Quantitative proteomic analysis in ATII cells reveals the different capacities of RAS and TGF-β to induce EMT

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Abstract

Alveolar type II (ATII) epithelial cells function as stem cells, contributing to alveolar renewal, repair and cancer. Therefore, they are a highly relevant model for studying a number of lung diseases, including acute injury, fibrosis and cancer, in which signals transduced by RAS and transforming growth factor (TGF)-β play critical roles. To identify downstream molecular events following RAS and/or TGF-β activation, we performed proteomic analysis using a quantitative label-free approach (LC-HDMS^E) to provide in-depth proteome coverage and estimates of protein concentration in absolute amounts. We chose ATII$^{ER:KRASV12}$ as an experimental cell line in which RAS is activated by adding 4-hydroxytamoxifen (4-OHT). Proteomic analysis of ATII cells treated with 4-OHT or TGF-β demonstrated that RAS activation induces an epithelial–mesenchymal transition (EMT) signature. In contrast, under the same conditions, activation of TGF-β signaling alone only induces a partial EMT. EMT is a dynamic and reversible biological process by which epithelial cells lose their cell polarity and down-regulate cadherin-mediated cell–cell adhesion to gain migratory properties, is involved in embryonic development, wound healing, fibrosis and cancer metastasis. Thus, these results could help to focus research on the identification of processes that are potentially driving EMT-related human disease.

1   Introduction

Fibrosis and lung cancer present a large medical burden with lung cancer being the leading cause of death in the United States in both men and women (Torre et al., 2016). Fibrotic lung diseases, such as idiopathic pulmonary fibrosis (IPF), present a growing problem with limited treatments and poor

36   prognosis (Richeldi et al., 2017; Hill et al., 2019a). Gas exchange in the lungs happens in alveolar
37   sacs which are lined with two epithelial cell types (squamous alveolar type I, ATI and surfactant-
38   secreting alveolar type II, ATII cells) (Pérez-Gil, 2008). It has been proposed that local signals are
39   able to regulate ATII epithelial cells, which are able to function as stem cells contributing to alveolar
40   renewal, repair and cancer (Desai et al., 2014). The integrity of these cell types is critical in the
41   modulation of a number of diseases including cancer and fibrosis (Desai et al., 2018).

42   Certain signaling pathways have been demonstrated to be key in mediating a number of processes
43   in lung-related disease. These signaling pathways have a diverse range of functions and subsequently
44   affect a diverse range of biological processes. They are also known to interact with each other to
45   influence their effects (Vallath et al., 2014; Aschner and Downey, 2016). Epidermal growth factor
46   receptor (EGFR) is a transmembrane receptor tyrosine kinase activated by members of the EGF
47   family (Linggi and Carpenter, 2006) and its pathway has been previously implicated in a number of
48   lung pathologies, including cancer and fibrosis (Burgel and Nadel, 2008; Vallath et al., 2014; Yao et
49   al., 2018). RAS is a downstream effector of EGFR and mutations in RAS have been widely reported
50   in a number of cancers and other diseases (Downward, 2003; Tzouvelekis et al., 2013; Stella et al.,
51   2014). RAS proteins switch between a GTP-bound "on-state" and a stable, GDP-bound "off-state";
52   this is mediated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors
53   (GEFs) which convert from active to inactive and inactive to active respectively (Simanshu et al.,
54   2017). Once active RAS stimulates a number of downstream signaling cascades which include
55   mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) (Downward,
56   2003). RAS signaling has been implicated in a number of cellular processes including proliferation,
57   differentiation and apoptosis (Downward, 1998; Cox and Der, 2003; Sun et al., 2015).

58   Transforming growth factor (TGF)-β is a well-studied, multifunctional profibrotic cytokine known
59   to play an important role in developmental biology (Shull et al., 1992; Kulkarni et al., 1993), cancer
60   (Massagué, 2008), fibrotic disease (Leppäranta et al., 2012; Yao et al., 2018) and many other lung
61   pathologies (Saito et al., 2018). Key mediators of the TGF-β family are Smads, which act together to
62   regulate transcription (Massagué, 1998). In turn, these are involved in a number of cellular context-
63   dependent processes including cell growth arrest (Mukherjee et al., 2010), proliferation (Carl et al.,
64   2016; Cheng et al., 2016), apoptosis (Schuster and Krieglstein, 2002), and epithelial–mesenchymal
65   transition (EMT) (Guo, 2017; Goldmann et al., 2018).

66   Our study utilized quantitative proteomic analysis to provide a comprehensive and unbiased
67   investigation into the role of RAS and TGF-β signaling in ATII cells. In this study we identified
68   EMT as a key pathway induced upon RAS-activation in ATII cells, but not in TGF-β-treated ATII
69   cells in the given time. ATII cells with both RAS- and TGF-β signaling induced, leads to an EMT
70   proteomic signature, which is mainly driven by RAS signaling. These findings are key in further
71   understanding the mechanisms underlying lung disease.

72   **2   Materials and Methods**

73   **2.1   Cell culture and reagents**

74   ATII$^{ER:KRASV12}$ cells (Molina-Arcas et al., 2013; Coelho et al., 2017; Yao et al., 2018) were
75   cultured in DCCM-1 (Biological Industries Ltd) supplemented with 10% newborn calf serum
76   (NBCS) (Life Technologies), 1% penicillin, 1% streptomycin and 1% L-glutamine (all from Sigma
77   Aldrich). Cells were kept at 37 °C and 5% CO_2. To induce RAS activation in ATII$^{ER:KRASV12}$ cells,
78   250 nM 4-OHT (Sigma-Aldrich) was added. TGF-β1 was from PeproTech. No mycoplasma

79 contamination was detected in the cell lines used. For wet lab experiments, treatment with both 4-
80 OHT (250 nM) and TGF-β1 (5 ng/ml) was for 24 hrs, unless otherwise indicated in figure. For
81 proteomics cell culture, ATII$^{ER:KRASV12}$ cells were cultured as indicated above. Each treatment group
82 (control, 4-OHT, TGF-β1 and combined (TGF-β1 & 4-OHT)) was conducted in triplicate and cells
83 were treated for 24 hrs with indicated treatments prior to cell lysis.

84 ## 2.2   Western blot analysis

85 Western blot analysis was performed with lysates from cells with urea buffer (8 M Urea, 1 M
86 Thiourea, 0.5% CHAPS, 50 mM DTT, and 24 mM Spermine). Primary antibodies were from: Santa
87 Cruz (E-cadherin, sc-21791; ZEB1, sc-25388; Snail2, sc-10436), Abcam (β-tubulin, ab6046), Cell
88 Signaling Technology (phospho-AKT, 9271; phospho-ERK, 9101; Snail1, 3879; Snail2, 9585;
89 Phospho-Smad2, 3104; β-tubulin, 86298), and BD Transduction Laboratories (E-cadherin, 610405).
90 Signals were detected using an Odyssey imaging system (LI-COR), and evaluated by ImageJ 1.42q
91 software (National Institutes of Health).

92 ## 2.3   qRT-PCR

93 Total RNA was isolated using RNeasy mini kit (Qiagen) according to manufacturer's instructions
94 and quantified using a Nanodrop Spectrophotometer 2000c (Thermo Fisher Scientific). Real-time
95 quantitative RT-PCR was carried out using gene-specific primers (QuantiTect Primer Assays,
96 Qiagen) for *CDH1* (E-cadherin) (QT00080143), *SNAI1* (Snail1) (QT00010010), *SNAI2* (Snail2)
97 (QT00044128), *ZEB1* (QT00008555), *ZEB2* (QT00008554), *TWIST* (QT00011956), *VIM*
98 (QT00095795), *TGFBR1* (QT00083412), *TGFBR2* (QT00014350), *TGFBR3* (QT00083223) or
99 *ACTB* (β -actin) (QT01680476) with QuantiNova SYBR Green RT-PCR kits (Qiagen). Relative
100 transcript levels of target genes were normalized to *ACTB* (β-actin).

101 ## 2.4   Proteomics sample preparation

102 Samples were lysed in 0.1M ammonium bicarbonate containing 1% SDS followed by pulsed
103 sonication. Lysates were then centrifuged at 13,000 x g, for 20 min at 4 °C, supernatant removed and
104 protein concentration measured using a DirectDetect spectrometer (Merck, UK).

105 Methanol/chloroform extraction was then performed on 25μg of protein for each lysate. Samples
106 were prepared in 100μl using lysis buffer (0.1M ammonium Bicarbonate). Samples were reduced
107 with 1mM Dithiothreitol (DTT, Thermo Scientific) at 56°C for 60 min. Samples were subsequently
108 alkylated using 5.5 mM Iodoacetamide (Sigma) and incubated in the dark at room temperature for 45
109 min. Samples were digested by addition of 2μg sequencing grade modified trypsin (Promega) and
110 incubation overnight at 37°C. After digestion, samples were lyophilized in vacuo.

111 Enolase protein digest internal standard (Waters) was spiked into each cellular peptide sample at a
112 concentration of 150 fmol prior to isoelectric focusing by OFFGEL fractionation using a 3-10 pH
113 gradient (Agilent) into 12 peptide fractions. Each fraction was purified using an Empore™ C18 solid
114 phase extraction plate (ThermoScientific Pierce) to remove residual salts, buffers and contaminants
115 before lyophilization and resuspension in loading buffer (3% acetonitrile + 0.1% formic acid) for
116 mass spectrometry analysis.

117 Separations were performed using a nanoAcquity UPLC system (Waters). Peptide digests were
118 injected onto a Symmetry C18, 180μm x 20mm trapping cartridge (Waters). After 5 min washing of

119 the trap column, peptides were separated using a 75µm i.d. x 500mm, 1.7µm BEH130 C18, column
120 (Waters) using a linear gradient of 5 to 40% B (buffer A = 0.1% formic acid in water, buffer B =
121 0.1% formic acid in acetonitrile) over 90 min with a wash to 85% B at a flow rate of 300 nl/min. All
122 separations were automated, performed on-line and sprayed directly into the nanospray source of the
123 mass spectrometer.

124 All mass spectrometry was performed using a Waters G2-S Synapt HDMS mass spectrometer
125 operating in MS^e mode. Data was acquired from 50 to 2000 m/z with ion mobility enabled using
126 alternate low and high collision energy (CE) scans. Low CE was 5V and elevated, ramped from 20-
127 40V. The lock mass Glu-fibrinopeptide, $(M+2H)^{+2}$, m/z = 785.8426) was infused at 300 nl/min at a
128 concentration of 200 fmol/µl and acquired every 13 seconds.

129 Raw mass spectra were processed using ProteinLynx Global Server Ver 3.0 (Waters, Manchester,
130 UK) enabled using an in-house developed script and the data processed to generate reduced charge
131 state and de-isotoped precursor and associated product ion mass lists. These peak lists were searched
132 against the human UniProt protein sequence (downloaded March 2017). A maximum of one missed
133 cleavage was allowed for tryptic digestion and the variable modification was set to contain oxidation
134 of methionine and carboxyamidomethylation of cysteine was set as a fixed modification. The false
135 discovery rate (FDR) was estimated with decoy-fusion database searches and were filtered to 1%
136 FDR.

137 **2.5   Proteomics sample normalization and imputation**

138 Raw data was imported into RStudio software (version 1.1.456) for down-stream analysis.
139 Expression values of proteins in femtomole (fmol) were extracted and normalized using the variance
140 stabilization normalization (Vsn) method (Valikangas et al., 2018a). The Vsn method was
141 implemented using the justvsn function of the Vsn package in R. This method was utilized as a recent
142 review of normalization methods for use in quantitative label-free proteomics determine it reduced
143 variation most between technical replicates compared to other tested methods (Huber et al., 2002).
144 Normalized data were transformed into log2 format. Missing values were imputed using local least
145 squares imputation (Lls) with parameter value 150 (Valikangas et al., 2018b), using pcaMethods
146 package (Stacklies et al., 2007).

147 Proteins had to be observed in at least 2 of 3 replicates to be used in experiment. Samples were
148 normalized and missing values imputed, before PCA (Supplementary Figure. S3) validated samples
149 clustered appropriately, differentially expressed proteins were analyzed by hierarchal clustering and
150 visualized on a heatmap (Supplementary Figures. S1b & S2b).

151 The limma package (v. 3_34.2) was utilized to detect differentially expressed proteins (Ritchie et
152 al., 2015). Differentially expressed proteins (DEPs) were determined by P value < 0.05 and log$_2$ [Fold
153 Change] > 0.5. All expression data after limma is available in Supplementary Tables S1, S3 and S5.
154 These are the top table export from limma, further details about these can be found limma user guide
155 (Ritchie et al., 2015).

156 **2.6   Pathway analysis**

157 Prior to pathway analysis protein symbols were converted to gene symbols in RStudio v 3.6.1.
158 Enrichment analyses were generated by Metascape (Zhou et al., 2019), targets were analyzed with
159 hallmark annotation.

160   **2.7   Statistical analysis and repeatability of experiments**

161   Proteomics experiments were performed in triplicate. Unless otherwise noted, data are presented as
162   mean and s.d., and a two-tailed, unpaired or paired Student's *t*-test was used to compare two groups
163   for independent samples, with the n number for each experiment included in the figure legends. *P* <
164   0.05 was considered statistically significant.

165   **3   Results**

166   **3.1   RAS-activation in ATII cells induces EMT**

167   To characterize the proteome-wide altered expression in ATII cells, we performed quantitative
168   mass spectrometry. We utilized a RAS-inducible model in ATII$^{ER:KRASV12}$ cells (Molina-Arcas et al.,
169   2013; Coelho et al., 2017; Yao et al., 2018). KRASV12 (containing a single amino acid mutation in
170   KRAS, glycine to valine at position 12) fused to the oestrogen receptor (ER) ligand-binding domain
171   was introduced into ATII cells to generate ATII$^{ER:KRASV12}$, where RAS pathway is activated by
172   addition of 4-hydroxytamoxifen (4-OHT). Mass spectrometry identified 1844 proteins, of these 182
173   were determined to be differentially expressed proteins (DEPs) (P value < 0.05, log$_2$(fold
174   change[FC]) ≥ 0.5) (Supplementary Table S1).

175   RAS signaling is important in a number of different processes. In order to determine which
176   pathways are altered when ATII cells undergo RAS activation, pathway analysis was conducted (Fig.
177   1a). Hallmark epithelial mesenchymal transition (EMT) was identified as one of the key pathways
178   altered upon RAS activation in ATII cells (Fig. 1a, q-value = 0.0007, shared genes = 8). A full list of
179   altered pathways, is included in Table 1, including shared genes identified in analysis.

180   RAS-activation has previously been demonstrated to be instrumental in the induction of EMT
181   (Wang et al., 2010; Arner et al., 2019). To evaluate the role of RAS-activation in ATII cells on EMT
182   processes, a selection of previously described Hallmark EMT proteins (Subramanian et al., 2005)
183   were investigated in our proteomics dataset. Of the identified 1844 proteins, 33 identified were EMT-
184   related proteins, as assessed by the Hallmark EMT list. Of the 33 EMT proteins found, 8 were
185   differentially expressed between control and RAS-activated, as indicated by red text (P value < 0.05,
186   log$_2$ FC ≥ 0.5) (Fig. 1b). All 33 EMT-proteins identified in proteomics data were visualized on a
187   heatmap, a clear separation is observed between RAS-activated (4-OHT) and Control (Fig. 1b),
188   suggesting a clear role for EMT in RAS-activated ATII cells. Further, all samples have been
189   visualized on a volcano plot (Supplementary Fig. S1a) and a heat map showing DEPs
190   (Supplementary Fig. S1b). Significantly up- and down- regulated EMT-proteins have been identified
191   (Supplementary Fig. S1a; Supplementary Table. S2).

192   **3.2   TGF-β does not induce a Hallmark EMT in proteomics pathway analysis**

193   Given the extensive role of TGF-β in numerous lung pathologies, determining which pathways are
194   altered in alveolar epithelial cells could be key in elucidating disease pathology. Proteomic analysis
195   was also conducted on the ATII cells treated with and without TGF-β (5 ng/ml for 24 hrs). Mass
196   spectrometry identified 1858 proteins, of which 90 were differentially expressed (DEPs) (P Value <
197   0.05, Log$_2$FC ≥ 0.5) (Supplementary Table S3).

198   Downstream pathway analysis of TGF-β treated ATII cells did not indicate Hallmark EMT to be
199   significantly induced (Fig. 2a, q value = 0.12). All significantly altered pathways have been
200   indicated, in addition to Hallmark EMT. A full list of altered pathways is included in Table 2,

201   including shared genes found in the analysis. Hallmark EMT processes did share three common
202   proteins with the dataset, but these were not significantly altered.

203   The same group of Hallmark EMT proteins, as previously described, were compared to identified
204   proteins and 38 EMT proteins were identified, 3 were differentially expressed (P value < 0.05, $\log_2$
205   FC ≥ 0.5) (all were up-regulated) (Supplementary Table. S4). A heat map illustrates all Hallmark
206   EMT proteins which were identified in the proteomics dataset based on hierarchal clustering and
207   visualized on a heatmap, DEPs are highlighted by red text (Fig. 2b). Clustering TGF-β samples based
208   on Hallmark EMT proteins did not cause defined separation, with Control_3 clustering with TGF-β
209   samples. Further, there was not a defined split in up- and down- regulated EMT proteins. A volcano
210   plot demonstrates all differentially expressed proteins (Supplementary Fig. S2a), the three EMT-
211   proteins which were significantly altered are labelled (THBS1, ITGAV and TGFB1). Further a heat
212   map shows all DEPs upon TGF-β treatment (Supplementary Fig. S2b).

213   3.3   RAS-activation dominates over TGF-β in driving EMT in ATII cells

214   Given the different EMT response to RAS and TGF-β signalling activation we wanted to determine
215   the effect of dual treatment and to elucidate if one pathway dominated in EMT-induction. In ATII
216   cells with RAS and TGF-β signalling active, mass spectrometry identified 1793 proteins, of which
217   123 were differentially expressed between control and treatment (P value < 0.05, $\text{Log}_2\text{FC} \geq 0.5$)
218   (Supplementary Table. S5).
219
220   We previously observed Hallmark EMT to be a significant pathway for RAS-activated (Fig. 1a, q-
221   Value= 0.0005) but not in TGF-β treatment alone (Fig. 2a, q-Value = 0.12), hence we wanted to
222   evaluate the effect RAS-activation together with TGF-β treatment on ATII$^{ER:KRASV12}$ cells. The same
223   group of Hallmark EMT proteins, as previously described, were compared to identified proteins and
224   35 EMT proteins were identified, 5 were differentially expressed (P value < 0.05, $\log_2$ FC ≥ 0.5)
225   (Supplementary Table. S6). Pathway analysis identified Hallmark EMT (Fig. 3a, q-Value = 0.01)
226   with 5 shared proteins. A full list of altered pathways is included in Table 3, including shared genes
227   found in the analysis. When all Hallmark EMT proteins are visualized using a heat map based on
228   hierarchal clustering, a more defined separation is observed, where DEPs are signified by red text
229   (Fig. 3b), compared with TGF-β treatment alone (Fig. 2b). Dendrograms show a clear divide between
230   control and RAS-activated/TGF-β treatment, however Control_3 appears to cluster separately from
231   other control samples. We previously confirmed a global view of sample uniformity by PCA
232   (Supplementary Fig. S3). These results suggest that hallmark EMT is also induced in RAS-
233   activated/TGF-β treated ATII$^{ER:KRASV12}$ cells.
234
235   Quantitative proteomic analysis identified different signatures of EMT in each treatment (RAS-
236   activated, TGF-β treatment alone and combined RAS activation with TGF-β treatment) and we
237   wanted to determine if a certain signalling pathway dominated. A chi-squared test (Fig. 3c)
238   demonstrated that when comparing A and B (A- RAS-activation vs RAS- activation with TGF-β, or
239   B- TGF-β alone vs RAS- activation with TGF-β), there is a significant number of uniquely
240   differentially expressed proteins (P value = 0.0494). This analysis suggested that RAS-activation (4-
241   OHT) dominates in the combined treatment of ATII$^{ER:KRASV12}$ cells.

242   3.4   RAS-activation, but not TGF-β, results in reduction in E-cadherin in ATII cells

243   We first demonstrated the presence of TGF-β receptor 1 (*TGFBR1*) and 2 (*TGFBR2*) in ATII cells
244   (Supplementary Fig. 4). In addition, phospho-Smad2 (p-Smad2) levels were increased upon TGF-β
245   treatment (Fig. 4a). These results suggested an intact TGF-β signalling in these cells. To confirm the
246   findings from our proteomic analysis, which identified induction of Hallmark EMT in RAS-activated
247   samples but not in TGF-β treatment alone, we sought to validate protein levels of a number of EMT
248   markers by western blot (Fig. 4a). At the time points utilised (t=24h) protein levels of E-cadherin, a
249   key cell-cell adhesion molecule and an epithelial marker, was significantly reduced upon RAS-
250   activation and RAS-activation together with TGF-β treatment. However, where ATII$^{ER:KRASV12}$ cells
251   were treated with just TGF-β, a reduction in E-cadherin was not observed. The levels of tight
252   junction proteins ZO1 and ZO2 were also investigation in our proteomics dataset (Supplementary
253   Fig. 5). No significant differences were observed in ZO1, however ZO2 was significantly reduced
254   upon RAS-activation and TGF-β treatment. We then examined the levels of a number of EMT-
255   Transcription Factors (EMT-TF) were altered in all treatments; as previously described (Yao et al.,
256   2018) upon RAS-activation ZEB1 was increased, TGF-β treatment led to up-regulation of Snail1 and
257   dual activation of RAS and TGF-β treatment led to up-regulation of both ZEB1 and Snail1. Further,
258   after 24 hrs, phase contrast images of RAS-activated ATII$^{ER:KRASV12}$ cells show the change to a
259   spindle-like morphology from the cuboidal morphology of controls, within the same time frame less
260   pronounced changes are observed by TGF-β treatment (Fig. 4b).

261   Having examined the effect of each treatment at one time point (t=24h), we next evaluated whether
262   alterations in E-cadherin protein levels were time-dependent. A time course of RAS-activated (4-
263   OHT) and TGF-β treated ATII$^{ER:KRASV12}$ cells determined protein levels of E-cadherin at a number of
264   times points up to 72 hrs (Fig. 4c). E-cadherin protein levels remain unaltered with TGF-β treatment
265   at all time points examined, however reduction in E-cadherin is evident in RAS-activated ATII cells
266   from 12 hrs, with a large reduction observed at 36 hrs. To further elucidate the potential role of TGF-
267   β in EMT in ATII$^{ER:KRASV12}$ cells, mRNA levels of a number of EMT markers were evaluated (Fig.
268   4d). Interestingly, levels of *VIM* (Vimentin), a structural protein mainly expressed in mesenchymal
269   cells, were increased with TGF-β. Whilst, *CDH1* (E-cadherin) levels increased upon TGF-β
270   treatment. We evaluated a number of EMT-TFs and of these only *SNAI1* (Snail1) was increased.
271   *SNAI1* is important in the direct repression of E-cadherin, however levels of *CDH1* (E-cadherin) and
272   protein levels of E-cadherin are not down-regulated, suggesting *SNAI1* may mediate some form of
273   partial EMT in these cells.

274   **4   Discussion**

275   ATII epithelial cells are able to function as stem cells and subsequently contribute to a number of
276   processes such as renewal, repair and the development of cancer (Desai et al., 2014). A number of
277   key signaling pathways have been implicated in lung disease and to better understand their roles we
278   performed quantitative label-free approach (LC-HDMS^E), to compare the capacity of these signaling
279   pathways. ATII cells with RAS activated and/or TGF-β treatment were utilized and hallmark
280   pathways identified. RAS-activation alone was sufficient to induce an EMT signature. In
281   comparison, activation of TGF-β signaling was insufficient to induce an EMT signature in the time
282   period. When both RAS- and TGF-β- signaling were activated an EMT signature was induced and
283   this was driven by RAS-signaling.

284   EMT is a well-established concept, it requires the involvement of a number of signaling pathways
285   (Thiery and Sleeman, 2006) and the induction of EMT varies significantly in different tissues and
286   diseases (Kalluri and Weinberg, 2009). As described, a number of signaling factors are involved in

287   the induction of EMT, including TGF-β, fibroblast growth factor (FGF), Wnt/β-catenin and EGF. In
288   turn, these can regulate the expression of a group of EMT specific transcription factors termed EMT-
289   TF which are capable of orchestrating the induction of EMT, these include Snail1/2, ZEB1/2 and
290   some basic helix-loop-helix (bHLH) factors. EMT-TFs have been demonstrated to be potent
291   repressors of E-cadherin (Peinado et al., 2007; Nieto, 2011), which is considered to be a key step in
292   the induction of EMT (Wheelock et al., 2008; Lamouille et al., 2014).

293   Previous studies have begun to compare the signaling mechanisms which drive EMT in malignancy
294   (Taube et al., 2010), here we examine the mechanisms which drive EMT in the lung. EMT has roles
295   in developmental biology, cancer and fibrosis and wound healing (Thiery et al., 2009; Barriere et al.,
296   2015). In cancer, EMT has been demonstrated as a mechanism which allows cancer cells to
297   metastasize to secondary tumor sites (Li and Balazsi, 2018). In fibrosis, the role of EMT is
298   controversial. IPF is a chronic, interstitial lung disease which lacks both effective treatment and a
299   clear understanding of underlying disease mechanisms (Richeldi et al., 2017; Hill et al., 2019a). A
300   number of studies have proposed that fibroblasts are derived from epithelial cells by EMT. However,
301   recent studies in a number of fibrotic models have suggested that although EMT may not contribute
302   directly to the fibroblast pool, it does augment fibrosis through secreted factors (Grande et al., 2015;
303   Lovisa et al., 2015; Yao et al., 2018; Hill et al., 2019a; Hill et al., 2019b). Both EGFR (Burgel and
304   Nadel, 2008; Cully and Downward, 2008; Vallath et al., 2014) and TGF-β have been demonstrated as
305   key players in lung disease (Tatler and Jenkins, 2012; Saito et al., 2018).

306   TGF-β is responsible for a wide range of functions (Schuster and Krieglstein, 2002; Mukherjee et
307   al., 2010; Carl et al., 2016; Cheng et al., 2016; Guo, 2017; Goldmann et al., 2018) and elucidating
308   pathways which are altered in the lung upon its activation could be key in determining underlying
309   mechanisms of lung disease. A number of studies have demonstrated TGF-β-induced EMT in the
310   lung (Kasai et al., 2005; Chen et al., 2016). These studies have modelled EMT using A549 lung cells
311   which harbor a *KRAS* mutation (Yoon et al., 2010), and also utilized a longer treatment time (Kasai et
312   al., 2005; Chen et al., 2016). Primary ATII cells treated with TGF-β for 1 week were unable to
313   induce EMT as assessed by reduction in E-cadherin, whereas treatment with EGF led to a reduction
314   in E-cadherin protein levels suggesting induction of EMT (Yao et al., 2018). A recent microarray
315   study also utilizing primary alveolar epithelial cells suggest EMT induction upon TGF-β treatment,
316   however there was no reduction in traditional EMT markers E-Cadherin or tight junctions
317   (Goldmann et al., 2018). *KRAS* mutations occur in around 25-35% of cases of lung cancer (Kempf et
318   al., 2016). Taking all these findings together we suggest that in a *KRAS*-driven environment, as is
319   common in many cancers, EMT is induced more potently by TGF-β. Here, we compare RAS-
320   activated ATII [ER:KRASV12] cells together with TGF-β treatment with TGF-β alone; and it appears that
321   EMT is induced to a greater extent than TGF-β alone. 'Hallmark EMT' was identified as a top hit in
322   the RAS-active proteomics analysis, and western blot analysis suggests that EMT was induced more
323   potently than the TGF-β treated ATII [ER:KRAS V12] cells. Here we demonstrated in ATII cells that in
324   this time period (24 hrs) there was no significant reduction in E-cadherin protein level. Using
325   unbiased, quantitative proteomic analysis we demonstrated that TGF-β was unable to induce a
326   complete EMT signature in ATII cells in the given period and pathway analysis did not identify EMT
327   as a pathway in ATII cells with TGF-β.

328   In comparison, RAS pathway activation was able to induce a hallmark EMT signature in ATII
329   cells. In lung fibrosis, the EGFR pathway, upstream of RAS, has been implicated with a number of
330   studies demonstrating mice expressing TGF-α develop lung fibrosis (Korfhagen et al., 1994; Hardie
331   et al., 1997) and those lacking EGFR to be resistant to bleomycin induced fibrosis (Madtes et al.,

332 1999). Clinically, IPF patients have been demonstrated to have EGFR mutations (Stella et al., 2014)
333 or increased expression of EGFR (Tzouvelekis et al., 2013). We have previously demonstrated in
334 ATII cells that EMT is induced via the EGFR-RAS-ERK pathway via ZEB1, and this mediated
335 fibrosis via paracrine signaling (Yao et al., 2018). We propose that in the same manner that RAS-
336 induced EMT in ATII $^{ER:KRAS\ V12}$ cells is via ZEB1, partial EMT is induced by TGF-β by Snail1
337 (Dang et al., 2011; Batlle et al., 2013; Li et al., 2014). As such, identifying the underlying
338 mechanisms and more potential biomarkers/drug targets is clinically beneficial. Unbiased proteomic
339 analysis of RAS-activated ATII cells identified a hallmark EMT signature, validated by protein and
340 mRNA levels of a number of EMT markers. These findings are consistent with published literature
341 on RAS-induced EMT in ATII cells (Yao et al., 2018).

342 Given the important roles of both RAS signaling and TGF-β in the pathogenesis of a number of
343 lung diseases, including cancer and fibrosis, understanding the capacity of their signaling to induce
344 different processes is particularly important (Kempf et al., 2016; Richeldi et al., 2017). Both have
345 been proposed as potent inducers of EMT (Lamouille et al., 2014; Nieto et al., 2016), and it has been
346 suggested in other contexts that when working together these can cooperate to have a synergistic
347 effect through a crosstalk effect (Oft et al., 1996; Oft et al., 1998; Iglesias et al., 2000; Park et al.,
348 2000; Gotzmann et al., 2002; Janda et al., 2002; Bates and Mercurio, 2003; Safina et al., 2009), and
349 in the context of malignancy, RAS and TGF-β can act together to regulate epithelial cell plasticity
350 (Janda et al., 2002). In a recent study in mammary epithelial cells, it was shown that when exogenous
351 TGF-β was not present, inhibition of RAF/MEK/ERK was able to prevent EMT, but when exposed
352 to TGF-β it is able to shortcut MEK to transition to mesenchymal cells (McFaline-Figueroa et al.,
353 2019). An investigation into the transcriptomic response to TGF-β after 48h found in human primary
354 ATII cells both EMT and KRAS signaling to be induced (Goldmann et al., 2018). In ATII cells when
355 RAS and TGF-β signaling are both induced, a hallmark EMT signature was identified and this was
356 driven by RAS but a synergistic effect is not observed.

357 Taken together, these results indicate RAS activation to be a key inducer of EMT in the time period
358 tested, whereas TGF-β does not seem to induce a complete EMT. When taken in the context of
359 existing studies, it is clear that both time- and dose- of TGF-β treatment are particularly important
360 when determining effect on EMT phenotype. Both TGF-β and RAS-activation are key signaling
361 cascades in the pathogenesis of IPF, and despite studies in other contexts demonstrating a
362 cooperation between these pathways, in ATII it appears RAS-activation drives EMT. Whilst the exact
363 details of these mechanism are yet to be fully-elucidated in the context of IPF, understanding the
364 interactions between these pathways will be helpful in the discovery of potential drug targets which
365 may prevent the progression of fibrosis.

5   Conflict of Interest

6   Author Contributions

7   Funding

8   References

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603

604 **9   Figure Legends**

605 9.1   **Figure 1 RAS-activation in ATII cells induces Hallmark EMT proteomic signature** (a)
606   Pathway analysis in Metascape (Metascape.com) using hallmark EMT dataset analyzing
607   differentially expressed proteins (DEPs) in ATII[ER:KRASV12] cells treated with 250 nM 4-OHT or
608   control for 24 hrs, all non-significant pathways have been removed from diagram. (b) Heatmap
609   and hierarchical cluster analysis of ATII[ER:KRASV12] cells treated with 250 nM 4-OHT (RAS-
610   activated) compared with control for 24 hrs, a panel of hallmark EMT proteins were evaluated.
611   P-Value: (blue: P-value <0.05, pink: P-value≥ 0.05). Log_2 fold change: (blue: $Log_2FC \geq 0.5$,
612   pink: $Log_2FC <0.5$,). Red indicates differentially expressed protein (DEP). Where Log_2FC
613   denotes absolute Log_2 fold change.

614 9.2   **Figure 2 TGF-β in ATII cells does not induce a classical EMT programme at a given time**
615   **point.** (a) Pathway analysis in Metascape (Metascape.com) using hallmark EMT dataset
616   analyzing differentially expressed proteins (DEPs) in ATII[ER:KRASV12] cells treated with 5ng/ml
617   TGF-β or control for 24hrs, all non-significant pathways have been removed from diagram
618   with the exception of epithelial mesenchymal transition (EMT) which was not significant and
619   has been highlighted in bold. (b) Heatmap and hierarchical cluster analysis of ATII[ER:KRASV12]
620   cells treated with 5ng/ml TGF-β or control treated for 24 hrs, a panel of hallmark EMT proteins
621   were evaluated. P-Value: (blue: P-value <0.05, pink: P-value≥ 0.05). Log_2 fold change: (blue:
622     $Log_2FC \geq 0.5$, pink: $Log_2FC <0.5$). Red indicates differentially expressed protein (DEP).
623   Where Log_2FC denotes absolute Log_2 fold change.

624 9.3   **Figure 3 RAS-activation together with TGF-β are capable of inducing EMT and RAS**
625   **signaling appears to drive EMT.** (a) Pathway analysis in Metascape (Metascape.com) using

626 hallmark EMT dataset analyzing differentially expressed proteins (DEPs) in ATII[ER:KRASV12]
627 cells treated with a combined treatment of 250 nM 4-OHT and 5ng/ml TGF-β or control for
628 24hrs, all non-significant pathways have been removed from diagram. (b) Heatmap and
629 hierarchical cluster analysis of ATII[ER:KRASV12] cells treated with a combined treatment of 250
630 nM 4-OHT and 5ng/ml TGF-β or control for 24hrs, a panel of hallmark EMT proteins were
631 evaluated. P-Value: (blue: P-value <0.05, pink: P-value ≥ 0.05). Log_2 fold change: (blue:
632 Log_2FC ≥ 0.5, pink: Log_2FC <0.5,). Red indicates differentially expressed protein (DEP).
633 Where Log_2FC denotes absolute Log2 fold change. (c) Diverging bar chart indicating number
634 of uniquely differentially expressed proteins in indicated treatments (Chi-squared test, P-value
635 = 0.0494).

636 9.4 **Figure 4 Unlike like RAS-activation, TGF-β is insufficient to induce EMT-like changes at**
637 **protein level at 24hrs in ATII[ER:KRASV12] cells.** (a) Protein expression of E-cadherin, ZEB1,
638 Snail1 Snail2, p-ERK, p-AKT, p-Smad2 in ATII[ER:KRASV12] cells with indicated treatments at
639 24hr. β-tubulin was used as a loading control. n=2 (b) Phase contrast images of ATII[ER:KRASV12]
640 cells with indicated treatment for 24hrs. (c) Protein expression of E-cadherin, p-ERK, p-Smad2
641 in ATII[ER:KRASV12] cells with 250 nM 4-OHT or 5ng/ml TGF-β at indicated time points, β-
642 tubulin was used as a loading control. n=2 (c) Fold change in the mRNA level of *CDH1* (E-
643 cadherin), *VIM* (Vimentin), *SNAI1*, *SNAI2*, *TWIST*, *ZEB1*, *ZEB2* in ATII[ER:KRASV12] cells with
644 indicated treatment for 24hrs. *ACTB* (β-actin)-normalized mRNA levels in control cells were
645 used to set the baseline value at unity. Data are mean ± s.d. n = 3 samples per group. ** P <
646 0.01. *** P < 0.001.

647 **10   Tables**

648 **10.1 Table 1**

649 **Table 1: Hallmark pathways identified using Metascape for differentially expressed proteins**
650 **between control and RAS-activated ATII cells.**

Hallmark pathways	Log (q-value)	Shared genes	Number of shared genes	UNI PROT ID
Hallmark myc targets v1	-5.900	CANX, EEF1B2, ETF1, XRCC6, KPNB1, MCM5, NAP1L1, PGK1, SYNCRIP, RUVBL2, EXOSC7, LSM2	12/200	P27824, P24534, P62495, P12956, Q14974, P33992, P55209, P00558, O60506, Q9Y230, Q15024, Q9Y333
Hallmark IL2 STAT5 signalling	-3.160	ENO3, ITGA6, ITGAV, PNP, PLEC, TGM2, NDRG1, AKAP2	8/200	P13929, P23229, P06756, P00491, Q15149, P21980, Q92597, Q9Y2D5

Hallmark epithelial mesenchymal transition	-3.160	CALU, ITGAV, TGM2, THBS1, TPM1, TPM2, PDLIM4, BASP1	8/200	O43852, P06756, P21980, P07996, P09493, P07951, P50479, P80723
Hallmark myogenesis	-3.160	ACTN3, DES, NQO1, ENO3, MYH2, MYH9, TPM2, TPM3	8/200	Q08043, P17661, P15559, P13929, Q9UKX2, P 35579, P07951, P06753
Hallmark MTORC1 signalling	-3.160	CANX, DHCR7, ETF1, PNP, PGK1, PSMB5, RRM2, STIP1	8/200	P27824, Q9UBM7, P62495, P00491, P00558, P28074, P31350, P31948
Hallmark androgen response	-3.949	ACTN1, XRCC6, ITGAV, TPD52, AKAP12, NDRG1, ADRM1	7/101	P12814, P12956, P06756, P55327, Q02952, Q92597, Q16186
Hallmark hypoxia	-2.510	ENO3, MYH9, PGK1, TGM2, TPD52, AKAP12, NDRG1	7/200	P13929, P35579, P00558, P21980, P55327, Q02952, Q92597
Hallmark mitotic spindle	-2.510	FLNB, MARCKS, MYH9, NUMA1, CLIP1, FSCN1, ANLN	7/200	O75369, P29966, P35579, Q14980, P30622, Q16658, Q9NQW6
Hallmark oxidative phosphorylation	-2.510	DLST, ETFB, IDH3A, NDUFV2, PMPCA, TOMM22, GRPEL1	7/200	P36957, P38117, P50213, P19404, Q10713, Q9NS69, Q9HAV7
Hallmark G2M checkpoint	-1.897	DR1, KPNB1, MARCKS, MCM5, NUMA1, SYNCRIP	6/200	Q01658, Q14974, P29966, P33992, Q14980, O60506
Hallmark DNA repair	-1.791	PNP, POLR2H, EDF1, ADRM1, AK3	5/150	P00491, P52434, O60869, Q16186, Q9UIJ7
Hallmark UV response up	-1.766	CDC5L, DNAJA1, POLR2H, STIP1, GRPEL1	5/158	Q99459, P31689, P52434, P31948, Q9HAV7

Hallmark interferon alpha response	-5.900	ADAR, CD47, TRIM25, IFITM2	4/97	P55265, Q08722, Q14258, Q01629
Hallmark reactive oxygen species pathway	-4.809	NQO1, TXN, OXSR1	3/49	P15559, P10599, O95747

651 **10.2 Table 2**

652 **Table 2: Hallmark pathways identified using Metascape for differentially expressed proteins**
653 **between control and TGF-β-treated ATII cells.**

Hallmark pathway	Log (q-value)	Shared genes	Number of shared genes	UNIPROT ID
Hallmark protein secretion	-4.507	KRT18, TPD52, STX7	3/96	P05783, P55327, O15400
Hallmark interferon alpha response	-4.507	CD47, TRIM25, IFITM2	3/97	Q08722, Q14258, Q01629
Hallmark androgen response	-2.115	ACTN1, ITGAV, TPD52, RRP12	4/101	P12814, P06756, P55327, Q5JTH9
Hallmark hypoxia	-2.115	ENO3, MYH9, PGK1, TGFBI,T PD52	5/200	P13929, P35579, P00558, Q15582, P55327
Hallmark apical junction	-2.115	ACTB, ACTN1, DLG1, MYH9, TGFBI	5/200	P60709, P12814, Q12959, P35579, Q15582
Hallmark glycolysis	-2.115	PGK1, PKM, PYGB, TGFBI, TXN	5/200	P00558, P14618, P11216, Q15582, P10599
Hallmark DNA repair	-1.763	POLR2H, RALA, ALYREF, SF3A3	4/150	P52434, P11233, Q86V81, Q12874

| Hallmark myc targets v1 | -1.475 | EEF1B2, KPNB1, PGK1, SYNCRIP | 4/150 | P24534, Q14974, P00558, O60506 |
| Hallmark epithelial mesenchymal transition | -0.916 | THBS1,ITGAV,TGFBI | 3/200 | P07996, P06756, Q15582 |

654

655 **10.3 Table 3**

656 **Table 3: Hallmark pathways identified using Metascape for differentially expressed proteins**
657 **between control and TGF-β-treated RAS-activated ATII cells.**

Hallmark pathways	Log (q-value)	Shared genes	Number of shared genes	UNIPROT ID
Hallmark IL2 STAT5 signalling	-4.682	ENO3, ITGA6, ITGAV, PNP, PLEC, TGM2, GSTO1, NDRG1, AKAP2	9/200	P13929, P23229, P06756, P00491, Q15149, P21980, P78417, Q92597, Q9Y2D5
Hallmark mitotic spindle	-3.986	FLNB, KIF11, LMNB1, MARCKS, NUMA1, FSCN1, SPTBN1, NDC80	8/200	O75369, P52732, P20700, P29966, Q14980, Q16658, Q01082, O14777
Hallmark androgen response	-3.900	CDK6, XRCC6, H1F0, ITGAV, AKAP12, NDRG1	6/101	Q00534, P12956, P07305, P06756, Q02952, Q92597
Hallmark G2M checkpoint	-2.564	KIF11, LMNB1, MARCKS, NUMA1, NDC80, TACC3	6/200	P52732, P20700, P29966, Q14980, O14777, Q9Y6A5
Hallmark hypoxia	-2.564	ENO3, PFKP, TGM2, AKAP12, NDRG1, CAVIN1	6/200	P13929, Q01813, P21980, Q02952, Q92597,Q6NZI2
Hallmark MTORC1 signalling	-2.564	CANX, IDH1, PNP, PSMD12, RAB1A, RRM2	6/200	P27824, O75874, P00491, O00232, P62820, P31350

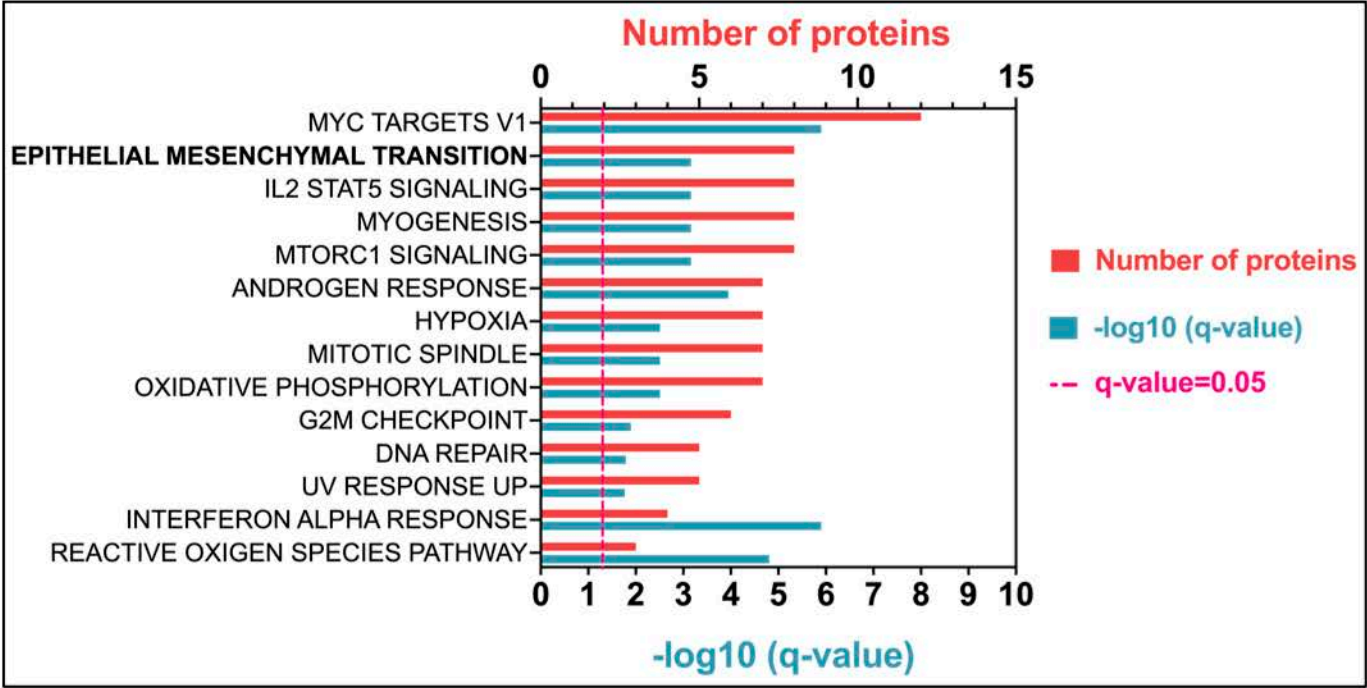|				
Hallmark interferon alpha response	-10.327	CD47, IFI35, IFIT3, IFITM2, NUB1	5/97	Q08722, P80217, O14879, Q01629, Q9Y5A7
Hallmark epithelial mesenchymal transition	-1.932	ITGA5, ITGAV, TGM2, TPM1, BASP1	5/200	P08648, P06756, P21980, P09493, P80723
Hallmark estrogen response early	-1.932	FLNB, KRT15,PODXL, TGM2, MYOF	5/200	O75369, P19012, O00592, P21980, Q9NZM1
Hallmark E2F targets	-1.932	XRCC6, LMNB1, NME1, RRM2, TACC3	5/200	P12956, P20700, P15531, P31350, Q9Y6A5
Hallmark myc targets v1	-1.932	CANX, EEF1B2, XRCC6, NME1, NPM1	5/200	P27824, P24534, P12956, P15531, P06748
Hallmark allograft rejection	-1.932	CD47, EIF5A, HLA-A, NME1, NPM1	5/200	Q08722, P63241, P01891, P15531, P06748
Hallmark DNA repair	-1.652	NME1, PNP, POLR2H, RALA	4/150	P15531, P00491, P52434, P11233
Hallmark UV response up	-1.601	DNAJA1, POLR2H, RRAD, GRPEL1	4/158	P31689, P52434, P55042, Q9HAV7
Hallmark interferon gamma response	-4.036	IFI35, IFIT3, IFITM2	3/200	P80217, O14879, Q01629

658

11 Table legends

11.1 **Table 1** Hallmark pathways identified using Metascape for differentially expressed proteins between control and RAS-activated ATII cells**.**

11.2 **Table 2** Hallmark pathways identified using Metascape for differentially expressed proteins between control and TGF-β-treated ATII cells.

11.3 **Table 3** Hallmark pathways identified using Metascape for differentially expressed proteins between control and TGF-β-treated RAS-activated ATII cells.
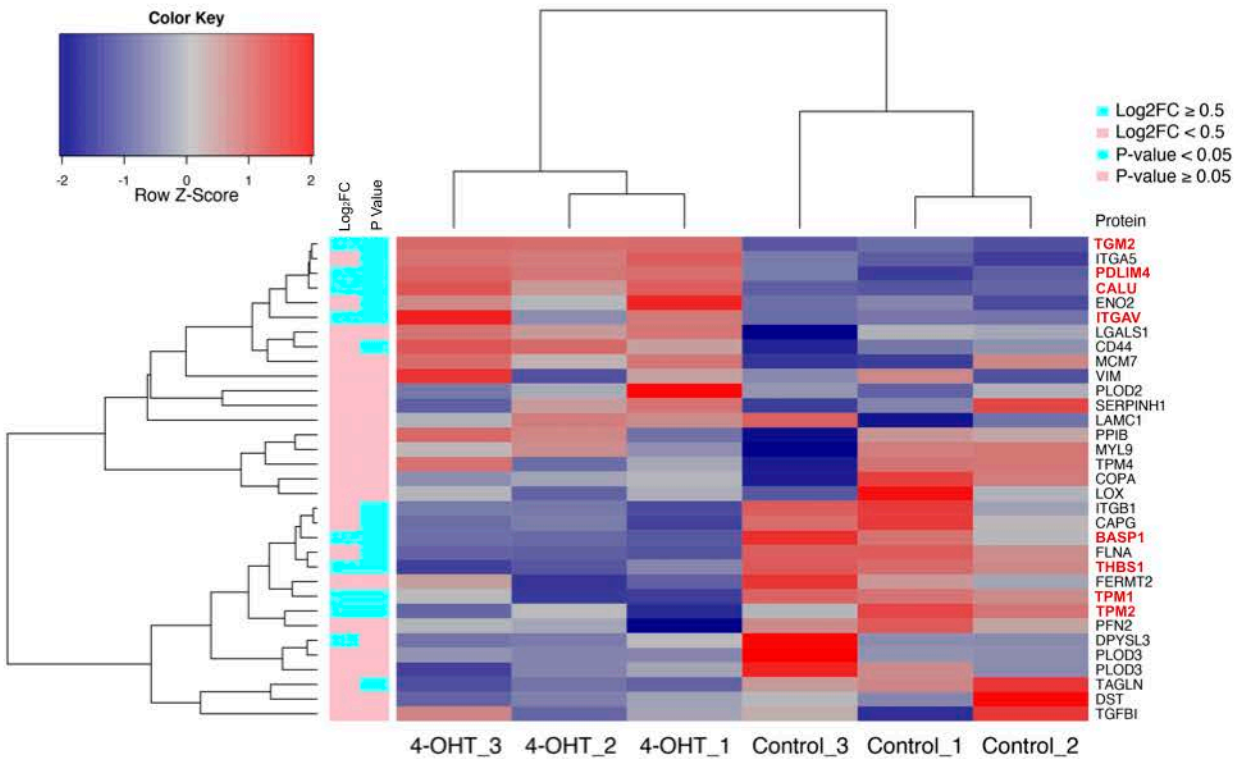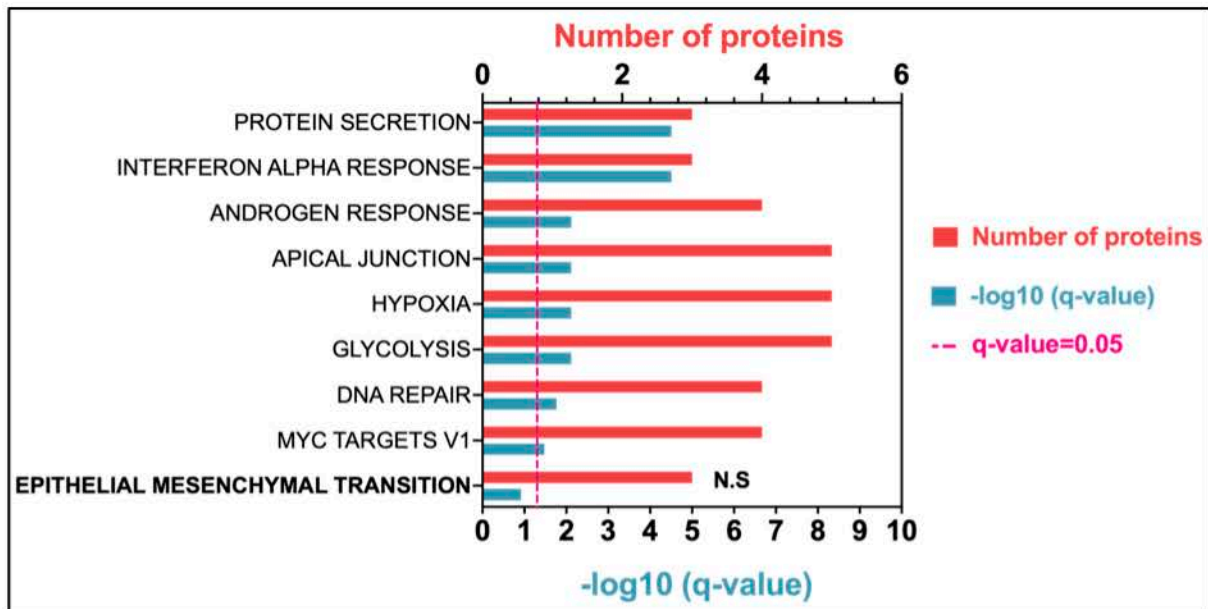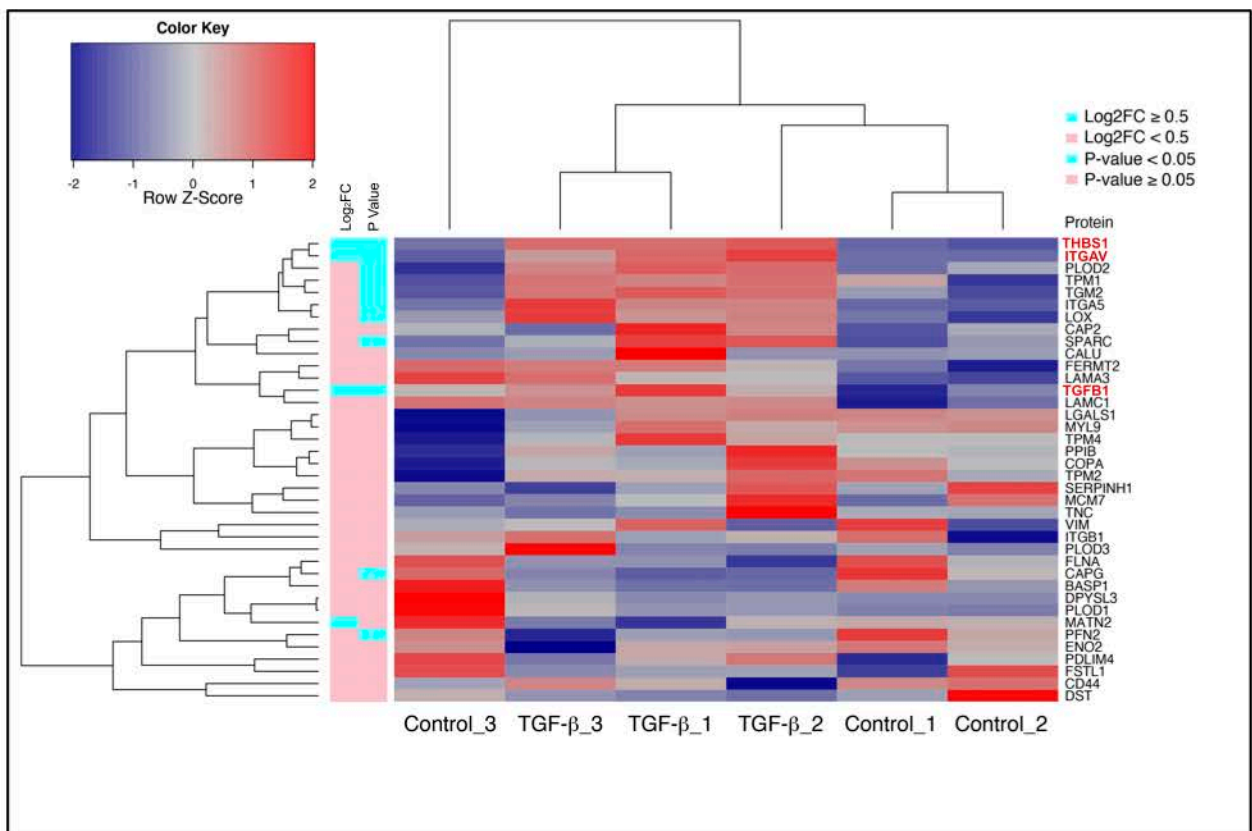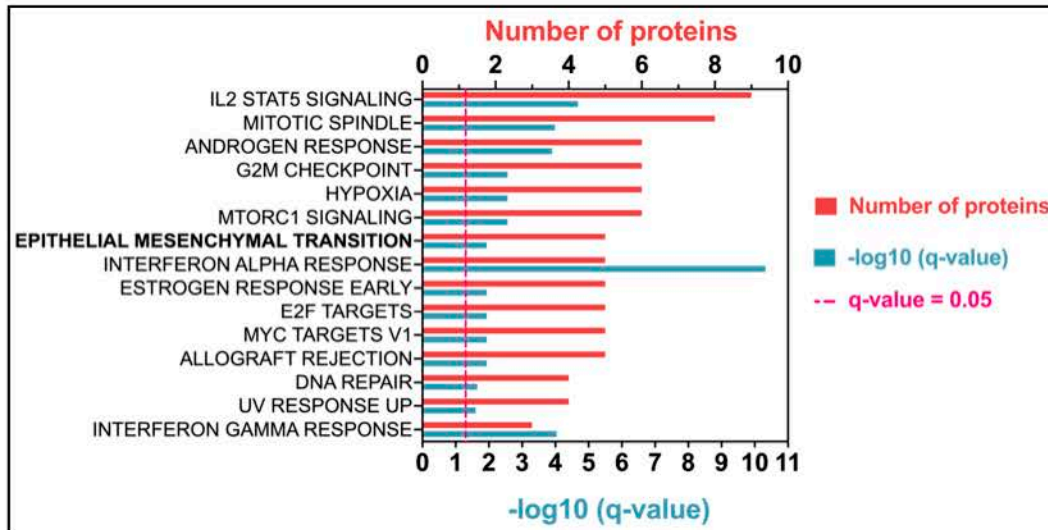
Figure 1

a

b

Figure 2

a

b

Figure 3

a

b

c

Figure 4

a

	control	4-OHT	TGF-β	4-OHT +TGF-β	
125-					E-cadherin
190-					ZEB1
25-					Snail1
25-					Snail2
40-					p-ERK
50-					p-AKT
50-					p-Smad2
50-					β-tubulin

b

Control   4-OHT

Control

TGF-β

ATII$^{ER:KRASV12}$

c

	TGF-β			4-OHT					
0	24	48	72	0	12	24	36	48	72	(hrs)
125-										E-cadherin
50-										p-Smad2
40-										p-ERK
50-										β-tubulin

d

Fold change in mRNA levels

□ control   ■ TGF-β

CDH1 ** | VIM *** | SNAI1 *** | SNAI2 | TWIST1 | ZEB1 | ZEB2