Two *in vitro* assays relevant to lung fibrosis for evaluating the potency and efficacy of prospective anti-fibrotic drugs

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1 Overview

Pathogenic mechanisms involved in fibrosis of various organs share many common features. Myofibroblasts are thought to play a major role in fibrosis through excessive deposition of extracellular matrix during wound healing processes. In lung fibrosis, resident fibroblasts are thought to differentiate into the more contractile myofibroblasts (Fibroblast-to-Myofibroblast Transition; FMT), secreting many extracellular matrix proteins. Also, the epithelial to mesenchymal transition (EMT) of bronchial epithelial cells may account for increased numbers of fibroblasts, which can subsequently transform into myofibroblasts. We have developed an optimized off-the-shelf panel of *in vitro* fibrosis assays, mimicking both FMT and EMT processes *in vitro* using lung cells derived from idiopathic pulmonary fibrosis (IPF) patients or healthy donors, to assess the translational potential of small molecules and/or other prospective drugs as novel therapies

2 Fibroblast-to-Myofibroblast Transition assay

A well-characterized hallmark of pathologic FMT is *de novo* formation of alpha-smooth muscle actin (αSMA) stress fibers. Since myofibroblasts localize at sites undergoing active matrix deposition and display elevated collagen synthetic capacity, myofibroblasts are considered to play a major role in the pathology of IPF. The well-established key fibrogenic mediator, transforming growth factor β1 (TGF-β1) induces FMT. In cells that have undergone FMT, increased expression of αSMA is observed. In vitro, increased αSMA expression positively correlates with contraction of myofibroblast-populated collagen gels, indicating that αSMA is a strong marker of myofibroblast differentiation and hence, a relevant readout for lung fibrosis. A validated, robust TGF-β1-induced FMT assay has been developed in IPF-derived fibroblasts to evaluate therapeutic candidates with various modes-of-action in this disease area.

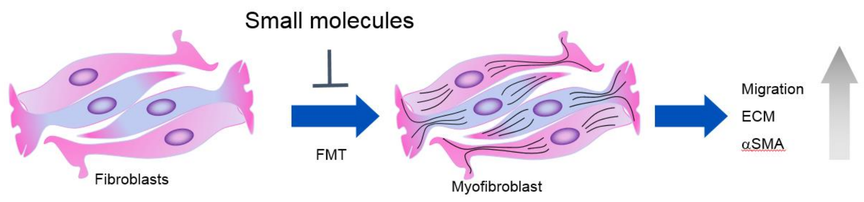
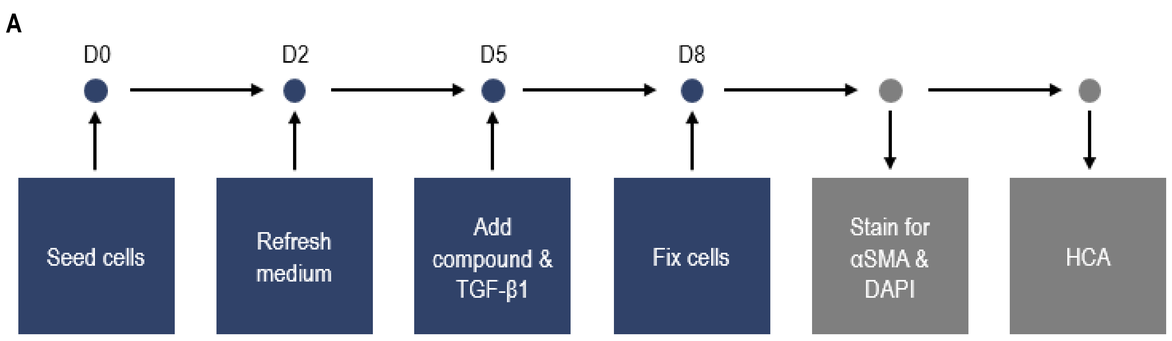


Figure 1. Schematic overview of TGF-β1-mediated trans-differentiation of fibroblasts to myofibroblasts (FMT) and modulation by small molecules.

Lung-derived primary human bronchial fibroblasts are seeded then refreshed in preparation for addition of small molecule compounds and the TGF-β1 trigger, After 3 days, the cells are fixed, then stained using αSMA and DAPI and imaged by high-content analysis (HCA).



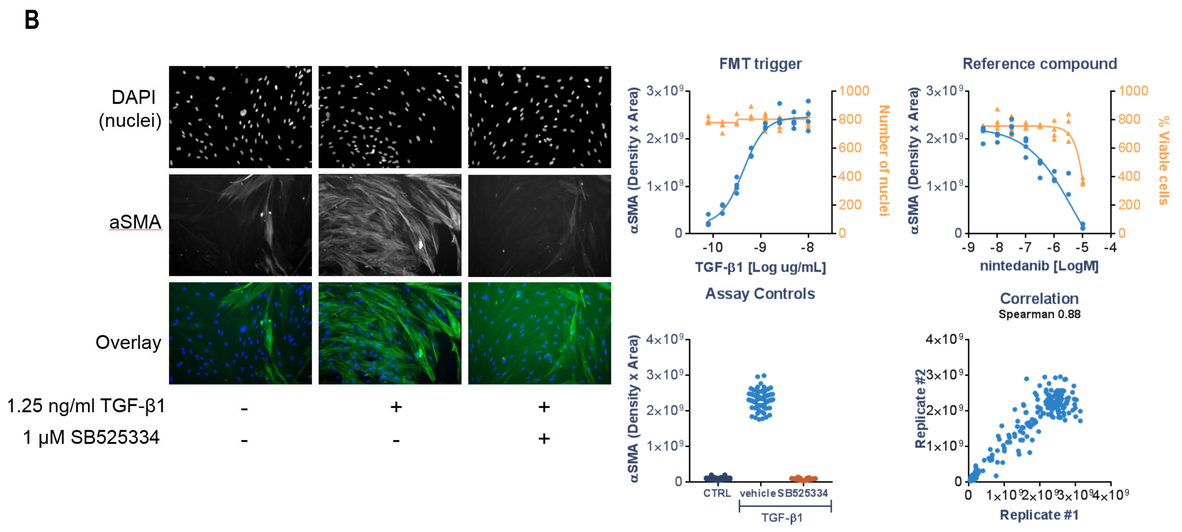


Figure 2. (A) Outline and timelines of the optimized FMT assay. **(B)** High content imaging of αSMA with an IN Cell Analyzer 2200. Exposure to TGF-β1 demonstrates clear concentration-dependent increase in αSMA levels. TGF-β1-mediated αSMA expression is fully inhibited by treatment with the ALK-5 inhibitor (SB525334) and reference compound nintedanib. IC_{50} values for nintedanib are consistent across different donors and strong Spearman rank correlation denotes consistency between biological replicates.

3 Epithelial-to-Mesenchymal Transition assay

EMT was proposed as a mechanism for collagen overproduction and increased number of fibroblast-like cells leading to fibrosis. Several studies demonstrated that EMT occurs in primary human bronchial epithelial cells (HBEC) exposed to TGF-β1. In cells that have undergone EMT, increased synthesis of fibronectin (FN1), an important component of the extracellular matrix, is considered a relevant readout for lung fibrosis. A validated, robust TGF-β1-induced EMT assay has been developed in IPF-derived HBEC to evaluate therapeutic candidates with various modes-of-action in this disease area.

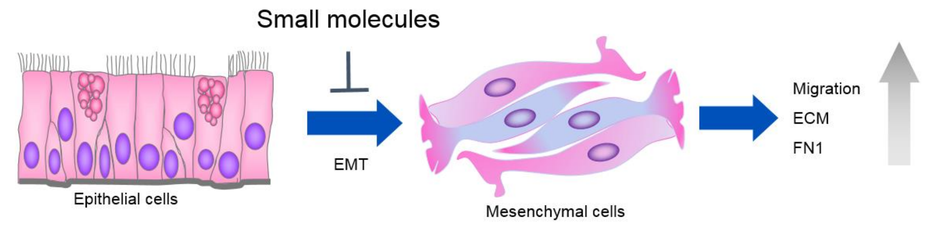
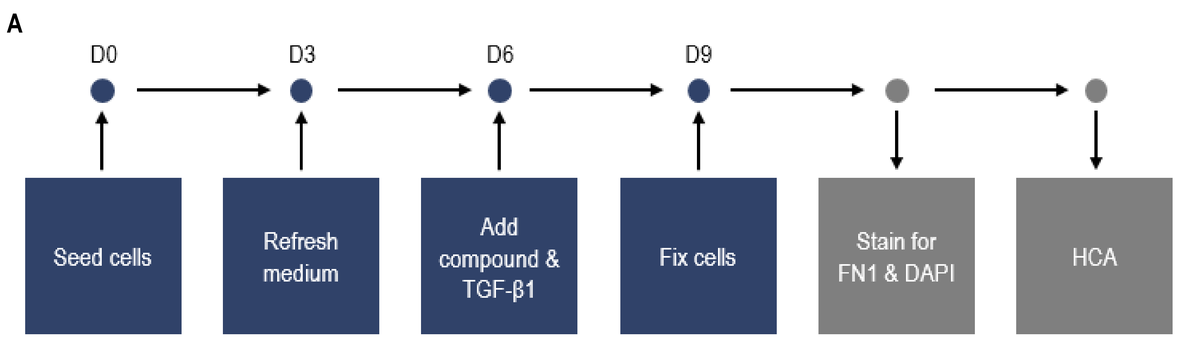


Figure 3. Schematic overview of TGF-β1-mediated trans-differentiation of epithelial cells to mesenchymal cells (EMT) and modulation by small molecules.

Lung-derived primary human bronchial epithelial cells are seeded then refreshed in preparation for addition of small molecule compounds and the TGF-β1 trigger, After 3 days, the cells are fixed, then stained using FN1 and DAPI and imaged by high-content analysis (HCA).



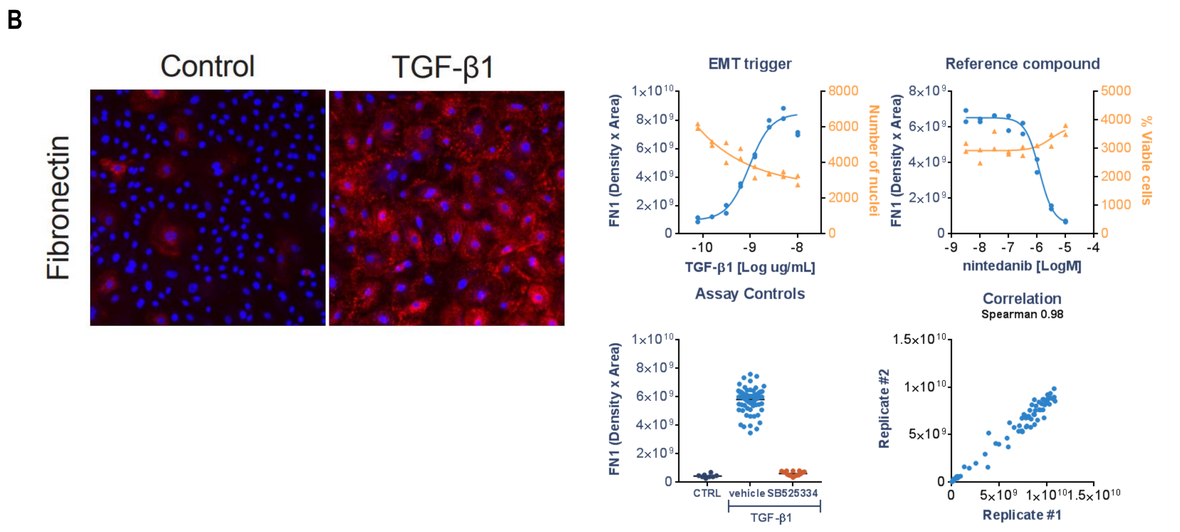


Figure 4. (A) Outline and timelines of the optimized EMT assay. **(B)** High content imaging of FN1 with an IN Cell Analyzer 2200. Exposure to TGF-β1 demonstrates clear concentration-dependent increase in FN1 levels. TGF-β1-mediated FN1 expression is fully inhibited by treatment with the ALK-5 inhibitor (SB525334) and reference compound nintedanib. IC_{50} values for nintedanib are consistent across different donors and strong Spearman rank correlation denotes consistency between biological replicates.

4 Conclusion

TGF-β1 sows a consistent and concentration-dependent induction of αSMA expression in lung fibroblasts and FN1 expression in HBE, indicative of triggering FMT and EMT, respectively. Among other effects, TGF-β1 induces G1 cell cycle arrest in epithelial cells through increased expression and/or stabilization of cyclin-dependent kinase inhibitors (CKI), resulting in a concentration-dependent decrease in number of nuclei upon stimulation with TGF-β1. TGF-β1 mediated FMT and EMT could be confined by treatment with the ALK-5 inhibitor SB525334, showing full inhibition of both αSMA and FN1 expression regardless the presence of TGF-β1. For both assays strong correlation denotes consistency between biological replicates. Therapeutic candidates can be evaluated in 8-point concentration response curves, using either cells derived from lungs from idiopathic pulmonary fibrosis patients or healthy donors, as is shown for the reference compound nintedanib. These results suggest that both the FMT and EMT assays can be a useful translatable tool for evaluating the potency and efficacy of prospective anti-fibrotic drugs

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