Fibroblast Growth Factor 2 Augments Transforming Growth Factor Beta 1 in Inducing Epithelial-Mesenchymal Transition in Human Lung Epithelial Cells

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Abstract

Background: Epithelial-mesenchymal transition (EMT) is a critical event in wound healing and tissue repair following injury. Transforming growth factor beta-1 (TGFβ1) plays an important role in inducing EMT in lung epithelial cells in vitro and in vivo. As fibroblast growth factor-2 (FGF2) reverses TGFβ1-induced collagen I (COL1A1) and α-smooth muscle actin (Actin alpha 2; ACTA2) expression in primary mouse and human lung fibroblasts, we set out this study to determine the effect of FGF2 on TGFβ1-induced EMT in human lung epithelial cells. **Methods:** BEAS-2B and A549 cells were treated with recombinant FGF2 (2 nM) with or without TGFβ1 (2 ng/ml) for up to 4 days. The phenotypic alterations associated with EMT were assessed by quantitative real-time PCR and E-cadherin protein expression levels was assayed by western blot and immunofluorescence staining. Cell migration was confirmed using wound-healing assay. **Results:** TGFβ1 treatment led to significantly reduced expression of E-cadherin (CDH1) and markedly induced expression of mesenchymal proteins such as N-cadherin (CDH2), tenascin C (TNC), fibronectin (FN), ACTA2 and COL1A1. TGFβ1 also induced a morphological change and a significant increase in cell migration. FGF2 did not significantly alter EMT gene expression markers on its own, however enhanced TGFβ1-induced suppression of CDH1 and upregulation of ACTA2, but did not alter TNC, FN and CDH2 gene expression levels induced by TGFβ1. FGF2 maintained TGFβ1-induced morphologic changes as well as increased the migration of TGFβ1-treated cells. Furthermore, FGF2 treatment significantly inhibited TGFβ1-induced COL1A1 expression in both BEAS-2B and A549 cells. FGFR-specific tyrosine kinase inhibitor PD173074 blocked the synergism between these two growth factors. **Conclusions:** This study suggests a synergistic effect between TGFβ1 and FGF2 in inducing EMT, which may play an important role in wound healing and tissue repair after injury. Our findings provide insight into the effects of FGF2 following lung injury and in pulmonary fibrosis.

Background

Lung epithelial cells are a frequent target of injury, and abnormalities in epithelial repair occur in many lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis, leading to accumulation of pathologic fibroblasts and myofibroblasts, collagen overproduction and tissue fibrosis [1]. Additionally, abnormalities in epithelial cell survival, apoptosis, proliferation, and migration contribute to pulmonary fibrosis [2].

Epithelial-mesenchymal transition (EMT) is a process by which differentiated epithelial cells lose their epithelial characteristics and reversibly acquire a migratory mesenchymal phenotype. EMT is characterized by a loss of polarity and loss of epithelial markers including junctional and cell-cell adhesion proteins such as zonula occludens-1 (ZO-1) and E-cadherin (CDH1), cytoskeletal reorganization, a transition to a spindle-shaped morphology, and acquisition of mesenchymal markers. Mesenchymal markers used to define EMT are α-smooth muscle actin (α-SMA), vimentin (VIM), collagen I (COL1A1), fibronectin (FN), connective tissue growth factor (CTGF), N-cadherin (CDH2), fibroblast-specific protein 1 (FSP1), the transcription factors Snail (SNAI1) and Slug (SNAI2), and matrix metalloproteinases (MMPs) [3–5].

EMT is classified into three subtypes based on different biological and functional consequences. Type I EMT occurs during organ development and is essential in gastrulation and neural crest cell migration. Type II EMT is associated with wound healing and organ fibrosis, where epithelial cells acquire a “myofibroblast” phenotype and migrate to heal injured tissues after trauma and/or inflammatory injury. If the injury is mild and acute, this process ends once the tissue is repaired and inflammation is attenuated. However, persistent EMT has been argued to promote myofibroblast differentiation leading to fibrosis [6, 7]. Type III EMT occurs in cancer metastasis, where neoplastic epithelial cells are transformed into invasive metastatic mesenchymal cells that underlie cancer progression [8, 9]. The mechanism underlying EMT is not well understood in the context of pulmonary fibrosis, and the role of EMT in the pathogenesis of different respiratory diseases such as asthma, COPD and pulmonary fibrosis is under debate [10, 11].

There are several growth factors that have been demonstrated to induce EMT in various cell types, including transforming growth factor beta-1 (TGF β 1), fibroblast growth factor-2 (FGF2), epidermal growth factor (EGF), insulin-like growth factor-2 (IGF-II), interleukin-1 (IL-1), hepatocyte growth factor (HGF), and Wnt ligands [12]. In addition, other factors that can enhance EMT include hypoxia [13, 14], high glucose, angiotensin II, albumin [15, 16], inflammatory cytokines [17] and matricellular proteins [18, 19].

TGF β 1 was first defined as a major inducer of EMT in normal mouse mammary epithelial cells *in vitro* [20], and has been implicated in mediating EMT *in vitro* in epithelial cells from kidney [21–23], eye [24, 25], and lung [26–37]. Among various FGFs, FGF2 and FGF4 are key regulators of EMT during development and cancer progression in the lung [38, 39]. It has been reported that FGF2 reduces E-cadherin in human ovarian cancer cells [40], and induces the expression of mesenchymal markers (VIM, FSP1, α -SMA and SNAI1) in corneal endothelial cells [41] and proximal tubular epithelial cells [39, 42]. A number of studies have shown the synergistic effect of combined treatment of TGF β 1 and FGF2 in inducing EMT in mouse normal mammary epithelial (NMuMG) cells [43], rat Hertwig’s epithelial root sheath (HERS) cells [44], mouse lung epithelial type II cell line MLE-12 [45], human non-small cell lung cancer cell lines NCI-H1975 and NCI-H165 [46], and human lung adenocarcinoma cell lines PC-9, HCC-827 and A549 [47, 48]. These studies only used the combination of TGF β 1 and FGF2 as profibrotic cytokines to induce type III EMT which is a key component of carcinogenesis. Therefore, inhibition of EMT induction may modify tumor progression and responsiveness to chemotherapy and/or immunotherapy for cancer. No studies have shown the synergistic effect of FGF2 and TGF β 1 in inducing type II EMT in lung epithelial cells that is associated with wound healing and tissue regeneration after injury.

We have previously shown that FGF2 is crucial for epithelial repair and recovery after bleomycin-induced lung injury in mice [49]. We have also found that FGF2 overexpression is protective against bleomycin-induced lung injury *in vivo* and inhibits TGF β 1-induced collagen I and α -SMA expression in primary mouse and human lung fibroblasts *in vitro*, suggesting that FGF2 is antifibrotic and protective against lung injury [50]. In this study we investigated the effect of FGF2 on TGF β 1-induced gene expression in both bronchial and alveolar lung epithelial cells *in vitro*. We hypothesized that FGF2 would induce EMT and may play an important role in wound healing and repair of lung epithelial cells after injury. *In vitro*, we

found that FGF2 did not inhibit the majority of TGF β 1-induced expression of EMT markers in human lung epithelial cells. This study provides insight into the potential use of FGF2 following lung injury and in pulmonary fibrosis.

Methods

Cell culture

Human virus-transformed bronchial epithelial cell line (BEAS-2B) and Human alveolar type II epithelial carcinoma cell line (A549) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA; #CRL9609, #CCL185, respectively). BEAS-2B cells were plated on pre-coated plates with a mixture of 0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I and 0.01 mg/ml bovine serum albumin (BSA) dissolved in BEBM medium and were grown in bronchial epithelial growth medium bullet kit (BEGM; Lonza, Walkersville, MD, USA). A549 cells were plated in Ham's F-12 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 U/ml). Cells were incubated in a humidified incubator at 37°C with 5% CO₂.

Epithelial-mesenchymal transition induction

To induce epithelial-mesenchymal transition, cells were plated at ~30-40% confluence in 6-well plates. After overnight culture, cells were treated with 2 ng/ml of TGF β 1 (Fisher Scientific, Fair Lawn, NJ, USA, #PHG9204), 2 nM of recombinant human low-molecular-weight FGF2 (PeproTech, Rocky Hill, NJ, USA, #100-18B) + 1 nM heparin sulfate (Fisher Scientific, #BP2524), or TGF β 1 + FGF2 + heparin for 4 days in complete medium. Medium with or without treatments was changed after 48 hours. The experiments were designed so that the cells reached confluence one day prior to harvesting and were conducted independently 3-6 times each in duplicate.

EMT assay in the presence of FGFR-specific tyrosine kinase inhibitor

BEAS-2B cells were incubated with TGF β 1 (2 ng/ml) alone, FGF2 (2 nM) alone, PD173074 (0.1 μ M, Cayman Chemical, Ann Arbor, MI, USA, #13032) alone or FGF2 (2 nM) + TGF β 1 +/- PD173074 for 4 days prior to collection of RNA.

RNA isolation and quantitative real-time PCR

Cells were lysed in RLT buffer and total RNA was prepared from the cells using the RNeasy plus mini kit (Qiagen, Valencia, CA, USA, #74136) according to the manufacturer's instructions. RNA concentration was determined utilizing a Nanodrop spectrophotometer. cDNA was made using the BioRad iScript Reverse Transcription Supermix for RT-qPCR kit (BioRad, Hercules, CA, USA, #170-8841). Quantitative RT-PCR was performed on an Applied Biosystems StepOne thermocycler using ABI Taqman® Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA, #4444557) and Taqman® gene expression assays.

All samples were normalized to *GAPDH* and then scaled relative to controls using the standard delta-Ct (Δ Ct) method. Data are reported as fold change over control.

Protein isolation and immunoblotting

Protein was extracted from cultured epithelial cells in radioimmunoprecipitation assay (RIPA) lysis buffer with freshly added 2% Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA, #P8340) and Phosphatase Inhibitor Cocktail I and II (Sigma-Aldrich, #P2850 and #P5726, respectively). Protein concentration was determined utilizing a Pierce Bicinchoninic acid (BCA) assay kit (Thermo Scientific, Rockford, IL, USA, #23225). Total protein (20-40 μ g) was separated on 4-20% polyacrylamide gels (BioRad) and transferred to PVDF membranes using a Trans-Blot Turbo transfer system (BioRad). Membranes were blocked for one hour at room temperature with gentle shaking in Tris-buffered saline with Tween-20 (TBST; 50 mM Tris, pH7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% BSA, and then probed with primary antibodies against E-Cadherin (BD Transduction Laboratories, Lexington, KY, USA, #610181) overnight at 4°C. Immunoblotting for β -tubulin (Abcam, Cambridge, MA, USA, #ab6046) was used as loading control. After three rinses in TBST, membranes were incubated for one hour at room temperature in horseradish peroxidase-linked secondary antibodies in TBST with 5% nonfat milk, rinsed again in TBST, and developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, #34096) or Pico (Thermo Scientific, #34580).

Migration assay

BEAS-2B cells were cultured in 6-well plates and grown in complete media. Cells were ~90% confluent at the time of scratch wounding. Five 1 mm diameter circular wounds were created using a custom-made rubber tool [51]. The non-adherent cells washed off with medium and fresh medium with the same treatments as described previously was added to the wells. The wound closure was measured immediately after scratch wounding (0 h), at 24 h, 48 h and 72 h or measured at 0 h, 24 h, 40 h and 48 h after the cells were treated for 3 days prior to wounding. Phase contrast light microscope pictures were taken using a digital camera attached to an inverted-stage microscope (Nikon, Morton Grove, IL). Wound areas were measured using Image J (NIH), and values were normalized to time = 0 values. Experiments were conducted independently 3 times each in triplicate.

Immunofluorescence staining for E-cadherin

BEAS-2B cells were grown on coated glass coverslips and stimulated with the same treatments for 4 days as described above. Cells were fixed with 4 % paraformaldehyde for 10 minutes at room temperature. After washing with ice-cold PBS, cells were blocked in 0.1% BSA and 5% serum prepared in PBS + 0.2% Tween-20 for 1h at room temperature. Coverslips were stained with monoclonal mouse anti-E-cadherin antibody (BD Transduction Laboratories, #610181) in a dilution of 1:50 made in blocking buffer and incubated overnight at 4°C, followed by the secondary antibody (goat anti-mouse conjugated with Alexa488Abcam, #ab150113) in a dilution of 1:500. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Scientific, #D1306) and coverslips mounted with SlowFade™ Gold Antifade

Mountant (Thermo Scientific, #S36936). Images were captured with 3i Marianas Yokogawa-type spinning disk confocal microscope and the two channels merged using Image J software.

Statistical Analysis

The data showed the mean $\pm$ standard deviation and the significant differences in mean values were determined using one-way ANOVA. A *p*-value of less than 0.05 was considered to be significant. Statistical analysis was performed using GraphPad Prism software.

Results

Recombinant FGF2 does not alter morphological changes in bronchial epithelial cells induced by TGF β 1.

Addition of FGF2 to BEAS-2B cells did not alter their cobblestone-like morphology (**Figure 1A, B**). Upon treatment with TGF β 1, cells developed a fibroblast-like shape (**Figure 1C**). Compared to cells treated with TGF β 1 alone, the addition of FGF2 maintained the fibroblast-like shape of BEAS-2B cells (**Figure 1 D**).

Recombinant FGF2 enhances TGF β 1 induced EMT-related gene expression. To determine the minimal concentration of TGF β 1 and the time point required to induce EMT in BEAS-2B cells, BEAS-2B cells were treated with 2 ng/ml or 5 ng/ml of TGF β 1 for 3, 4 and 5 days and total RNA was collected. Expression of *CDH1*, *ACTA2*, and *COL 1A1* mRNA were then assessed by qRT-PCR. We found that 2 ng/ml was sufficient to repress *CDH1* and induce *ACTA2* and *COL 1A1*, but *ACTA2* started to be only detectable after 4 days of treatment (data not shown). We then determined whether the addition of FGF2 to BEAS-2B cells leads to altered gene expression. BEAS-2B cells were treated for 4 days with FGF2 (2 nM), TGF β 1 (2 ng/ml), or TGF β 1 + FGF2. FGF2 treatment alone led to a non-significant decrease in *CDH1* (*P* value = 0.2225) and a non-significant increase in both *ACTA2* (*P* value = 0.2239) and *CDH2* (*P* value = 0.7515) when compared to control. We observed a significant decrease in *CDH1* (*P* value = <0.0001) (**Figure 2A**) and a significant increase in *ACTA2* (*P* value = 0.0142) (**Figure 2B**) and *CDH2* (*P* value = 0.0065) (**Figure 2C**) mRNA expression levels after treating BEAS-2B cells with TGF β 1. Addition of FGF2 to TGF β 1 resulted in a further significant decrease in *CDH1* expression (*P* value = 0.0470) (**Figure 2A**) and significant increase in *ACTA2* (*P* value = 0.0160) compared to TGF β 1 treatment alone (**Figure 2B**). Addition of FGF2 to TGF β 1 did not alter expression of *CDH2* (*P* value = 0.9950) induced by TGF β 1 alone (**Figure 2C**).

To confirm these mRNA changes, we assessed the effects of TGF β 1 and FGF2 on E-cadherin protein levels in BEAS-2B cells. Immunoblotting of total cell lysates obtained after 4 days of incubation with TGF β 1, FGF2, or TGF β 1 + FGF2 demonstrated that E-cadherin protein levels were not significantly altered by FGF2 treatment alone and were significantly decreased (*P* value = 0.0012) in response to TGF β 1 (**Figure 3A, B**). Addition of FGF2 to TGF β 1 led to further suppression of E-cadherin (**Figure 3A, B**). Immunofluorescence for E-cadherin revealed a loss of grid-like localization of E-cadherin at the cell-cell contact surface following FGF2 treatment and further loss of cell-cell contact induced by TGF β 1 that was not altered by the addition of FGF2 (**Figure 3C**).

FGF2 inhibits TGFβ1-induced collagen, but not fibronectin or tenascin C. We then tested whether FGF2 has an effect on expression of extracellular matrix (ECM) proteins such as fibronectin (*FN*), tenascin C (*TNC*) and collagen I (*COL 1A1*). BEAS-2B cells were treated with FGF2, TGFβ1, or TGFβ1 + FGF2 for 4 days prior to total mRNA collection. Treatment with FGF2 alone did not significantly alter expression of *FN*, *TNC*, or *COL 1A1*, but TGFβ1 treatment led to a highly significant induction of each of these genes (P values = <0.0001) (**Figure 4A-C**). FGF2 treatment had no effect on TGFβ1-induced expression of *FN* (**Figure 4A**) and *TNC* (**Figure 4B**), but interestingly there was a significant decrease in the expression of *COL 1A1* (P value = 0.0008) (**Figure 4C**) compared to TGFβ1 treatment alone unlike other EMT genes studied in this report.

Recombinant FGF2 has similar effects on EMT gene expression induced by TGFβ1 in A549 cells. We then tested whether the effect of FGF2 on TGFβ1-induced gene expression in BEAS-2B cells was unique to bronchial epithelial cells or conserved in multiple epithelial cell types in the lung. To do this we treated the alveolar epithelial A549 cells with FGF2, TGFβ1, or TGFβ1 + FGF2 for 4 days prior to collection of total mRNAs. We observed that FGF2 enhanced TGFβ1-induced downregulation of *CDH1* (P value = <0.0001) (**Figure 5A**) and upregulation of *ACTA2* (P value = 0.0013) (**Figure 5B**) compared to control. In addition, FGF2 suppresses TGFβ1-induced *COL 1A1* mRNA expression (P value = 0.0011) in A549 cells compared to TGFβ1 treatment alone similar to what was observed in BEAS-2B cells (**Figure 5C**).

Effect of FGF2 on TGFβ1-treated cells is inhibited by PD173074 in both types of epithelial cells. To confirm that the effect of FGF2 is mediated by FGF receptor (FGFR) signaling, BEAS-2B and A549 cells were treated with the FGFR-specific tyrosine kinase inhibitor PD173074 (0.1 μM) in combination with FGF2, TGFβ1, or TGFβ1 + FGF2. We found that PD173074 significantly blocked the effect of FGF2 on TGFβ1-induced repression of *CDH1* (P value = 0.0002) (**Figure 6A**) and induction of *ACTA2* (**Figure 6B**), and reversed FGF2-mediated inhibition of TGFβ1 induction of *COL 1A1* expression non-significantly (P value = 0.8644) in BEAS-2B cells (**Figure 6C**). Similarly, PD173074 inhibited FGF2 effect on TGFβ1-induced EMT in A549 cells significantly for *CDH1* (P value = <0.0001) (**Figure 6D**) and non-significantly for *ACTA2* (P value = 0.5619) (**Figure 6E**) and *COL 1A1* (P value = 0.0641) (**Figure 6F**). PD173074 alone did not alter the expression of the above genes in both types of epithelial cells.

FGF2 promotes epithelial cell migration alone or in combination with TGFβ1. As FGF2 appears to enhance EMT gene expression induced by TGFβ1, we then determined whether FGF2 enhances migration of BEAS-2B cells after injury. BEAS-2B cells were treated with FGF2, TGFβ1, or TGFβ1 + FGF2 immediately after wounding with a 1 mm circular rubber stylet. Treatment with TGFβ1 significantly reduced migration rates of BEAS-2B cells when given immediately after wounding at 48 h (P value = 0.0345) and 72 h (P value = 0.0422), however, both FGF2 alone and the addition of FGF2 to TGFβ1-treated cells caused a non-significant increase in migration rate (**Figure 7A, B**). Therefore, we treated BEAS-2B cells with FGF2, TGFβ1, or TGFβ1 + FGF2 for 3 days prior to wounding. Both FGF2 and TGFβ1 alone significantly increased migration at 24 h (P values = <0.0001), 40 h (P values = <0.0001 and 0.0222, respectively) and 48 h (P values = <0.0001 and 0.0042, respectively) after wounding compared to control, and this effect was substantially potentiated by addition of FGF2 to TGFβ1 (P values = <0.0001) (**Figure 8A, B**).

Discussion

This study has demonstrated that TGFβ1 induces EMT in human bronchial epithelial cells (BEAS-2B) and alveolar type II epithelial cells (A549), as shown by morphological and EMT-related gene expression. We found that FGF2 enhances TGFβ1 induced EMT-related gene expression except for collagen I, which is inhibited by FGF2. Additionally, epithelial migration was enhanced by pre-treatment with FGF2 and/or TGFβ1.

Several studies have demonstrated that human alveolar epithelial cells [26, 27, 30–36] and human bronchial epithelial cells [28, 29, 32, 37] undergo EMT in response to TGFβ1 *in vitro*. A number of studies have reported that FGF2 induces EMT in malignant pleural mesothelioma cells [52], tubular epithelial cells [39, 42] and lens epithelial cells [53]. Although the synergistic effect between TGFβ1 and FGF2 in inducing EMT [43, 44, 47] and proliferation [54–56] has been described in other cell types, to our knowledge, the synergistic effect between FGF2 and TGFβ1 in inducing type II EMT in lung epithelial cells *in vitro* had not been described previously.

In this study, we stimulated both BEAS-2B and A549 cells with TGFβ1, FGF2 or both for up to 4 days. In response to TGFβ1, BEAS-2B cells lost their cobblestone morphology and adopted an elongated spindle-like shape, and this shape was unaltered by the addition of FGF2. TGFβ1 treatment also led to downregulation of the epithelial cell-specific adherence junction protein *CDH1* and upregulation of the mesenchymal markers *ACTA2* and *CDH2*. FGF2 alone did not significantly alter *CDH1*, *ACTA2*, or *CDH2* expression, but had a significant additive effect on the changes in expression of these genes when added to TGFβ1-treated cells. Additionally, FGF2 augments the increase in the ECM mRNA expression of *FN* and *TNC* induced by TGFβ1. Similarly, we observed an identical pattern of EMT gene expression following stimulation with TGFβ1 +/- FGF2 in A549 cells. These results establish that FGF2 augments TGFβ1 in the induction of EMT in multiple lung epithelial cell lines *in vitro*.

In accordance with the present results, Shirakihara *et al.* [43] showed that there was a synergistic effect between FGF2 and TGFβ1 on the induction of EMT in the mouse normal mammary epithelial (NMuMG) cells, without evidence of induction of EMT by FGF2 alone. They found that the morphology of NMuMG cells clearly changed from a cobblestone-like shape to a fibroblastic spindle shape with TGFβ1 treatment. Although FGF2 alone did not alter this shape, the addition of FGF2 to TGFβ1-treated cells maintained the spindled-shape morphology. Li *et al.* [46] and Kurimoto *et al.* [48] indicated that treatment with FGF2 + TGFβ1 downregulated E-cadherin, upregulated vimentin and N-cadherin and increased migration ability in A549 cells. These findings suggest that the combination of FGF2 and TGFβ1 treatment and not FGF2 alone is an effective way of promoting the induction of an EMT phenotype.

The present study showed that FGF2 alone or in combination with TGFβ1, increased the migratory capacity of BEAS-2B cells after 24 h, 40 h and 48 h that were pre-treated for 3 days prior to wounding, however, the cell motility of immediately treated cells after wounding was not accelerated significantly when measured after 24 h, 48 h and 72 h. These results show that treatment with TGFβ1, FGF2, or both requires at least 3 days to promote increased motility and almost complete wound closure after injury.

These findings are consistent with a previous study which showed that TGFβ1 treatment alone increased cell motility, and the addition of FGF2 to TGFβ1-treated cells treated for 4 days strongly enhanced the motility of NMuMG cells [43]. These results match those observed in Chen *et al.* [44] study who also reported that treatment with TGFβ1, FGF2, or both generate EMT phenotype in rat Hertwig's epithelial root sheath (HERS) cells. They found that there was no significant difference in the EMT markers mRNA expression levels after 3 days and highly appeared after 7 days of induction. Also, they found the migratory capacity highly increased after 48h and 72 h of the pretreated cells with TGFβ1, FGF2, or both for 3 days. These findings suggest that generating a well-established EMT phenotype in epithelial cells using the combined treatments of TGFβ1 and FGF2 require prolonged induction.

Several studies have shown increased *COL 1A1* expression in BEAS-2B [28, 29, 57] as well as in A549 [26, 32, 35] epithelial cells in response to TGFβ1. This also accords with our observations, which showed that TGFβ1 treatment significantly increases *COL 1A1* expression in both BEAS-2B and A549 cells. However, interestingly, *COL 1A1* expression was dramatically suppressed with the addition of FGF2 to TGFβ1-treated BEAS-2B and A549 cells. These findings mirror those of our previous study demonstrating inhibition of TGFβ1-induced collagen expression by FGF2 in primary mouse and human lung fibroblasts *in vitro* [50]. These results may provide an important insight into the antifibrotic effect of FGF2 through suppression of TGFβ1-induced collagen expression in both lung fibroblast and epithelial cells.

The FGFR-specific tyrosine kinase inhibitor PD173074 was used to block the inductive effect of FGF2 on TGFβ1-treated cells. PD173074 inhibitor was reported to show both high affinity and selectivity for the FGF receptor (FGFR) family [58]. We found that the addition of PD173074 attenuates the inductive effect of FGF2 as indicated by reversing reduction of *CDH1* expression and *COL 1A1*, and the induction of *ACTA2*. This finding suggests that the effect of FGF2 is dependent upon canonical signaling through FGFRs.

Numerous studies have demonstrated that EMT is implicated in pulmonary fibrosis and cancer metastasis in mouse models [59–61] and in humans [62, 63]. The importance of EMT in pulmonary fibrosis has been challenged by other studies in animal models [64–66] and humans [67]. Whilst this study did not investigate the cellular mechanism underlying the effect of FGF2 in lung epithelial cells, it does demonstrate that FGF2 has an antifibrotic effect in part through decreasing collagen expression in epithelial cells and promotion of TGFβ1-induced gene expression required for migration of epithelial cells in wound repair after injury.

Conclusions

In conclusion, the data presented in this report suggest that FGF2 is synergistic with TGFβ1 to drive type II EMT associated with wound healing and tissue repair after injury. The mechanism driving this synergy needs more investigation to identify possible therapeutic targets and the future uses of FGF2 following lung injury and in pulmonary fibrosis.

Abbreviations

ACTA2: Actin alpha 2; BCA: Bicinchoninic acid; BEGM: Bronchial epithelial growth medium; BSA: Bovine serum albumin; CDH1: E-cadherin; CDH2: N-cadherin; COL1A1: Collagen I; CTGF: Connective tissue growth factor; DAPI: 4',6-diamidino-2-phenylindole; EGF: Epidermal growth factor; EMT: Epithelial-mesenchymal transition; FGF2: Fibroblast growth factor-2; FN: Fibronectin; FSP1: Fibroblast-specific protein 1; HGF: Hepatocyte growth factor; IGF-II: Insulin-like growth factor-2; IL-1: Interleukin-1; MMPs: Matrix metalloproteinases; RIPA: Radioimmunoprecipitation assay; SNAI1: Snail; SNAI2: Slug; TBST: Tris-buffered saline with Tween-20; TGF β 1: Transforming growth factor beta-1; VIM: Vimentin; ZO-1: Zonula occludens-1; α -SMA: α -smooth muscle actin.

Declarations

Availability of data and materials

The datasets used and analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

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Author Contributions

LMFEB performed all experiments, analyzed data, and wrote the manuscript. NMS, MLS, and HSH contributed to manuscript revision. RDG designed, supervised all experiments, and contributed to manuscript revision.

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Figures

Figure 1

Morphological changes induced by TGF β 1 and FGF2. BEAS-2B cells were grown in complete growth media (Control) or stimulated with TGF β 1 (2 ng/ml), FGF2 (2 nM) + heparin sulphate (1 nM), or TGF β 1 + FGF2 + heparin sulphate for 4 days. Representative phase contrast images (10 $\times$ original magnification) show the morphological change of BEAS-2B cells from cobblestone-like shape as in the control (A) and FGF2 treatment alone (B) to the fibroblast-like shape in the presence of TGF β 1 (C) which is unaltered after adding FGF2 (D).

Figure 2

Figure 2

Recombinant FGF2 enhances TGFβ1-induced EMT gene expression in BEAS-2B cells. BEAS-2B cells were incubated for 4 days in the absence or presence of 2 ng/ml TGFβ1 alone, 2 nM FGF2 alone, or both TGFβ1 and FGF2. Quantitative real-time PCR analysis (n = 6) shows that FGF2 when in combination with TGFβ1, downregulates the epithelial marker CDH1 (A) and induces an increase in ACTA2 (B) and CDH2 (C) mRNA expression levels. Δ Ct values were normalized to GAPDH and expressed as fold change from untreated controls in the same sample. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test; ns = not significant, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$.

Figure 3

Figure 3

TGFβ1 induced decrease in E-Cadherin protein expression is augmented by FGF2. (A) Total cell lysates from BEAS-2B cells stimulated for 4 days with or without TGFβ1 (2 ng/ml) alone, FGF2 (2 nM) alone or TGFβ1 and FGF2, were immunoblotted for E-cadherin. Blots were reprobed for β-tubulin as a loading control. (B) Densitometry for E-cadherin was normalized to β-tubulin and is expressed as fold change from untreated control. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test; ns = not significant, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$. (C) Representative images showing immunofluorescent staining for E-cadherin in BEAS-2B cells stimulated with or without TGFβ1 (2 ng/ml) alone, FGF2 (2 nM) alone or TGFβ1 and FGF2 for 4 days. The confocal images were obtained at 20× original magnification for E-cadherin (green) and DAPI (blue).

Figure 4

Figure 4

Recombinant FGF2 inhibits TGFβ1 induced collagen, but not fibronectin or tenascin-C. BEAS-2B cells were incubated for 4 days in the absence or presence of 2 ng/ml TGFβ1 alone, 2 nM FGF2 alone, or both TGFβ1 and FGF2. Quantitative real-time PCR analysis (n = 3-6) shows that FGF2 maintained the effect of TGFβ1-induced expression of FN (A) and TNC (B) and downregulate COL1A1 (C) mRNA expression levels. Δ Ct values were normalized to GAPDH and expressed as fold change from untreated controls in the same sample. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test; ns = not significant, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$.

Figure 5

A549 Cells

Figure 5

The effect of recombinant FGF2 on TGFβ1-induced EMT gene expression is conserved in A549 cells. A549 cells were grown in complete Ham's F12 media (Control) or stimulated with TGFβ1 (2 ng/ml), FGF2 (2 nM) + heparin sulphate (1 nM), or TGFβ1 + FGF2 + heparin sulphate for 4 days. Quantitative real-time PCR analysis (n = 3) shows that FGF2 has a similar effect of TGFβ1 in decreasing CDH1 (A) and increasing ACTA2 (B) as well as inhibiting COL1A1 (C) mRNA expression levels. ΔCt values were normalized to GAPDH and expressed as fold change from untreated controls in the same sample. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test; ns = not significant, ** indicates p < 0.01, *** indicates p < 0.001, and **** indicates p < 0.0001.

Figure 6

Figure 6

The effect of recombinant FGF2 on TGFβ1 induced EMT gene expression in lung epithelial cells is blocked by the FGFR-specific tyrosine kinase inhibitor PD173074. BEAS-2B cells were incubated with TGFβ1 (2 ng/ml), FGF2 (2 nM) alone, PD173074 (0.1 μM) alone or FGF2 (2 nM) + TGFβ1 +/- PD173074 for 4 days. Quantitative real-time PCR analysis (n =3-6) shows that PD173074 inhibit the effect of FGF2 on TGFβ1-treated cells in repression of CDH1 (A), induction of ACTA2 (B) and blocking of COL1A1 (C) mRNA expression levels. A549 cells were incubated with TGFβ1 (2 ng/ml), FGF2 (2 nM) alone, PD173074 (0.1 μM) alone or FGF2 (2 nM) + TGFβ1 +/- PD173074 for 4 days. Quantitative real-time PCR analysis (n =3) shows that PD173074 inhibit the effect of FGF2 on TGFβ1-treated cells in repression of CDH1 (D), and partially inhibit the induction of ACTA2 (E) and blocking of COL1A1 (F) mRNA expression levels. ΔCt values were normalized to GAPDH and expressed as fold change from untreated controls in the same sample. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test; ns = not significant, * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, and **** indicates p < 0.0001.

Figure 7

Figure 7

FGF2, but not TGFβ1, increases BEAS-2B migration of epithelial cells when added immediately after wounding. BEAS-2B were grown in complete media to confluence, and 1mm diameter circular wounds were generated and the cells were treated immediately with TGFβ1 (2 ng/ml), FGF2 (2 nM), or TGFβ1 + FGF2 (n =3). (A) Wound area was imaged at 0 h, 24 h, 48 h and 72 h then the % of wound closure was measured using ImageJ software. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test; Red asterisks mark significant differences for FGF2 vs. TGFβ1, blue asterisks mark significant differences for TGFβ1 + FGF2 vs. FGF2, and green asterisks mark significant differences for TGFβ1 vs. control. * indicates $p < 0.05$, *** indicates $p < 0.001$ and **** indicates $p < 0.0001$. (B) Representative phase contrast images (10× original magnification) of the same area were taken immediately after wounding (0 h) as well as 24 h, 48 h and 72 h later.

Figure 8

Figure 8

Pre-treatment with FGF2 and TGFβ1 significantly increases migration rates BEAS-2B cells after wounding. (A) BEAS-2B cells were treated with TGFβ1 (2 ng/ml), FGF2 (2 nM), or both TGFβ1 and FGF2 for 3 days (n =3). The cells then wounded and the % of wound closure was measured at 0 h, 24 h, 40 h and 48 h later. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test; Red asterisks mark significant differences for FGF2 vs. control, blue asterisks mark significant differences for TGFβ1 + FGF2 vs. FGF2, blue circles mark significant differences for TGFβ1 + FGF2 vs. TGFβ1, green asterisks mark significant differences for TGFβ1 vs. control and black asterisks mark significant differences for control vs TGFβ1 + FGF2. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$. (B) Representative phase contrast images (10× original magnification) of the same area were taken immediately after wounding (0 h) as well as 24 h, 40 h and 48 h later.