# ACTIVATION OF SOMATOSTATIN RECEPTORS ATTENUATES PULMONARY FIBROSIS

On line supplementary data

Raphael Borie [1], Aurélie Fabre [1,2], Fabienne Prost [1], Joëlle Marchal-Somme [1], Rachida Lebtahi[3], Sylvain Marchand-Adam[1,4] , Michel Aubier[1,4], Paul Soler [1], Bruno Crestani [1,4]

## Methods

### Quantitative PCR

RNA extraction was performed using the NucleoSpin® RNA II kit (Macherey Nagel, Hoerdt, France) on left mouse lungs using standard protocols. Reverse transcription was performed with Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Cergy-Pontoise, France) with standard protocols. Primers for polymerase chain reaction are presented in table E1. PCR were carried out in a 20 µl final reaction volume containing 5 µl cDNA, 2 pM oligonucleotide primers (table E1), 1 µl Sybr Green Jumpstart Taq Ready Mix® (Sigma-Aldrich Chimie, Lyon, France), and 0.2 µl rox (Sigma). Amplification reactions (Mx3000P thermocycler, Stratagene, La Jolla, USA) used one step at 94°C for 2 min, and 40 cycles consisting each of denaturation at 94°C for 30s, annealing at 60°C for 1 min. For the detection of TGF-β1, CTGF and sst3 mRNAs, a specific extension phase at 72°C for 30 s was applied. All samples were measured in duplicates. Negative controls with distilled water were included. Messenger RNAs extracted from mouse liver were used as positive controls. The relative expression of the gene of interest was expressed as a ratio to the house keeping gene, coding for the Ribosomal Protein L13 (RPL13) in a relative number of copies calculated with a standard dilution ($10^{-1}$-$10^{-4}$) performed for each reaction, as previously described (E1).

Table E1 : Primers for polymerase chain reaction

| Genes | Sequences | Reference Genbank® |
|---|---|---|
| Mouse sst1 | Forward : TGGAGGCGTTTTCCGTAATG<br>Reverse : CAGTGCCCGGCCTCAA | **NM_009216** |
| Mouse sst2A | Forward : ACCGGAAAAACCAAAACTAAATCAA<br>Reverse : CACGAACTAGCTACTTGGGCTTACA | **NM_016738** |
| Mouse sst3 | Forward : GCCGCACTGGGCTTCTTT<br>Reverse : GCACCTTTACCACAATGAGCAA | **NM_009218** |
| Mouse sst4 | Forward : CCTGCTAACTGCTGGCATGAA<br>Reverse : CACTGACACCAGGAGAAACTGAAGT | **NM_009219** |
| Mouse sst5 | Forward : TGATCATCTCTCTCACCAAAAC<br>Reverse : AGACTGACAAAAATGATGCCACAGT | **AF_035777** |
| Mouse TGF-β1 | Forward : AATATAGCAACAATTCCTGGCGTTA<br>Reverse : GCAGTGAGCGCTGAATCGA | **NM_011577** |
| Mouse CTGF | Forward : CTGAGGTGAGTCTCCTGGAACAGT<br>Reverse : CACATGCTCAGCTCTCGCTAGA | **NM_010217** |
| Mouse KGF | Forward : AATCAGTTCTTTGAAGTTGCAATCCT<br>Reverse : AACAGCTACAACATCATGGAAATCAG | **NM_008008** |
| Mouse HGF | Forward : GTGGATGCCAAGCCAAGCT<br>Reverse : CAGTAGGGTGGATGGTTAGTTTGAA | **NM_010427** |
| Mouse alpha-2 collagen-1 | Forward : GGCTATGACTTTGGTTTTGAAGGA<br>Reverse : CGTTGTCGTAGCAGGGTTCTTT | **NM_007743** |
| Mouse RPL13 | Forward : GTGGTCCCTGCTGCTCTCAA<br>Reverse : CGATAGTGCATCTTGGCCTTTT | **NM_016738** |
| Human alpha-1 collagen-1 | Forward : AGCCACCAGCCCCTCACT<br>Reverse : CGAGGTAGTCTTTCAGCAACACAGT | **NM_000088** |
| Human Ubiquitin C | Forward 5_CACTTGGTCCTGCGCTTGA-3<br>Reverse TTTTTTGGGAATGCAACAACTTT | **NM_004181** |

Immunohistochemical detection of somatostatin receptors

Mice sst2 receptor was detected in human lung samples with a rabbit polyclonal anti-mouse sst2A antibody (ref SS-800, Biotrend, Cologne, Germany) (1/400 dilution). Two negative controls were used: absorption of the antibody with the peptide antigen (ref SS-801, Biotrend) and incubation of the tissue sections with non immune rabbit serum instead of the SS-800 antibody.

We detected sst2 receptors in frozen human lung tissue samples obtained from 3 controls and 5 patients with IPF. A polyclonal rabbit antibody to human somatostatin receptor type 2B (ref SS-860, Biotrend) was used (1/1000 dilution). Absorption of the antibody with the peptide antigen used for antibody generation (ref SS-861, Biotrend) and incubation of the tissue sections with non immune rabbit serum were used as negative controls. The SST2 receptor was detected by immunohistochemistry in fibroblasts from normal and idiopathic pulmonary fibrosis lung cultured on Lab-Tek slides at 80% confluence (Nunc, Naperville, IL).

Proliferation of and collagen production by human lung fibroblasts

Human lung fibroblasts derived from four control lungs [E1] were cultured with 1% Fetal Clone II (Hyclone, Perbio, Brebieres, France) for 48 hours with or without recombinant human TGF-β1 (10 ng/ml) (R&D) and SOM230 ($10^{-10}$M, $10^{-8}$M or $10^{-6}$M). Iincorporation of bromodeoxyuridine (BrdU colorimetric assay, Roche Diagnostics, Meylan, France) was used to estimate cell proliferation. Alpha-1 collagen-1 mRNA content was quantified by quantitative real time PCR and expressed as a ratio to the ubiquitin C mRNA. [E1].

Results

Treatment with SOM230 increased the expression of hepatocyte growth factor and keratinocyte growth factor.

Hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) exert potent antifibrotic effects in the lung [E2]. As previously shown by Adamson and Kawoska [E3], we observed an increase of lung HGF mRNA and KGF mRNA contents with a maximum on day 7 following bleomycin instillation (figure E1).

Treatment with SOM230 increased HGF mRNA content on day 3 (HGF/RPL13 mRNA ratio = 5.3 [4.2-7.7] in blm-SOM mice versus 1.5 [1-1.9] in blm-vehicle mice, n=6, *P*=0.002) (figure E1). Similarly, treatment with SOM230 increased KGF mRNA content on day 3 (KGF/RPL13 mRNA ratio = 5.5 [2-6] vs 2.3 [1-3.3] in blm-vehicle mice, n=6 in each group, *P*=0.01) (figure 6).

Effect of SOM230 in naive mice

Lung histology (figure E2) and bronchoalveolar lavage cytology (data not shown) were not different in mice treated with vehicle or SOM230. Similarly, SOM230 did not modify lung collagen content on day 14 (74µg/lung [52-76] in vehicle treated mice versus 66 µg/lung [59-72]). Alpha2-collagen 1 mRNA, TGFβ, HGF and KGF mRNA levels were similar in vehicle and SOM230 mice (data not shown). However, SOM230 decreased lung CTGF mRNA content by 30% at all time points (statistically significant on day 3 and day 14, figure E2).

SOM230 inhibited human lung fibroblasts proliferation in vitro and reduced collagen-1 mRNA expression

In a further set of expermiments, we asked wether some of the effect of SOM230 may be a direct effect on fibroblasts. We assessed the effect of SOM230 on human lung fibroblasts proliferation, either at baseline or after incubation with TGF-β1 (10 ng/ml) (Figure E3A). In these experiments, TGF-β1 reduced BrdU incorporation in cultured lung fibroblasts by 30%. SOM230 consistently inhibited BrdU incorporation in lung fibroblasts, and the percentage of

inhibition was very similar in fibroblasts cultured in basal conditions or in the presence of TGF-β1.The maximal inhibition (-24%) was observed with the lowest doses of SOM230 ($10^{-10}$M and $10^{-8}$M). The highest dose of SOM230 ($10^{-6}$M) had no inhibitory effect.

We evaluated the effect of SOM230 on collagen-1 expression by lung fibroblasts in vitro (Figure E3B). SOM230 did not modulate the basal level of alpha-1 collagen-1 mRNA content. As expected, TGF-β1 increased alpha-1 collagen-1 mRNA contents, SOM230 reduced by 20 to 30% TGF-β induced collagen expression. The maximal inhibition was observed with the lowest dose of SOM230 ($10^{-10}$ M).

## FIGURE LEGENDS

**Figure E1:** SOM230 promotes an anti-fibrotic environment in the lung

Panels A and B: Treatment with SOM230 increased the expression of HGF mRNA and KGF mRNA on day 3 when compared with bleo+vehicle mice and controls. (n=5 to 6 animals for each experimental condition) **P<0.05*.

The centerline of the box denotes the median, the extremes of the box denote the interquartile range and the bars denote the highest and lowest values.

**Figure E2:** Effect of SOM230 in naïve mice**.**

Panel A and B: Representative lung sections in naive mice treatd 14 days with vehicle (panel A) or SOM230 (25 µg/kg/day) (panel B) (hematoxyline-eosin-staining, original magnification x80). SOM230 did not modify lung histology.

Panel C: CTGF mRNA content was assessed by quantitative real time PCR and expressed as the ratio to RPL13 mRNA content in the lung of controls mice, and in naive mice treated with vehicle or SOM230 for 3, 7 and 14 days. Whereas CTGF mRNA content was not modified with vehicle, SOM230 decreased CTGF mRNA expression after 3 and 14 days of treatment, and tended to decrease CTGF mRNA content after 7 days of treatment.

(Data are mean and standard deviation; n=3 to 4 animals for each experimental condition) **P<0.05*.

**Figure E3:** Effect of SOM230 on human lung fibroblasts, derived from a control lung

Panel A: Human lung fibroblasts, derived from four control lungs**,** were cultured for 48 hours with 1% fetal clone. Cell proliferation was assessed by measuring the incorporation of BrdU and expressed as a percentage of the basal condition (see methods). TGF-β1 (10 ng/ml) reduced lung fibroblast proliferation *in vitro*. SOM230 inhibited fibroblasts proliferation

cultured in basal conditions or in presence of TGF-β1. The maximal inhibition was observed with the lowest dose of SOM230 (n=3 experiments).

Panel B : Human lung fibroblasts, derived from four control lungs, were cultured for 48 hours with 1% fetal clone and stimulated with TGF-β1 for 24 hours. The expression of alpha-1 collagen-1 (A1COL1) mRNA was quantified by PCR and expressed as a ratio to the ubiquitin mRNA. TGF-β1 strongly increased the expression of A1COL1 mRNA. At the lowest dose ($10^{-10}$M), SOM230 reduced by 25% A1COL1 mRNA level whereas it had no effect at higher concentrations.

Data are means and standard deviation. * *P<0.05*

References

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E3. Adamson IY, Bakowska J. Relationship of keratinocyte growth factor and hepatocyte growth factor levels in rat lung lavage fluid to epithelial cell regeneration after bleomycin. *Am J Pathol*. 1999;**155**:949-54.