was blocked by MEK inhibition. We further examined if induction of ECM production by IGFBP-5 is IGF-I-independent. Paracrine/autocrine IGF-I was blocked using IGF-I neutralizing antibody and fibroblasts were stimulated

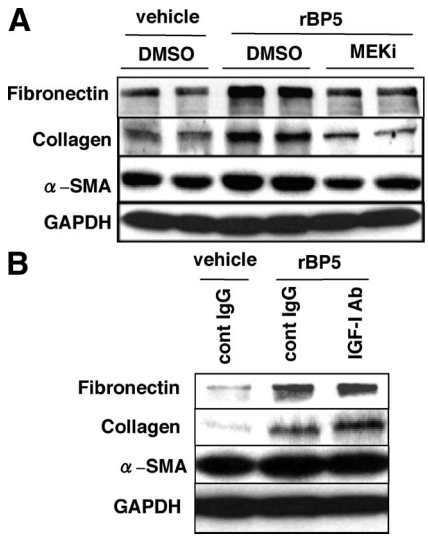

Figure 2. IGFBP-5 induces ECM and α-SMA production in a MAPK-dependent and IGF-I-independent manner. **A:** Fibroblasts were stimulated with 500 ng/ml IGFBP-5 or vehicle following a 1-hour incubation with MEK inhibitor U0126 or DMSO as a vehicle control. **B:** Fibroblasts were treated as in A following a 1 hour incubation with neutralizing anti-IGF-I antibody. ECM and α-SMA expression were analyzed by Western blot.

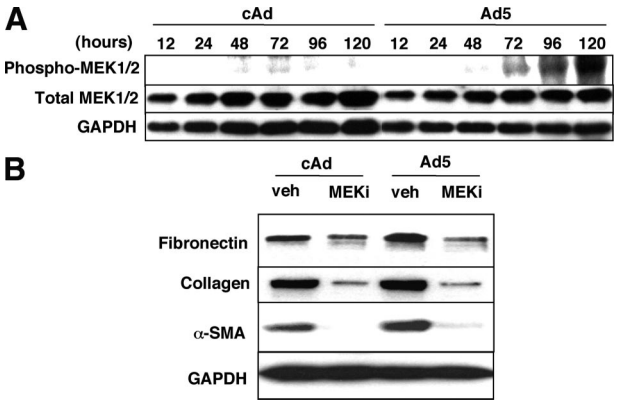

Figure 3. IGFBP-5-induced ECM and α-SMA production is regulated by the MAPK pathway. **A:** Fibroblasts were infected with Ad-IGFBP-5 (Ad5) or cAd. Activation status of MEK1/2 was analyzed by Western blot. **B:** Fibroblasts were treated as in **A** following a 1-hour incubation with 10 μmol/L MEK inhibitor U0126 (Meki) or DMSO as a vehicle control (veh). ECM and α-SMA levels were analyzed by Western blot.

with IGFBP-5. Neutralizing IGF-I activity had no effect on IGFBP-5 induction of ECM, suggesting that IGFBP-5's fibrotic activity is IGF-I independent (Figure 2B).

Endogenous Adenovirally Mediated IGFBP-5 Expression has a Similar Effect on Signaling and ECM Production as Exogenous Recombinant Protein

The endogenous versus exogenous effects of IGFBP-5 have been a subject of debate.[26] Having shown that exogenous or recombinant IGFBP-5 activates the MAPK signaling cascade, we sought to determine whether endogenously expressed IGFBP-5 exerts similar effects. Primary fibroblasts expressing adenovirally encoded IGFBP-5 were compared with control infected fibroblasts. Adenovirally expressed IGFBP-5 induced phosphorylation of MEK in a time-dependent manner (Figure 3A). As shown in Figure 3B, induction of fibronectin, type I collagen α1 chain, and α-SMA by endogenously expressed IGFBP-5 was also blocked by MEK inhibition. Thus, activation of MAPK signaling and up-regulation of ECM are comparable in Ad-IGFBP-5 and recombinant IGFBP-5 treated fibroblasts, indicating that IGFBP-5 was capable of stimulating fibroblasts in a paracrine/autocrine manner irrespective of its mode of administration.

IGFBP-5 Translocation to the Nucleus is MAPK Pathway-Independent

IGFBP-5 has been shown to translocate to the nucleus in human breast cancer cells.[27] We have reported nuclear localization of IGFBP-5 in primary fibroblasts from fibrotic lungs, which express high levels of IGFBP-5, but not in normal lung fibroblasts.[17] We therefore examined IGFBP-5 nuclear localization in primary lung fibroblasts expressing IGFBP-5 (Ad-IGFBP-5), IGFBP-3 (Ad-IGFBP-3), or control (cAd). As shown in Figure 4A, IGFBP-5 levels increased in cytoplasmic and nuclear fractions of

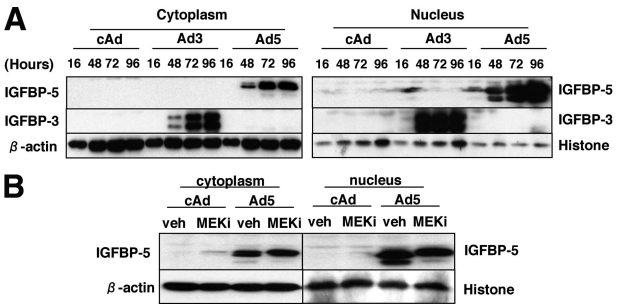

Figure 4. Nuclear translocation of IGFBP-5 is not regulated by MAPK signaling in primary human lung fibroblasts. **A:** Fibroblasts were infected with Ad-IGFBP-5 (Ad5), Ad-IGFBP-3 (Ad3), or cAd at an MOI of 50. Cytoplasmic and nuclear fractions were extracted. Expression of IGFBP-3 and -5 was examined by Western blot. β-actin is shown as a loading control for cytoplasmic extracts, and histone H3 for nuclear extracts. **B:** Fibroblasts were infected with Ad-IGFBP-5 or cAd at an MOI of 50 following pretreatment with MEK inhibitor (MEKi) or DMSO as vehicle control (veh). Cytoplasmic and nuclear fractions were extracted. Expression of IGFBP-5 was examined by Western blot.

Ad-IGFBP-5-treated cells in a time-dependent manner. Nuclear translocation of IGFBP-5 was not blocked by MEK inhibition (Figure 4B), suggesting that nuclear translocation of IGFBP-5 is not necessarily regulated by the MAPK pathway.

Egr-1 Expression is Induced by IGFBP-5 in an IGF-I-Independent MAPK-Dependent Manner

To further delineate the pathways involved in IGFBP-5-induced ECM production, we examined the role of Egr-1, a transcription factor known to orchestrate the effects of several pro-fibrotic growth factors, in mediating the ECM-promoting effects of IGFBP-5. *In vitro*, up-regulation of Egr-1 was confirmed in Ad-IGFBP-5-treated human lung fibroblasts compared with cAd-treated fibroblasts at both the mRNA (Figure 5A) and protein levels (Figure 5B). Similarly to IGFBP-5 induction of ECM production, Egr-1 expression was regulated by MAPK activation since the MEK inhibitor U0126 abrogated Egr-1 induction by IGFBP-5 (Figure 5C). Exogenous administration of physiological concentrations of IGFBP-5, similarly to endogenously expressed and secreted IGFBP-5, also induced the expression of Egr-1 (Figure 5D). Furthermore, IGFBP-5 induction of Egr-1 expression was not affected by neutralizing IGF-I activity (Figure 5D). Thus, induction of Egr-1 expression by IGFBP-5 is likely IGF-I-independent and MAPK-dependent. We also examined the expression of Egr-1 *in vivo*. Egr-1 was up-regulated in the lungs of mice treated with Ad-IGFBP-5 (Figure 5E). Egr-1 was expressed in almost all cellular components in the lung, which was anticipated since Egr-1 is a ubiquitous protein.[14] Thus, IGFBP-5 induced Egr-1 expression both *in vitro* and *in vivo*.

Increased Expression of Egr-1 in vivo *in IPF Lung Tissues and* in vitro *in Primary Fibroblasts*

Egr-1 expression was also examined in early passage primary fibroblasts cultured from lung tissues of patients with IPF. As shown in Figure 6A and B, Egr-1 levels were significantly higher in IPF lung fibroblasts, as compared with healthy donor fibroblasts (4.2-fold ± 1.0, $P < 0.05$) and paralleled IGFBP-5 expression.[17] To determine whether increased Egr-1 levels in IPF fibroblasts *in vitro* reflect levels *in vivo*, Egr-1 was detected by immunohistochemistry in lung tissues of patients with IPF and those from healthy donors. Figure 6C shows increased levels of Egr-1 *in vivo* in IPF lung tissues. Egr-1 expression was ubiquitous in IPF lungs and included areas reminiscent of fibroblastic foci (inset). Thus Egr-1 is increased *in vivo* in IPF lung and *in vitro* in primary fibroblasts derived from IPF lung tissues.

Egr-1 Translocates to the Nucleus and Co-localizes with IGFBP-5

Egr-1 is a transcription factor that localizes to the nucleus to exert its effects.[14] We determined whether Egr-1 translocates to the nucleus in response to IGFBP-5. As shown in Figure 7A, Egr-1 levels are increased in nuclear extracts of Ad-IGFBP-5-treated fibroblasts, as compared with cAd-treated fibroblasts. In contrast to IGFBP-5 nuclear translocation (Figure 4B), the translocation of Egr-1 to the nucleus was blocked by the MEK inhibitor U0126, suggesting that nuclear localization of Egr-1 is regulated

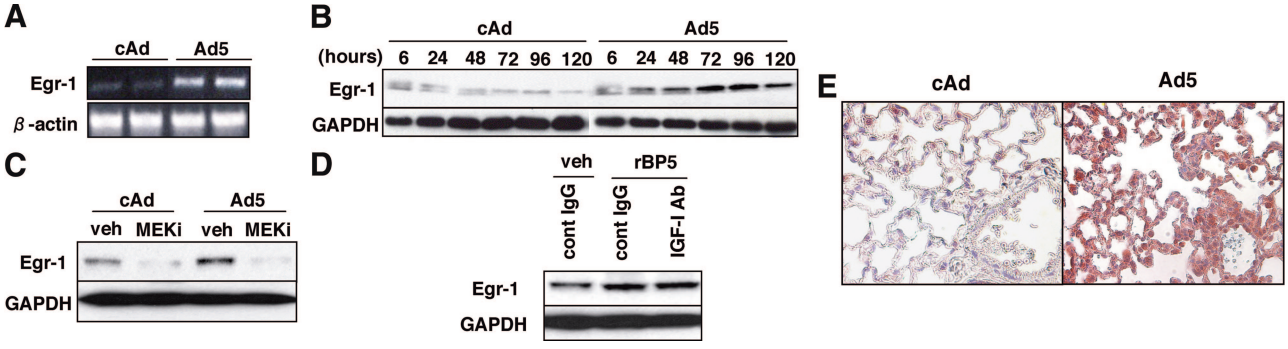

Figure 5. Egr-1 is up-regulated by IGFBP-5 in a MAPK-dependent manner. Fibroblasts were infected with Ad-IGFBP-5 or cAd. **A:** Egr-1 mRNA expression was analyzed by RT-PCR. **B:** Egr-1 protein levels were examined by Western blot in cellular lysates. **C:** Fibroblasts were incubated with 10 μmol/L MEK inhibitor U0126 (Meki) or DMSO as a vehicle control (veh) before infection. **D:** Fibroblasts were treated with IGFBP-5 or vehicle following a 1 hour-incubation with neutralizing anti-IGF-I antibody. **E:** Expression of Egr-1 was examined by immunohistochemistry in mouse lung 3 days after treatment with Ad-IGFBP-5 (Ad5) or control adenovirus (cAd). Magnification, ×400.

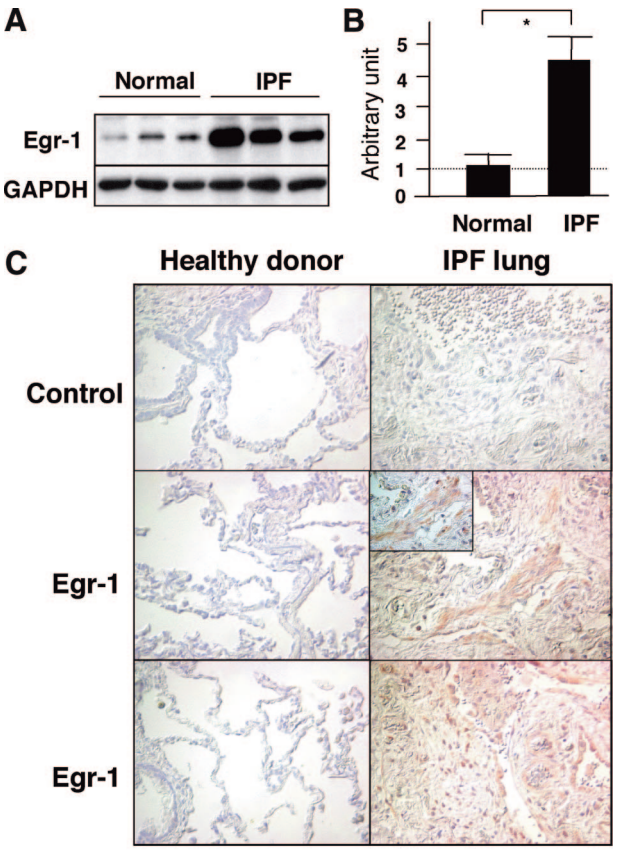

Figure 6. Increased Egr-1 expression in IPF. **A:** Egr-1 levels in primary fibroblasts from three patients with IPF and three healthy donors were analyzed by Western blot. **B:** Graphical summary of Egr-1 levels. Intensity of the bands on Western blot was analyzed using ImageJ and the ratio of Egr-1 to GAPDH was calculated. $^*P < 0.05$. **C:** Egr-1 levels were detected using immunohistochemistry in lung tissues of three patients with IPF and three normal donors. Magnification: ×400; ×800 (**Inset**).

by MAPK signaling. We further examined localization of IGFBP-5 and Egr-1 by immunofluorescence. IGFBP-5 (green) and Egr-1 (red) colocalized (yellow) in the peri- and intranuclear regions of fibroblasts (Figure 7B). Interestingly Egr-1 was also up-regulated in the nuclei of non-IGFBP-5 expressing cells suggesting that the IGFBP-5 expressed from adjacent transduced cells could exert paracrine effects (Figure 7B, arrows). To determine whether IGFBP-5 and Egr-1 interact in IGFBP-5 expressing fibroblasts, immunoprecipitation using anti-Egr-1 antibody was performed. IGFBP-5 co-precipitated with Egr-1, indicating that IGFBP-5 and Egr-1 form protein complexes (Figure 7C).

Response of Wild-Type and Egr-1 KO Mouse Fibroblasts to IGFBP-5 Stimulation

We previously reported that IGFBP-5 triggers pulmonary fibrosis in wild-type C57BL/6J mice.[19] To determine whether primary human and mouse lung fibroblasts respond similarly to IGFBP-5 stimulation *in vitro*, we expressed IGFBP-5 in primary lung fibroblasts from wild-type mice. Using immunofluorescence, we demonstrate that the distribution of IGFBP-5 and Egr-1 in mouse fibroblasts (Figure 8A and B) is comparable with that seen in human fibroblasts (Figure 7). Activation of intracellular MAPK-signaling induced by exogenous IGFBP-5 was similar in both wild-type and Egr-1 KO mouse fibroblasts (Figure 8C), suggesting that MAPK pathway activation occurs upstream of Egr-1.

Egr-1 Regulates Fibronectin Expression but not Translocation of IGFBP-5 into the Nucleus

Fibronectin is one of the important components of ECM that is regulated by IGFBP-5 *in vitro* and *in vivo*.[17–19] We examined whether IGFBP-5-induced fibronectin expression was regulated by Egr-1. As shown in Figures 9A and B, baseline and IGFBP-5-induced expression of fibronectin was suppressed in Egr-1 KO mouse fibroblasts, as compared with wild-type mouse fibroblasts. We examined the kinetics of IGFBP-5-induced nuclear translocation of Egr-1 and its induction of fibronectin production in primary mouse fibroblasts (Figure 9C). Egr-1 nuclear localization occurred as early as 24 hours and noticeably at

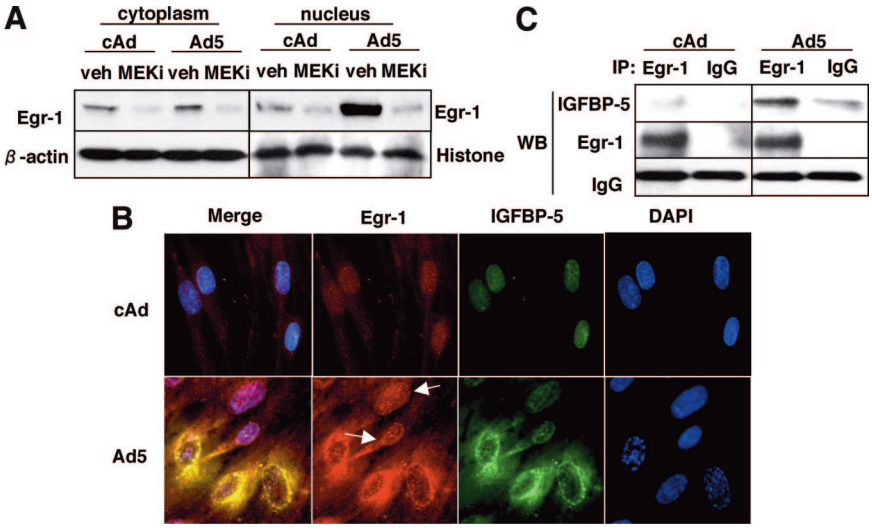

Figure 7. IGFBP-5-induced Egr-1 translocates to the nucleus in a MAPK-dependent manner, co-localizes and forms a complex with IGFBP-5. **A:** Primary fibroblasts were infected with Ad-IGFBP-5 (Ad5) or cAd following pretreatment with MEK inhibitor (MEKi) or DMSO as a vehicle (veh). Cytoplasmic and nuclear fractions were extracted after 72 hours. Egr-1 expression was examined by Western blot. β-actin is shown as a loading control for cytoplasmic extracts, and histone H3 for nuclear extracts. **B:** Expression of Egr-1 (red) and IGFBP-5 (green) was examined by immunofluorescence. DAPI was used to identify nuclei. **Arrows** show paracrine induction of Egr-1 in non-IGFBP-5-expressing cells. Magnification: ×1200. **C:** Protein-protein interaction between IGFBP-5 and Egr-1 was examined by immunoprecipitation with anti-Egr-1 antibody or rabbit IgG (IgG).

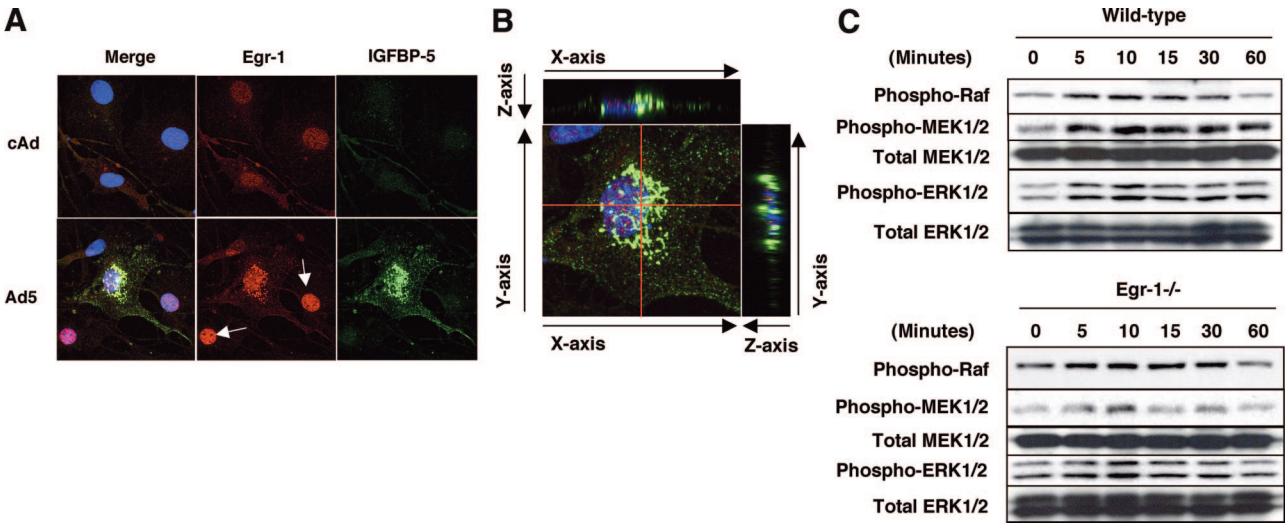

Figure 8. IGFBP-5 exerts similar effects in primary mouse and human lung fibroblasts. **A:** IGFBP-5 co-localizes with Egr-1 and IGFBP-5 induces Egr-1 in non-IGFBP-5-expressing mouse fibroblasts in a paracrine manner. Expression of Egr-1 (red) and IGFBP-5 (green) was examined by immunofluorescence. DAPI was used for nuclear counterstaining. **Arrows** show paracrine induction of Egr-1 in non-IGFBP-5-expressing cells. Images were taken at ×1200 magnification on a confocal microscope. **B:** IGFBP-5 and Egr-1 co-localize in the peri- and intranuclear cellular regions. Images were reconstructed with X-Z and Y-Z axis using MetaMorph. Yellow signal denotes co-localization. Images were taken at ×2000 on a confocal microscope. **C:** IGFBP-5 activates MAPK signaling in wild-type and Egr-1 KO mouse lung fibroblasts. Fibroblasts were stimulated with 500 ng/ml recombinant IGFBP-5. Protein phosphorylation was analyzed by Western blot.

48 hours after Ad-IGFBP-5 infection, while fibronectin production increased at 72 hours. Thus, the nuclear translocation of Egr-1 preceded the increase in fibronectin levels. These data parallel the kinetics observed in primary human lung fibroblasts (Figure 5B) and further support our data indicating that IGFBP-5 induces fibronectin via Egr-1-dependent pathways. We also examined the translocation of IGFBP-5 in Egr-1 KO mouse fibroblasts. Nuclear translocation of IGFBP-5 was not blocked in Egr-1 KO fibroblasts (Figure 9D), suggesting that ECM production and nuclear shuttling of IGFBP-5 occur via Egr-1-dependent and independent pathways, respectively. To determine whether expression of Egr-1 in Egr-1 KO fibroblasts can rescue IGFBP-5 induction of fibronectin, we infected Egr-1 KO fibroblasts with an adenovirus expressing mouse Egr-1, followed by an infection with Ad-IGFBP-5 (Figure 9E). Our data show that IGFBP-5 induction of fibronectin and its deposition in the ECM can be rescued in Egr-1 KO cells upon expression of Egr-1, thus restoring the ECM-promoting effects of IGFBP-5. These data further support the dependence of IGFBP-5 on Egr-1 as a mediator of fibronectin induction.

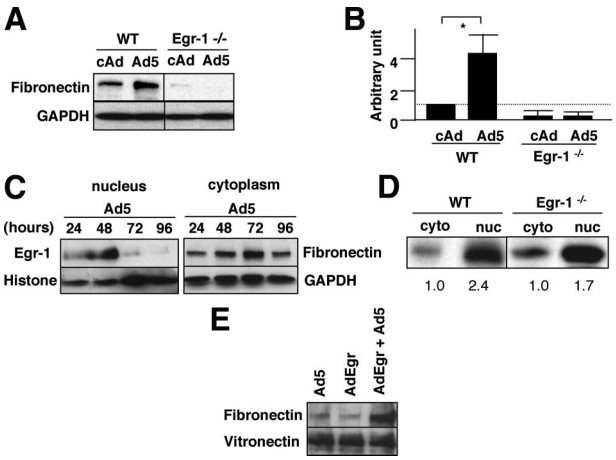

Figure 9. IGFBP-5's effects are Egr-1-dependent and -independent. **A:** Fibronectin induction by IGFBP-5 is abolished in Egr-1 KO mouse fibroblasts. Fibroblasts were infected with Ad-IGFBP-5 (Ad5) or cAd. Fibronectin expression was analyzed by Western blot. **B:** Graphical summary from three independent experiments. Intensity of the bands on Western blot was analyzed using ImageJ and the ratio of fibronectin to GAPDH was calculated; $^{*}P < 0.05$. **C:** Fibroblasts were infected with Ad-IGFBP-5, and cytoplasmic and nuclear fractions were extracted at 24 to 96 hours. Expression of Egr-1 (nuclear) and fibronectin (cytoplasmic) were examined by Western blot. Histone and GAPDH were used as loading controls in nuclear and cytoplasmic extracts, respectively. **D:** Fibroblasts were infected with Ad-IGFBP-5. Cytoplasmic and nuclear fractions were extracted after 72 hours. Expression of IGFBP-5 was analyzed by Western blot. Signal intensity was quantified using ImageJ. The number denotes ratio of IGFBP-5 in nuclear extract to that in cytoplasmic extract. **E:** Egr-1 KO lung fibroblasts were infected with Ad-IGFBP5, Ad-Egr-1, or both at a multiplicity of infection of 25 each. Extracellular matrix was harvested after 72 hours. Fibronectin deposition was assessed in the extracellular matrix using Western blot. Vitronectin served as a loading control.

IGFBP-5-Induced Cell Migration is Regulated by Egr-1

We have previously demonstrated prominent infiltration of mononuclear cells in IGFBP-5 expressing mouse lungs.[19] This was due, in part, to induction of cell migration by IGFBP-5.[19] To determine whether Egr-1 modulates the migratory activity of IGFBP-5, we compared the migration of fibroblasts and splenocytes from wild-type and Egr-1 KO mice. IGFBP-5-treated wild-type fibroblasts exhibited significantly increased migration (Figure 10A and B). On the other hand, the migratory activity of Egr-1 KO mouse fibroblasts in response to IGFBP-5 was suppressed (Figure 10B). A similar tendency was observed when splenocytes from wild-type and Egr-1 KO mice were used in migration assays (Figure 10C). The lack of migration of Egr-1 KO cells in response to IGFBP-5 suggests that Egr-1 mediates the chemoattractant activity of IGFBP-5.

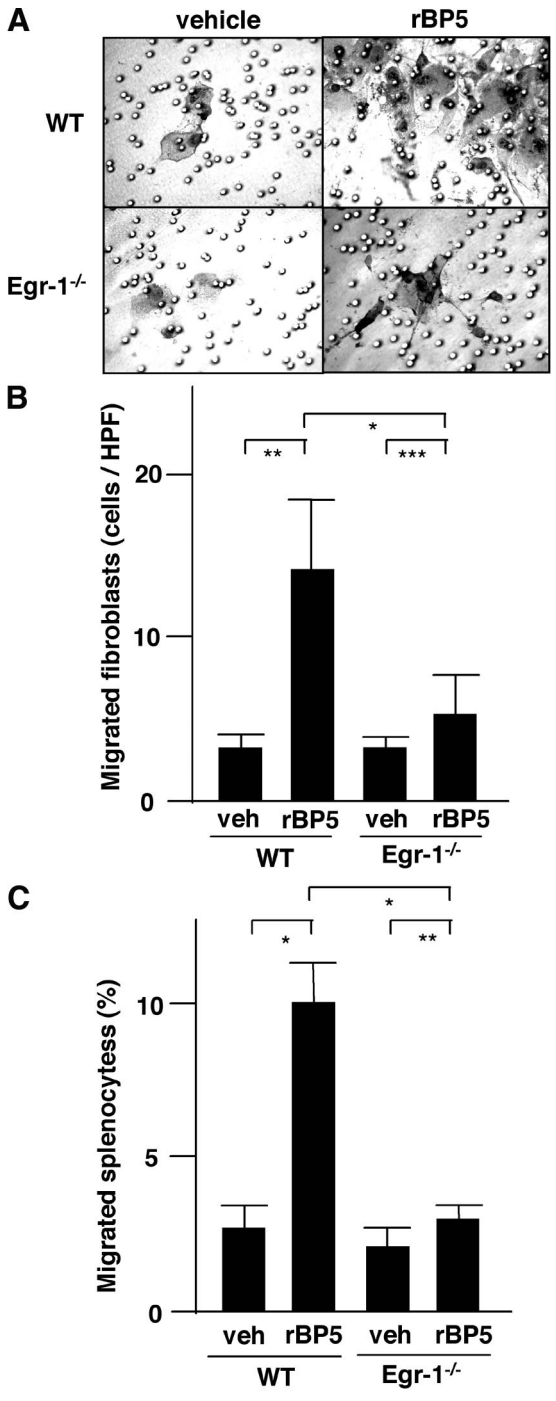

Figure 10. IGFBP-5 induces migration of mouse lung fibroblasts and splenocytes in an Egr-1-dependent manner. **A:** Primary mouse lung fibroblasts from wild-type and Egr-1 KO mice were used in migration assays with a vehicle control (veh) or recombinant IGFBP-5 (rBP5). **B:** Migration of lung fibroblasts from wild-type and Egr-1 KO mice. Data represent mean +SD of 15 independent fields. $^{*}P < 9.0 \times 10^{-4}$, $^{**}P < 5.0 \times 10^{-5}$, $^{***}P < 0.05$. **C:** Migration of splenocytes from wild-type and Egr-1 KO mice. Data represent mean + SD of six independent experiments. $^{*}P < 0.003$, $^{**}P < 0.006$.

Discussion

We have previously reported the induction of a fibrotic phenotype by IGFBP-5 *in vitro* in human fibroblasts and *in vivo* in fibrotic skin and lung tissues.[17–19] In IGFBP-5-expressing mouse lungs, fibrosis was prominent in the interstitial areas.[19] However, the mechanism regulating IGFBP-5-induced fibrosis has not been delineated. In this study, we identified the intracellular events mediating IGFBP-5 effects. IGFBP-5 activated the MAPK pathway and induced ECM and α-SMA production in an IGF-I-independent manner. IGFBP-5 also translocated to the nucleus of fibroblasts in a MAPK-independent manner. We also examined the effects of IGFBP-5 on Egr-1, a master transcription factor that mediates the pro-fibrotic effects of several growth factors, and the role of Egr-1 in mediating the ECM-promoting effects of IGFBP-5. Egr-1 was induced by endogenous IGFBP-5 expression and exogenous administration of recombinant protein in a MAPK-dependent manner. Egr-1 levels were also increased in primary lung fibroblasts from patients with IPF and colocalized with and bound IGFBP-5 in and around the nucleus. Both baseline and IGFBP-5-induced fibronectin expression were blocked in Egr-1 KO fibroblasts, but fibronectin expression was rescued by overexpressing Egr-1. IGFBP-5 activated MAPK signaling independently of Egr-1. Moreover, IGFBP-5 induced migration of wild-type mouse fibroblasts and splenocytes was abrogated in Egr-1 KO cells. Taken together, our findings suggest that the fibrotic phenotype induced by IGFBP-5 occurs in a MAPK-dependent and an IGF-I-independent manner, and that Egr-1 mediates IGFBP-5 induction of fibronectin and cell migration.

Studies examining signaling cascades activated by IGFBP-5 have been limited to mouse and human osteoblasts and smooth muscle cells.[22,25] In these cells, IGFBP-5 activates p38 kinase and p44/p42 MAPK.[22,25] Our results suggest that IGFBP-5 exerts its fibrotic effects on fibroblasts through MAPK activation, independently of IGF-I. Thus, MAPK activation by IGFBP-5 can now be added to the list of IGF-I-independent IGFBP-5 effects, which include stimulation of growth and IGF-I secretion by human intestinal smooth muscle cells,[22] migration of porcine vascular smooth muscle cells,[21] proliferation and activation of osteoblasts[28–29] and osteoclasts,[30] and more recently induction of cellular senescence[31] and formation of hair shaft medulla.[32]

IGFBP-5 contains a nuclear localization sequence, which targets the protein to the nuclear compartment. IGFBP-5 nuclear localization has been reported in human breast carcinoma cell lines,[27] Chinese hamster ovary cells,[33] and porcine vascular smooth muscle cells.[34] We previously reported nuclear localization of IGFBP-5 in primary fibroblasts from fibrotic lung tissues, but not those from normal tissues.[17] We now show that IGFBP-5 translocates to the nucleus of IGFBP-5-expressing cells in a time-dependent manner. Nuclear compartmentalization of IGFBP-5 may mediate its recently identified transactivation function[35] and induce expression of ECM genes via transcription factors such as Egr-1.

In our study, the MAPK pathway was activated by IGFBP-5 and mediated IGFBP-5-induced ECM production. We propose that the MAPK signaling cascade is specifically activated by IGFBP-5. The MAPK pathway is also activated by several other known mediators of fibrosis. Previous reports show that connective tissue growth

factor is induced by TGF-β and endothelin-1 via activation of p44/p42 MAPK signaling and endothelin-1 stimulates ECM production via p44/p42 MAPK activation.[8–9] Moreover, the TGF-β receptor type I-dependent fibrogenic gene program is mediated via activation of p44/p42 MAPK signaling.[10] Activation of p44/p42 MAPK signaling was recently shown to mediate adenosine A2A receptor-induced fibrosis.[11] The role of other, possibly autocrine and/or paracrine, mediators of ECM production that depend on MAPK is supported by data shown in Figure 3B, where MAPK inhibitor alone was able to decrease baseline production of ECM. These factors likely sustain ECM production via activation of the MAPK signaling cascade. An alternate explanation is that the concomitant use of adenoviruses and MAPK chemical inhibitors can exert nonspecific effects on ECM production. This is not likely to be a confounding factor when recombinant proteins are used as stimuli since we have recently reported that the same chemical inhibitor, U0126, does not block IGF-II stimulation of ECM production.[36] The role of MAPK activation as a mediator of IGFBP-5 effects is also supported by our recent findings demonstrating that IGFBP-5 triggers the migration of peripheral blood mononuclear cells in an IGF-I-independent MAPK-dependent manner.[37] Collectively, these observations suggest that the MAPK pathway is a convergent pathway for multiple mediators of fibrosis, including IGFBP-5.

We show that IGFBP-5 induces Egr-1 expression. Egr-1 was induced not only in IGFBP-5-expressing cells, but also in neighboring non-IGFBP-5-expressing cells, supporting a paracrine role for IGFBP-5. Similar paracrine activity has been described for other growth factors such as fibroblast growth factor-2, which also induces Egr-1 via activation of MAPK signaling in a paracrine manner.[38] Egr-1 was first discovered as an immediate-early gene induced in growth-quiescent fibroblasts on addition of serum.[39] Egr-1 orchestrates transcriptional responses in fibrosis and inflammation and induces or represses at least 80 different genes, including platelet-derived growth factor and TGF-β.[40–41] Egr-1 is activated by multiple extracellular stimuli such as growth factors, cytokines, and environmental stresses.[15] It is conceivable that the fibrotic phenotype triggered by IGFBP-5 is enhanced or maintained as a result of up-regulation of other pro-fibrotic growth factor genes via Egr-1. Our studies demonstrate nuclear localization of IGFBP-5 and Egr-1 and the formation of complexes. Although we have not directly demonstrated that IGFBP-5/Egr-1 complexes are functional, IGFBP-5, which exerts IGF-independent transcriptional regulatory activity,[35] likely promotes Egr-1 mediated transcription of downstream genes associated with fibrosis via its transactivating domain.

We have shown that IGFBP-5-induced Egr-1 expression and localization to the nucleus are regulated by MAPK activation. Egr-1 serves as a convergence factor for multiple signaling cascades and, similarly to IGFBP-5, has a nuclear localization signal.[42] Activation of p44/p42 MAPK plays an important role in the accumulation of Egr-1 in the nucleus.[43] We have also shown that IGFBP-5 induces fibronectin production via activation of MAPK signaling and Egr-1. These findings are supported by those of Gaggioli et al who demonstrated that hepatocyte growth factor-induction of fibronectin in melanoma cells occurs in a MAPK-Egr-1-dependent fashion.[44] The fibronectin gene is also transactivated by Egr-1 in human glioblastoma cells.[45] IGFBP-5 is a novel pro-fibrotic factor and IGFBP-5-induced Egr-1 production and the resulting fibrotic phenotype are regulated by MAPK signaling. Thus, the activation of MAPK signaling and induction of Egr-1 appear to be an important pathway for fibrosis involving ligands such as IGFBP-5 and TGF-β.[13]

Sustained increased Egr-1 levels have been reported in chronic lung diseases such as emphysema[46] and chronic obstructive pulmonary disease.[47] We recently reported increased Egr-1 in fibrotic lung tissues of patients with systemic sclerosis.[48] Additionally, Egr-1 mRNA and protein expression is increased in skin fibroblasts from patients with diffuse systemic sclerosis[49] and *in vivo* in systemic sclerosis-affected skin.[13,48] Egr-1 levels are increased in synovial fibroblasts in the collagen-rich subsynovial space in patients with rheumatoid arthritis.[50] Egr-1 is also associated with wound healing in a rodent model.[51] We now show increased Egr-1 in IPF lung tissues and primary lung fibroblasts derived from them. It is possible that sustained increased Egr-1 expression in IPF lung tissues and fibroblasts is one of the mechanisms responsible for the maintenance of remodeling in this and other fibrosing disorders.

The central role of Egr-1 in mediating fibrotic responses is demonstrated by the ability of Egr-1 to rescue the phenotype of Egr-1 null fibroblasts and restore the fibrotic effects of IGFBP-5 (Figure 9E). Taken together, our findings in the context of the published reports suggest that up-regulation of Egr-1 by IGFBP-5 could directly regulate transcription of ECM genes and further support the role of Egr-1 as a "master regulator" of transcription in fibrosis.

Our study demonstrates that IGFBP-5 has a chemoattractant activity for human and mouse fibroblasts. We have recently reported IGFBP-5's chemoattractant activity for human peripheral blood mononuclear cells.[19,37] IGFBP-5 has also been shown to induce migration of mouse embryonic cells,[52] porcine vascular smooth muscle cells,[21] and rat glomerular mesengial cells.[53] We and others have shown that activation of MAPK and c-Raf also plays an important role in the regulation of cell migration.[36,54–55] Thus our results combined with these reports suggest that IGFBP-5 is an important mediator of cell migration. Furthermore, we demonstrate that IGFBP-5-induced migration of fibroblasts and splenocytes requires Egr-1. The dependence of cell migration on Egr-1 has been previously suggested in TGF-β1-induced pulmonary fibrosis and parenchymal inflammatory cell accumulation.[56]

In summary, we have identified MAPK activation and Egr-1 expression as mechanisms mediating IGFBP-5-induced fibrosis. Since IGFBP-5 is overexpressed in fibrotic diseases such as IPF and systemic sclerosis,[17,18,57] newly identified downstream mediators of IGFBP-5 action may potentially serve as novel therapeutic targets to halt the progression of fibrosis.

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