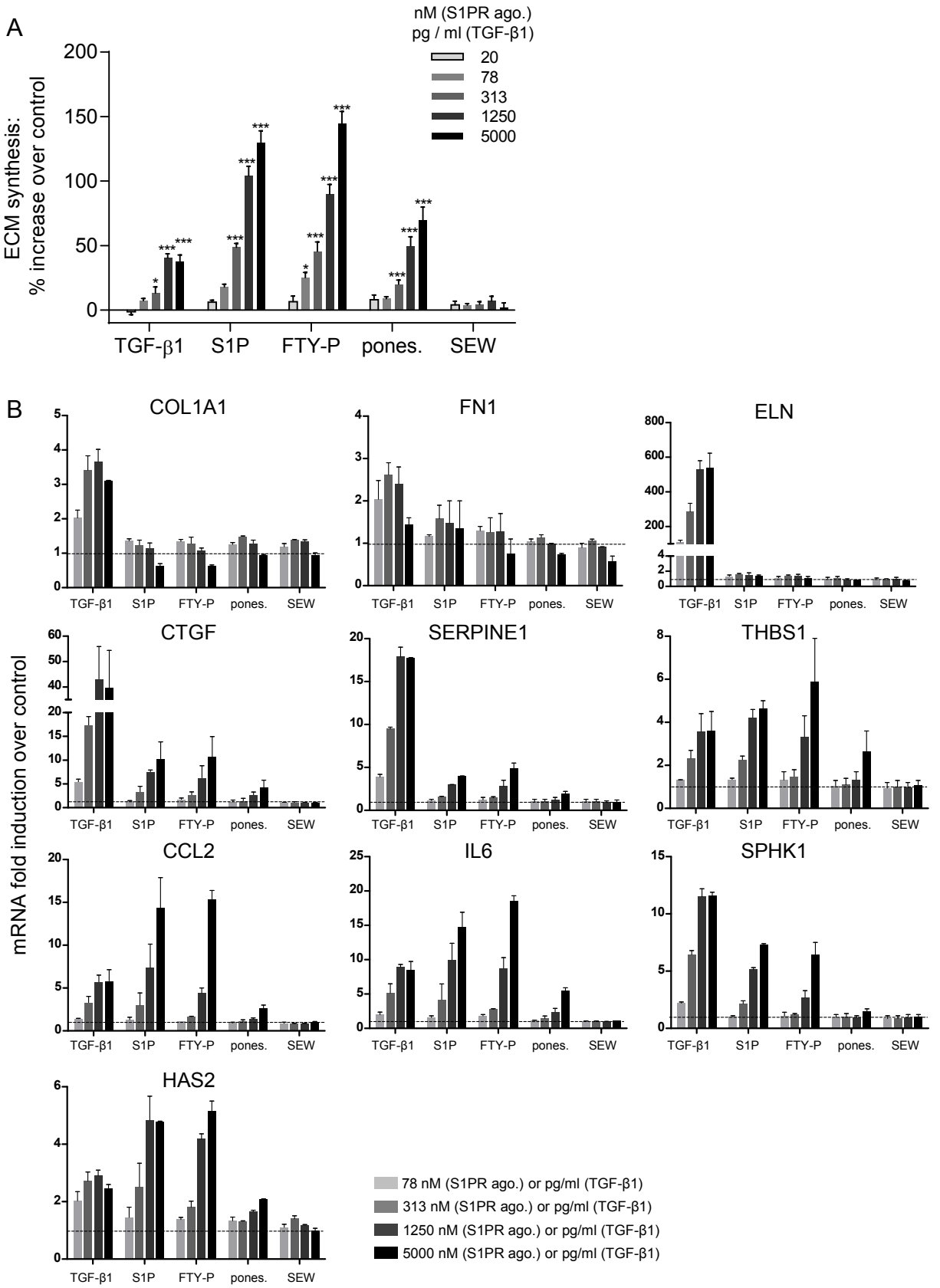
Sphingosine-1-phosphate (S1P) receptor agonists mediate pro-fibrotic responses in normal human lung fibroblasts via $S1P_2$ and $S1P_3$ receptors and Smad-independent signaling

Supplemental Information

Katrin Sobel, Katalin Menyhart, Nina Killer, Bérengère Renault, Yasmina Bauer, Rolf Studer, Beat Steiner, Martin H. Bolli, Oliver Nayler and John Gatfield

Supplementary Figure 1

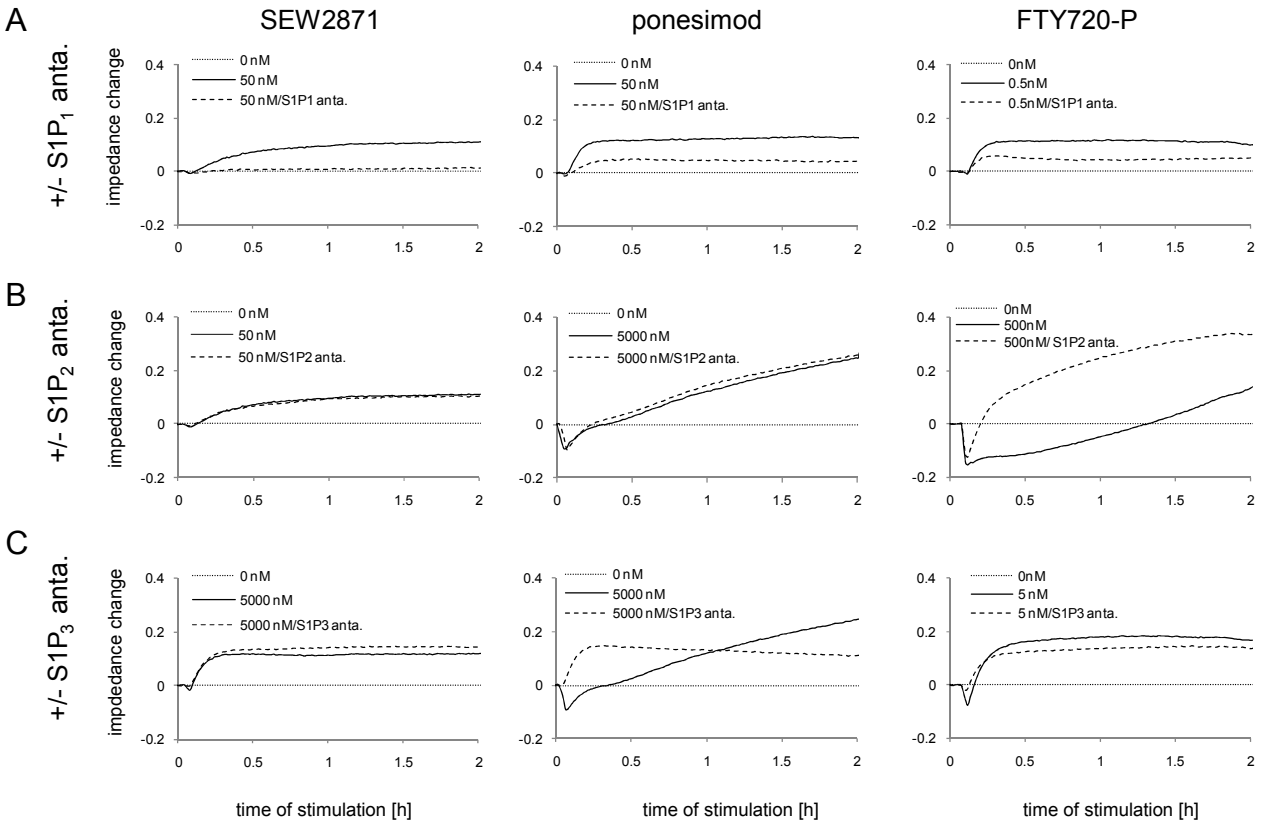


Suppl. Fig. 1: Analysis of ECM synthesis and regulation of pro-fibrotic gene expression in NHLF isolated from a second, independent donor

(A) NHLF were stimulated with TGF-β1 (20-5000 pg/ml) or S1PR agonists (20-5000 nM) and ECM synthesis was measured after 24 h with the ^{3}H-proline incorporation assay. Data represent mean + SEM of 5 independent experiments, * = $p < 0.05$, *** = $p < 0.001$, one-way ANOVA, Dunnett post test. **(B)** qPCR of pro-fibrotic gene expression after stimulation of NHLF with TGF-β1 (78-5000 pg/ml) or S1PR agonists (78-5000 nM) for 8 h. Normalization was performed using B2M, HPRT1, 18s and PPIA, which were selected by GENORM application (1). Data are mean + SEM of two independent experiments. Dashed lines show the control level of gene expression.

(1) Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034.

Supplementary Figure 2



Suppl. Fig. 2 Analysis of S1PR subtype signaling in NHLF using impedance assays
NHLF were pre-incubated with or without 1 µM $S1P_1R$ antagonist W146 (A), 0.2 µM $S1P_2R$ antagonist JTE-013 (B) or 1.25 µM $S1P_3R$ antagonist TY-52156 (C) for 1 h before stimulation with various concentrations of SEW2871, ponesimod or FTY720-P. Signaling was monitored over 2 h. Data in A, B show representative experiments, n=3.
(A) The $S1P_1R$ antagonist reduced the increase in impedance induced by SEW2871 (50 nM), ponesimod (50 nM) and FTY720-P (0.5 nM), confirming that the increase in impedance reflects the $S1P_1R$ response.
(B) Pre-incubation with the $S1P_2R$ antagonist did not affect SEW2871 (50nM)- and ponesimod (5 µM)-induced impedance responses, demonstrating that no $S1P_2R$ activation was measurable. In contrast, the FTY720-P (500 nM)-induced prolonged decrease in impedance was completely reversed to increase above baseline in the presence of $S1P_2R$ antagonist, while the first, rapid decrease was unaffected. This demonstrates that the prolonged decrease in impedance represents $S1P_2R$ activation.
(C) Pre-incubation with the $S1P_3R$ antagonist did not affect the SEW2871 (5 µM)-induced increase in impedance. In contrast, the rapid ponesimod (5 µM)-induced decrease was completely inhibited by the antagonist. Also the rapid decrease induced by 5 nM FTY720-P, a concentration that did not yet induce a $S1P_2$ response, was inhibited in presence of $S1P_3R$ antagonist. These data confirm that the first rapid decrease represents $S1P_3R$ activation.

Supplementary Table 1
List of TaqMan assays from Applied Biosystems that were used for mRNA detection.

Gene	Assay number (Applied Biosystems)
18S	4319413E
B2M	4310886E
CCL2	Hs00234140_m1
COL1A1	Hs00164004_m1
CTGF	Hs00170014_m1
ELN	Hs00355783_m1
FN1	Hs01565277_m1
HAS2	Hs01052031_m1
HPRT1	4326321E
IL6	Hs00985639_m1
PPIA	4326316E
SERPINE1	Hs01126606_m1
SPHK1	Hs00184211_m1
$S1P_1$	Hs00173499_m1
$S1P_2$	Hs00244677_s1
$S1P_3$	Hs00245464_s1
$S1P_4$	Hs02330084_s1
$S1P_5$	Hs00258220_s1
THBS1	Hs00962908_m1