Transcription factors involved in stem cell maintenance are downstream of Slug/Snail2 and repressed by TGF-β in bronchial epithelial progenitors from Chronic Obstructive Pulmonary Disease

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Abstract

Objectives: Patients with Chronic Obstructive Pulmonary Disease (COPD) have a bronchial epithelium with many anomalies and basal/progenitor cells showing a decrease of self-renewal and differentiation potential. The objective of this study was to identify deregulations in the genetic program of COPD bronchial progenitors that could account for their exhaustion. The transcription factor Slug/Snail2 is highly expressed in bronchial progenitors and we aimed at identifying genes downstream of Slug whose expression is deregulated in COPD progenitors.

Results: We knocked down Slug in primary basal cells from COPD subjects and, since COPD subjects have higher levels of Transforming Growth Factor (TGF)-β and Slug is regulated by TGF-β, we selected genes downstream of Slug involved in differentiation that respond to TGF-β. We identified transcription factors involved in stem cell maintenance downstream of Slug and repressed by TGF-β in COPD but not normal progenitors. We found that the effect of TGF-β on the expression of these genes is correlated to Slug knockdown effect. We also found a correlation between the mRNA levels of Slug and these genes only in presence of TGF-β. These results reveal that stem cell maintenance genes are deregulated in COPD bronchial progenitors, Slug and TGF-β being involved in that deregulation.

Key words: COPD, Bronchial epithelium, Primary progenitors, TGF-β, Slug/Snail2, Somatic stem cell maintenance genes, Transcription factors, Microarray analysis

Introduction

Subjects with chronic obstructive pulmonary disease (COPD), a respiratory disease mainly caused by cigarette smoke and characterized by a progressive and irreversible loss of respiratory capacity, have a remodeling of their airways with many anomalies of the epithelium that increase with disease progression (1, 2). These anomalies are found all along the airway epithelium as cells keep the memory of the exposure to cigarette smoke establishing a "field of injury", and would result from an imbalance of the fate of basal cells, the airway epithelium adult stem/progenitor cells that can self-renew and/or differentiate to repair the epithelium after injury (3, 4). A decrease in the count and functional ability of basal progenitors has been reported in smokers with a higher decrease in COPD smokers, leading to a statistically significant difference between non-smokers and smokers-COPD that correlates with the loss of respiratory capacity (5, 6).

Slug/Snail2 belongs to the Snail family of transcription factor and is involved in epithelial-mesenchymal transition (EMT). In contrast to other EMT-inducing transcription factors, Slug is highly expressed in adult basal/progenitor cells in both mouse and human normal airway epitheliums (7, 8). We hypothesized that Slug is involved in the fate of basal/progenitor cells, and, as such, deregulation of its function could lead to the decrease in count and functional ability of progenitors observed in COPD. To test this hypothesis, we exploited the Gene Expression Omnibus (GEO) dataset GSE123129 that we generated from a microarray analysis of Slug knockdown in basal/progenitor cells of COPD and normal subjects (9). Transforming Growth Factor (TGF)-β is found at higher levels in COPD lung tissues and, as it has been shown to regulate Slug expression and to play a role in stem/progenitor cell fate, we also used the GEO dataset GSE122957 to select genes downstream of Slug in COPD that respond to TGF-β (9-12).

Main text

Material and methods

Study subjects and cell isolation

Human lung tissues, smokers (n=6) and COPD-smokers (n=6) (see additional file 2) were obtained from patients undergoing lung lobectomy for peripheral lung carcinoma for removal of a primary lung tumor. COPD patients were diagnosed according to the GOLD (Global initiative for chronic Obstructive Lung Disease) guidelines. Lung tissues used in this study were dissected as far away as possible from the tumor. Primary human bronchial epithelial cells were isolated from a piece of large bronchus according to standard protocol.

Cell culture

Primary human bronchial epithelial cells (Passage 1 or 2) were expanded on flasks coated with collagen I (BD Biocoat) in bronchial epithelial growth medium (BEGM), composed of bronchial epithelial basal medium (BEBM) supplemented with the SingleQuots kit (Lonza) and incubated at 37°C in 5% de CO2. TGF-β1 (Peprotech) was added at a concentration of 1ng/ml for 2 days.

RNA extraction

Cells were rinsed with phosphate buffer saline (PBS) before to be homogenized in RNA lysis buffer (NucleoSpin-RNA Kit, Macherey-Nagel), supplemented with 1% β-Mercaptoethanol. Lysates were vortexed and proceeded immediately or snapped Frozen and stored at -80˚C. Total RNA was extracted on column using the NucleoSpin-RNA Kit (Macherey-Nagel). DNaseI treatment was done directly on the column. RNA concentration was determined using a NanoDrop.

cDNA synthesis

87 For each sample, 0.3µg of total RNA was reverse transcribed as previously described (13) with
88 the following modifications: briefly, total RNA was annealed with 0.1mg/ml oligo(dT)15 primer
89 (Promega) and cDNA synthesis was performed with M-MLV Reverse Transcriptase (Promega)
90 for 1h at 42˚C. A control without reverse transcriptase was done for each series of cDNAs.

91 **Quantitative Real-Time Polymerase Chain Reaction (qPCR)**

92 Quantitative PCR was done on the QuantStudio6 Flex with the QuantStudio Real-Time PCR
93 software using SYBR green PCR master mix (Applied BioSystems). Primers (see additional file
94 3) were designed to be used with an annealing temperature of 60°C and an elongation time of
95 1min. For a given gene target, cDNA volume used was chosen to be in a Ct range from 20 to 30,
96 using 0.125µM each forward and reverse primer. Glyceraldehyde-3-Phosphate Dehydrogenase
97 (GAPDH) was used to normalize cDNA amounts between samples.

98 **Gene expression profiling**

99 Microarray analysis was performed on biological triplicate samples. Total RNA were amplified
100 and labeled before hybridization onto Affymetrix human Gene 2.1ST GeneChip according to the
101 manufacturer, by the Genomics platform at Curie Institute, Paris (14). Array datasets were
102 controlled using Expression console (Affymetrix) and further analyses and visualization were
103 made using EASANA (GenoSplice, www.genosplice.com), which is based on the FAST DB
104 annotations (15, 16). Gene Array data were normalized using quantile normalization. Background
105 corrections were made with antigenomic probes and probes were selected as described previously
106 (17). Only probes targeting exons annotated from FAST DB transcripts were selected to focus on
107 well-annotated genes whose mRNA sequences are in public databases (16). Bad-quality selected
108 probes (e.g. probes labeled by Affymetrix as 'cross-hybridizing') and probes whose intensity
109 signal was too low compared to antigenomic background probes with the same GC content were

110 removed from the analysis. Only probes with a DABG P-value ≤ 0.05 in at least half of the arrays
111 were considered for statistical analysis (17). Only genes expressed in at least one compared
112 condition were analyzed. To be considered expressed, the DABG P-value had to be ≤ 0.05 for at
113 least half of the gene probes. We performed an unpaired Student's t-test to compare gene
114 intensities in the different biological replicates. Genes were considered significantly regulated
115 when fold-change was ≥1.2 and P-value ≤ 0.05. Significant KEGG and REACTOME pathways
116 and GO terms were retrieved using DAVID (18-20) from union of results of all, up- and down-
117 regulated genes separately. Data set GEO ID numbers are GSE122957 and GSE123129.

118 **Statistical Analysis**

119 Biological replicates were n ≥3 for the knockdown experiments and n ≥5 for expression analysis
120 with data generated by at least 2 independent experiments. For fold-change, mean is ±SEM and
121 statistical analysis was carried out by a one sample two-sided t-test. Correlations were computed
122 as Pearson correlation coefficients and P-value determined by two-sided test. Significance was
123 accepted when P-value < 0.05.

124

125 # Results

126 **Transcription factor genes involved in somatic stem cell maintenance are**
127 **repressed downstream of Slug in COPD progenitors**

128 When setting the minimum fold-change threshold at 1.2 with P-value ≤5.00E-0, 808 genes were
129 found upregulated in COPD cells knocked down for Slug, i.e. repressed downstream of Slug.
130 Among these, 307 genes are responding to both differentiation and TGF-β and we classified them
131 in 4 groups according to their combined type of response. Fig. 1a shows histograms representing
132 the number of genes for each group and small bars indicating the mean fold-increase induced by

133 Slug knockdown for these genes in COPD (red) and normal (blue) cells. The large majority of the
134 genes are upregulated during differentiation and repressed by TGF-β, and for these the mean fold-
135 increase induced by Slug knockdown is much higher in COPD cells. A search for enriched gene
136 pathways using KEGG, REACTOME and Gene Ontology (GO) databases (18-20) identified 2
137 pathways related to stem cells, Somatic stem cell population maintenance and transcriptional
138 regulation of pluripotent stem cell, comprising 5 genes coding all for transcription factors. We
139 first confirmed by RT-qPCR the fold-change induced by Slug knockdown in COPD and normal
140 cells and Fig. 1b shows the correlation with the values obtained in the microarray. The good
141 correlation (R^2 values of 0.7309 and 0.5519, respectively for normal and COPD), validates the
142 microarray analysis and confirms that all the genes are repressed downstream of Slug in COPD
143 cells while they are not downstream of Slug in normal cells, except for ELF5 that is also
144 repressed in normal cells but less than in COPD.
145

146 Transcription factor genes involved in stem cell maintenance are more 147 repressed by TGF-β in COPD progenitors

148 The transcription factor genes involved in stem cell maintenance, which we identified as
149 repressed downstream of Slug in COPD progenitors, were also found by microarray analysis
150 repressed by TGF-β. We confirmed that all 5 genes are repressed by TGF-β in COPD, with the
151 decrease of expression statistically significant except for STAT3. In contrast, different effects,
152 mostly mild, are observed in normal progenitors, none being statistically significant **(**Fig. 2a**).** By
153 plotting Slug knockdown effect with that of TGF-β on the expression of these genes, we find a
154 good correlation with a R^2 value of 0.6813 (Fig. 2b). Moreover, for all the genes, except STAT3,
155 a strong negative correlation coefficient is found between their expression levels and that of Slug

156 but only in presence of TGF-β. The correlation is statistically significant for SOX2 and ELF5
157 while it is not for EPAS1 and PBX1; however, for these later genes, the correlation strength
158 increases significantly in presence of TGF-β (Table1, additional file 1).
159 Taken together, these results show that in COPD progenitors, when compared to normal, genes
160 coding for transcription factors involved in stem cell maintenance that became direct or indirect
161 repressed targets of Slug, have their response to TGF-β deregulated and a link exists between the
162 repressive effect of TGF-β and that of Slug.
163

164 **Discussion**

165 We identified 5 genes coding for transcription factors involved in somatic stem cell maintenance
166 that are repressed downstream of Slug and by TGF-β in COPD bronchial basal/progenitor cells;
167 in contrast, in normal cells, these genes are less or not repressed by TGF-β, and 4 of them are not
168 downstream of Slug. In COPD, we find a correlation between the effect of Slug knockdown and
169 that of TGF-β on their expression, as well as between their mRNA levels and that of Slug when in
170 presence of TGF-β.
171 Previous works have shown at the cellular level that bronchial basal/progenitor cells from
172 COPD are deregulated when compared to normal, with a decrease in self-renewal and a loss in
173 differentiation potential leading to progenitor exhaustion (5, 6). Our work reveals that key
174 transcription factors involved in stem cell maintenance, direct or indirect repressed targets of Slug,
175 have their expression deregulated in COPD, being in particular repressed when in presence of
176 TGF-β. A high level of TGF-β is found in COPD airways and our results are in line with the
177 epithelial progenitor exhaustion described for this disease.

178 TGF-β represses these transcription factors with different efficiency, and it correlates with
179 the effect of Slug knockdown, showing a link in COPD bronchial progenitors between Slug
180 expression and the response of these genes to TGF-β. This is supported by the high negative
181 correlation coefficient between the mRNA levels of Slug and that of these transcription factors,
182 with the exception of STAT3, found in presence of TGF-β. For EPAS1 and PBX1, despite strong
183 correlation coefficients, P-values are above the significance threshold, showing only a tendency
184 for these genes. However, the small number of patients studied is likely to limit the statistical
185 significance. No such correlation is found for STAT3 but Slug knockdown has the least effect on
186 this gene and it is repressed by TGF-β only slightly and with no statistical significance. Each gene
187 have a different profile of regulation by Slug and TGF-β and it is likely that this reflects the
188 existence of other regulations, specific for each gene. TGF-β may coregulate Slug and these
189 genes or, as in other cells, Slug may act as a mediator of TGF-β to regulate these transcription
190 factors (21, 22).

191 Our work reveals that the genetic program of COPD bronchial progenitors is deregulated;
192 this deregulation involves a change in Slug downstream genes and in the response to TGF-β, with
193 consequences on self-renewal and differentiation potential. There is a higher level of TGF-β in
194 COPD and it increases with disease progression (23-25) and we can speculate that progenitor
195 deregulation worsen with the level of TGF-β, participating to COPD progression.

196

197 # Limitations

198 In the present work, the analysis of the stem cell maintenance genes was limited to the
199 respective effect of Slug knockdown and TGF-β on their expression, and the study of Slug and

200 TGF-β link was limited to expression correlation. Further research on functional analysis is
201 warranted to define the role of these stem cell maintenance transcription factors in bronchial
202 progenitors and the mechanisms involved in their regulation by Slug and TGF-β.
203

204 List of abbreviations

205 COPD Chronic obstructive pulmonary disease, TGF-β Transforming growth factor beta, EMT
206 epithelial-mesenchymal transition, GAPDH Glyceraldehyde-3-Phosphate Dehydrogenase
207

208 Declarations

209 Authors' contributions

210 PL conceptualized project, conceived and designed the study, conducted experiments, interpreted
211 the data, obtained financial support. PdlG designed the study, analyzed and interpreted the data.
212 AJ analyzed the data. CC and CBB performed the experiments. PL and PdlG wrote the
213 manuscript. All authors read and approved the final manuscript.

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217 France.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The data analyzed in this publication have been deposited in the NCBI's Gene Expression Omnibus (GEO) and will be accessible through GEO Series Accession Number: GSE122957 and GSE123129

Consent for publication

Not applicable.

Ethics approval and consent to participate

Human lung tissues were obtained from patients undergoing lung lobectomy for peripheral lung carcinoma after receiving written informed consent. The study was approved by the ethics committee of Paris Nord, IRB 00006477 Paris 7 University, France.

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329

330 # Figures, table and additional Files

331 **Fig. 1** Slug knockdown in COPD bronchial progenitors identifies repressed genes
332 involved in somatic stem cell maintenance. Primary bronchial epithelial basal cells from COPD
333 and normal subjects were knocked down for Slug, and RNA were analyzed by microarray on an
334 Affymetrix chip. Data are for n ≥ 3 each, normal and COPD. **a** Genes significantly upregulated by
335 Slug knockdown in COPD cells and responding to both differentiation and TGF-β were classified
336 in 4 groups according to their type of response. Histograms represent the number of genes in each
337 group with genes upregulated by TGF-β in red and genes downregulated by TGF-β in green, and
338 genes upregulated during differentiation as checkboard and genes downregulated during
339 differentiation as oblique lines. Horizontal bars represent the mean fold-increase for each group
340 of genes, in red for COPD cells and in blue for normal cells. **b** Validation of microarray by RT-
341 qPCR and comparison of Slug knockdown effect in COPD (red) and normal (blue) cells for the
342 genes coding for 5 transcription factors involved in somatic stem cell maintenance. Results are
343 Pearson correlations calculated with log2 (fold-change) and are presented as scatter plots with
344 regression line and R-square values.
345
346 **Fig. 2** Genes involved in stem cell maintenance are more repressed by TGF-β in COPD bronchial
347 progenitors. Primary bronchial epithelial basal cells, normal and COPD, were grown on filters
348 without TGF-β or in presence of 1 ng/ml of TGF-β. At confluence, RNA lysates were prepared
349 and mRNA analyzed by RT-qPCR. GAPDH was used to normalize cDNA amounts between
350 samples and results were calculated as a ratio on GAPDH. Data shown are for n ≥ 5. **a**
351 Comparison of TGF-β effect on the expression of stem cell maintenance genes normal and COPD
352 cells. Results are presented as the fold-change induced by TGF-β on mRNA expression with
353 mean ±SEM. **b** Correlation in COPD progenitors between Slug knockdown and TGF-β effect on

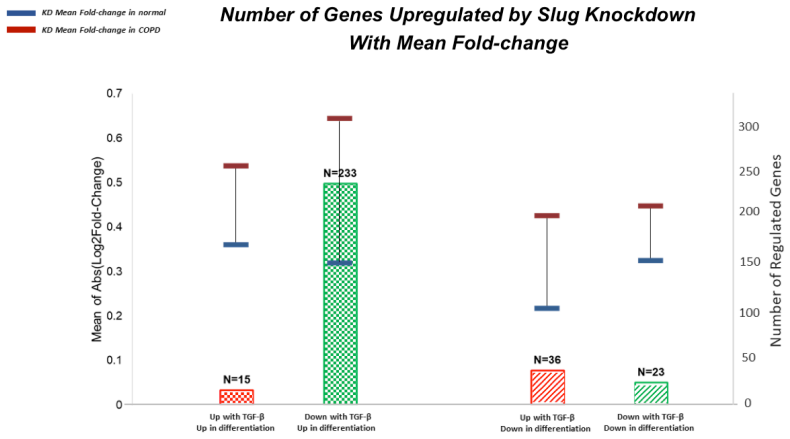
354 the expression of the 5 genes coding for transcription factors involved in somatic stem cell
355 maintenance. Results are Pearson correlations calculated with log2 (fold-change) and are
356 presented as scatter plots with regression line and R-square values. Statistical significance is at P-
357 value < 5.00E-02 *, < 1.00E-03 *** as indicated. ns: non significant.
358
359 **Table 1** Correlations between Slug and stem cell maintenance genes mRNA in COPD progenitors
360 treated with TGF-β

	Slug mRNA	
	no TGFβ	**+ TGFβ**
SOX2 mRNA	- 0.6566 (2.28E-01)	- 0.8951 (4.01E-02)
ELF5 mRNA	- 0.1203 (8.47E-01)	- 0.8976 (3.87E-02)
EPAS1 mRNA	- 0.4492 (4.47E-01)	- 0.7753 (1.23E-01)
PBX1 mRNA	- 0.6255 (2.59E-01)	- 0.7753 (1.23E-01)
STAT3 mRNA	- 0.1261 (8.39E-01)	0.1827 (7.68E-01)

361
362
363 Pearson correlation coefficients and associated P-value between expression levels.
364 Computation was performed on log2 of values.
365 Statistical significance was determined with a two-sided t-test.
366 Correlations are significant at P-value < 5.00E-02.
367
368 **Additional File 1** Correlation Graphs corresponding to data in Table 1
369 **Additional File 2** Characteristics of the study subjects.
370 Data are presented as Mean ±SD. Pack-year=1 year smoking 20 cigarettes per day. COPD,
371 chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s;
372 **Additional File 3** Sequences of primers used for qPCR

Figure 1

a


KD Mean Fold-change in normal
KD Mean Fold-change in COPD
Number of Genes Upregulated by Slug Knockdown
With Mean Fold-change
Mean of Abs(Log2Fold-Change)
Number of Regulated Genes
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
300
250
200
150
100
50
0
N=15
N=233
N=36
N=23
Up with TGF-β
Up in differentiation
Down with TGF-β
Up in differentiation
Up with TGF-β
Down in differentiation
Down with TGF-β
Down in differentiation

b

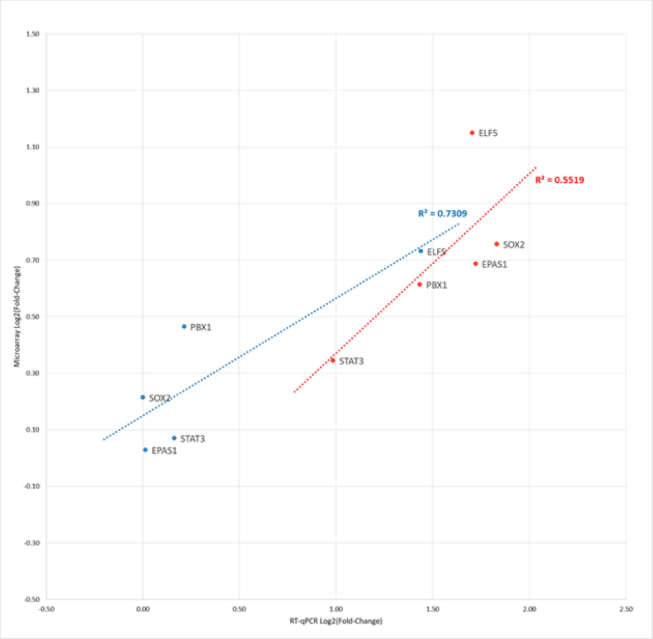
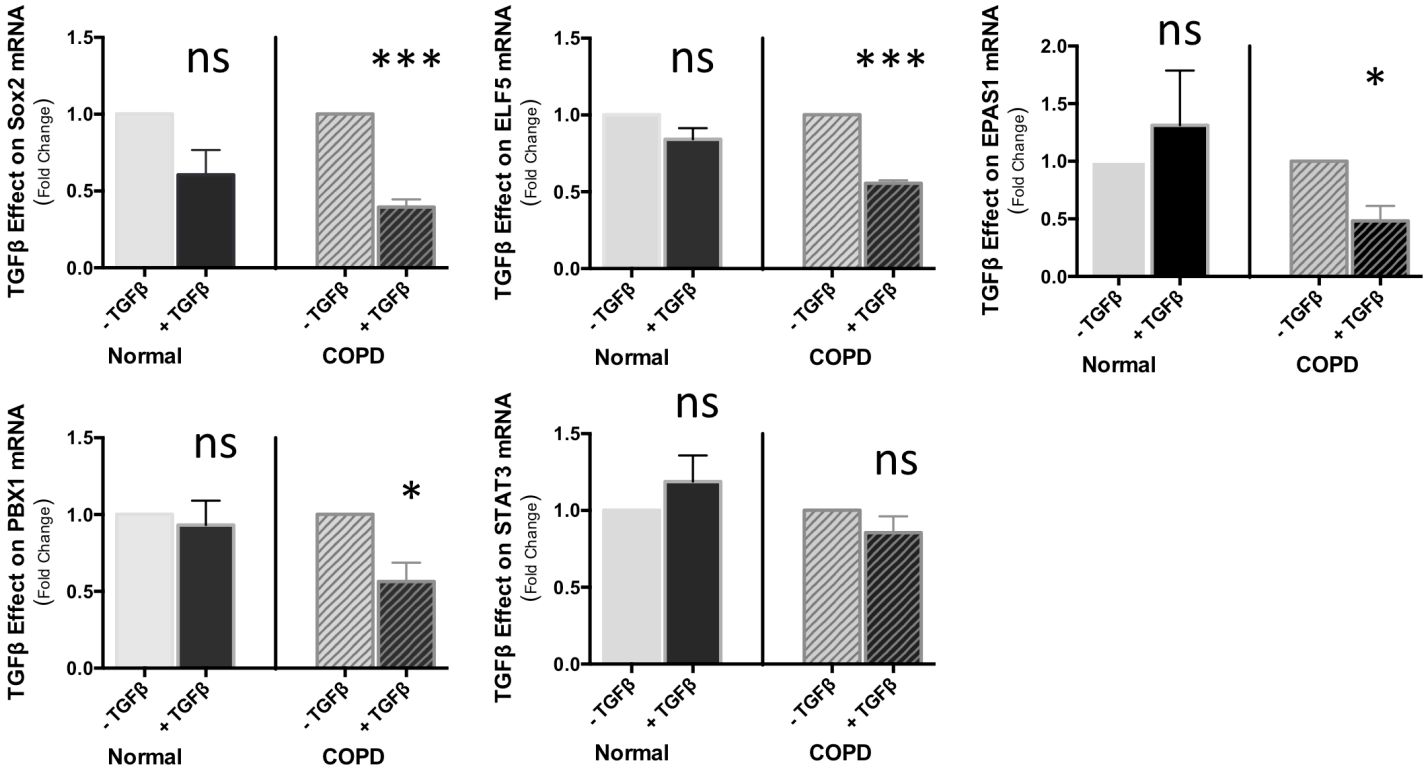
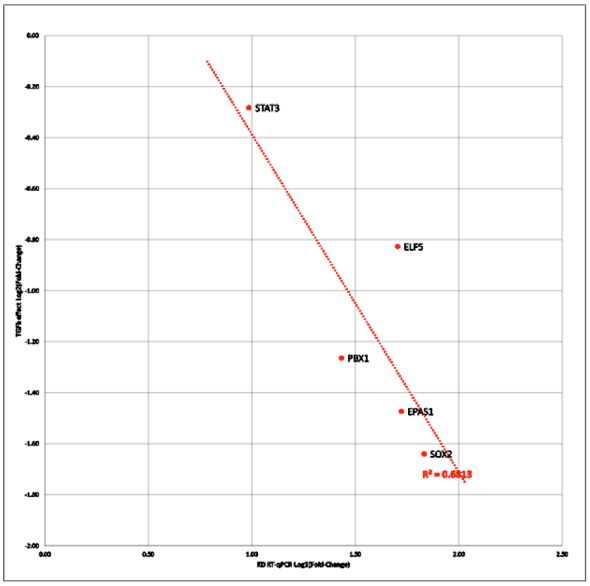

ELF5
SOX2
EPAS1
PBX1
STAT3
R² = 0.5519
R² = 0.7309
Microarray Log2(Fold-Change)
RT-qPCR Log2(Fold-Change)

Figure 2

a


TGFβ Effect on Sox2 mRNA
(Fold Change)
ns
***
TGFβ Effect on ELF5 mRNA
(Fold Change)
ns
***
TGFβ Effect on EPAS1 mRNA
(Fold Change)
ns
*
TGFβ Effect on PBX1 mRNA
(Fold Change)
ns
*
TGFβ Effect on STAT3 mRNA
(Fold Change)
ns
ns
- TGFβ
+ TGFβ
Normal
COPD

b


STAT3
ELF5
PBX1
EPAS1
SOX2
R² = 0.6813