Cell Oncol. (2017) 40:639–650
DOI 10.1007/s13402-017-0344-6

REPORT

Differential effect of TGFβ on the proteome of cancer associated fibroblasts and cancer epithelial cells in a co-culture approach - a short report

Maria Magdalena Koczorowska[1] · Charlotte Friedemann[1] · Klaus Geiger[2,3] · Marie Follo[2,3] · Martin Lothar Biniossek[1] · Oliver Schilling[1,4,5]

Accepted: 26 July 2017 / Published online: 14 August 2017

Abstract
Background Solid tumors contain various components that together form the tumor microenvironment. Cancer associated fibroblasts (CAFs) are capable of secreting and responding to signaling molecules and growth factors. Due to their role in tumor development, CAFs are considered as potential therapeutic targets. A prominent tumor-associated signaling molecule is transforming growth factor β (TGFβ), an inducer of epithelial-to-mesenchymal transition (EMT). The differential action of TGFβ on CAFs and ETCs (epithelial tumor cells) has recently gained interest. Here, we aimed to investigate the effects of TGFβ on CAFs and ETCs at the proteomic level.
Methods We established a 2D co-culture system of differentially fluorescently labeled CAFs and ETCs and stimulated this co-culture system with TGFβ. The respective cell types were separated using FACS and subjected to quantitative analyses of individual proteomes using mass spectrometry.
Results We found that TGFβ treatment had a strong impact on the proteome composition of CAFs, whereas ETCs responded only marginally to TGFβ. Quantitative proteomic analyses of the different cell types revealed up-regulation of extracellular matrix (ECM) proteins in TGFβ treated CAFs. In addition, we found that the TGFβ treated CAFs exhibited increased N-cadherin levels.
Conclusions From our data we conclude that CAFs respond to TGFβ treatment by changing their proteome composition, while ETCs appear to be rather resilient.

Keywords Cancer associated fibroblasts (CAFs) · Epithelial tumor cells (ETCs) · TGFβ · Tumor-stroma interaction

1 Introduction

Solid tumors usually contain multiple cellular components, collectively forming the tumor microenvironment [1]. Different cell subtypes in the tumor microenvironment exhibit distinct characteristics, which is illustrated by their diverse gene expression patterns. The latter include genes encoding cell surface receptors, which define differential sensitivities of the different cell subtypes to external stimuli [2]. Cancer associated fibroblasts (CAFs) display a spindle-shaped morphology and express specific markers such as alpha smooth muscle actin (αSMA) and fibroblast activation protein alpha (FAPα). CAFs are highly capable of secreting signaling molecules and, by doing so, contribute to key features of tumor biology such as growth, invasion and metastasis [3, 4]. Unlike epithelial tumor cells (ETCs), CAFs are considered to be highly responsive to growth factor stimuli [2]. Given their distinctive features, together with their high abundance within tumor masses, CAFs have emerged as prominent targets in the design of novel therapeutic strategies [5, 6].

Electronic supplementary material The online version of this article (doi:10.1007/s13402-017-0344-6) contains supplementary material, which is available to authorized users.

✉ Oliver Schilling
oliver.schilling@mol-med.uni-freiburg.de

[1] Institute of Molecular Medicine and Cell Research, University of Freiburg, Stefan-Meier-Str. 17, D-79104 Freiburg, Germany

[2] Lighthouse Core Facility, Zentrum für Translationale Zellforschung (ZTZ), Medical Center - University of Freiburg, Freiburg, Germany

[3] Department of Medicine I, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany

[4] BIOSS Centre for Biological Signaling Studies, University of Freiburg, Freiburg, Germany

[5] German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), Heidelberg, Germany

Transforming growth factor β (TGFβ) is a well-established inducer of epithelial-to-mesenchymal transition (EMT) [7]. Its action is exerted through binding to one of its receptors: TGFBR1, TGFBR2 or TGFBR3 [8], the presence of which determines the cellular responsiveness to TGFβ-induced signaling. Interactions between stromal and tumor cells may also yield indirect, downstream effects on other cells within the tumor microenvironment.

Inspired by recent interest in the differential action of TGFβ on CAFs and ETCs [2], we set out to investigate the effects of TGFβ on CAFs and ETCs at the proteomic level. To this end, we established a co-culture system combined with TGFβ stimulation, as well as FACS separation of the respective cell types (Fig. 1). Through independent analysis of the co-cultured cells we show that TGFβ treatment has a strong impact on the protein composition of the CAFs, but not the ETCs.

2 Materials and methods

2.1 Cell lines and culture

The generation of CT5.3 cancer associated fibroblasts (CAFs) with shRNA-silenced FAPα expression (CT5.3shFAP) or scrambled shRNA expression (CT5.3shctr) has been reported before [9–11]. P-56-HM epithelial cancer cells were derived from a distal bile duct adenocarcinoma and isolated by Bachem's outgrowth method [12] into Quantum 333 medium from a human ampullary adenocarcinoma in 2012, expanded and immortalized through SV40 transduction. HCT116 epithelial colon tumor-derived cells were purchased from the ATCC. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, PAN, Aidenbach, Germany) supplemented with 10% fetal calf serum (PAN) and 1% penicillin/streptomycin (Gibco/Invitrogen, Paisley, UK) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2 Cell line-specific fluorescent protein expression

CT5.3 cells were stably transfected with a pAcGFP1-Hyg-N plasmid (Genecopoeia) using SuperFect Transfection Reagent (Qiagen). After transfection cells were selected for 2 weeks using 300 μg/ml hygromycin B (EMD Millipore Corp., USA.). HCT116 cells were transfected with pmCherry-N1 as described above and selected for 2 weeks using 750 μg/ml Geniticin (Invitrogen). Both cell lines expressing fluorescent proteins (FPs) were subjected to FACS sorting to enrich for GFP or mCherry Red expressing cells at equal levels.

2.3 Direct co-culture and mono-culture approaches to TGFβ treatment

For the co-culture approach, CT5.3shFAPGFP cells (CAFs) and HCT116mCherry cells (ETCs) were mixed in a 1:1.2 ratio. At 70% confluence the cells were treated for 48 h with10 ng/ml recombinant human TGFβ1 (R&D Systems), whereas control cells remained untreated. Next, the cells were trypsinized and sorted on a FACS Aria III machine (BD Biosciences). The wavelengths used for GFP were 488 nm for excitation and 515–545 nm for emission, while those for mCherry were 561 nm for excitation and 600–620 nm for emission. In mono-culture approaches CAFs and ETCs (HCT116, SW480 or SW620) were grown until 70% confluency and treated with 10 ng/ml recombinant human TGFβ1 for 48 h. The cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1% NP-40) supplemented with protease inhibitors (5 mM EDTA, 0.01 mM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64) and 1 mM phenylmethanesulfonyl fluoride (PMSF)).

2.4 Quantitative proteome profiling

For proteomic analyses, samples were prepared as reported previously, using differential dimethylation [9]. For mass spectrometry analyses, a Q-Exactive plus system (Thermo Scientific, Bremen, Germany) was used again as reported before [9]. Likewise, LC-MS/MS data analysis was performed as reported before [9]. STRING [13] was used to annotate functional interactions of proteins and non-redundant Gene Ontology (GO) term enrichment analysis was performed using the TopGO R package.

2.5 Western blot analysis

Western blot analysis was performed as reported before [9] using the following primary antibodies: ant-FAPα (R&D, AF3715), anti-N-Cadherin (Cell Signaling 4061), anti-Phospho-Smad3 (S423/S425; R&D, AB3226) and anti-α-Tubulin (Sigma-Aldrich, T 6199), and the following secondary antibodies: anti-mouse (Dianova, 115-035-003), anti-rabbit (BioRad, 172-1019) and anti-sheep (Dianova, 713-035-147). Tubulin or GAPDH served as internal loading control standards for the Western blots. In case of CCM samples, Coomassie staining of the gels was used as an equal loading control.

3 Results and discussion

TGFβ is a powerful signal transducer, involved in the regulation of various hallmark cancer processes. In addition, TGFβ signaling has been associated with a poor prognosis in cancer

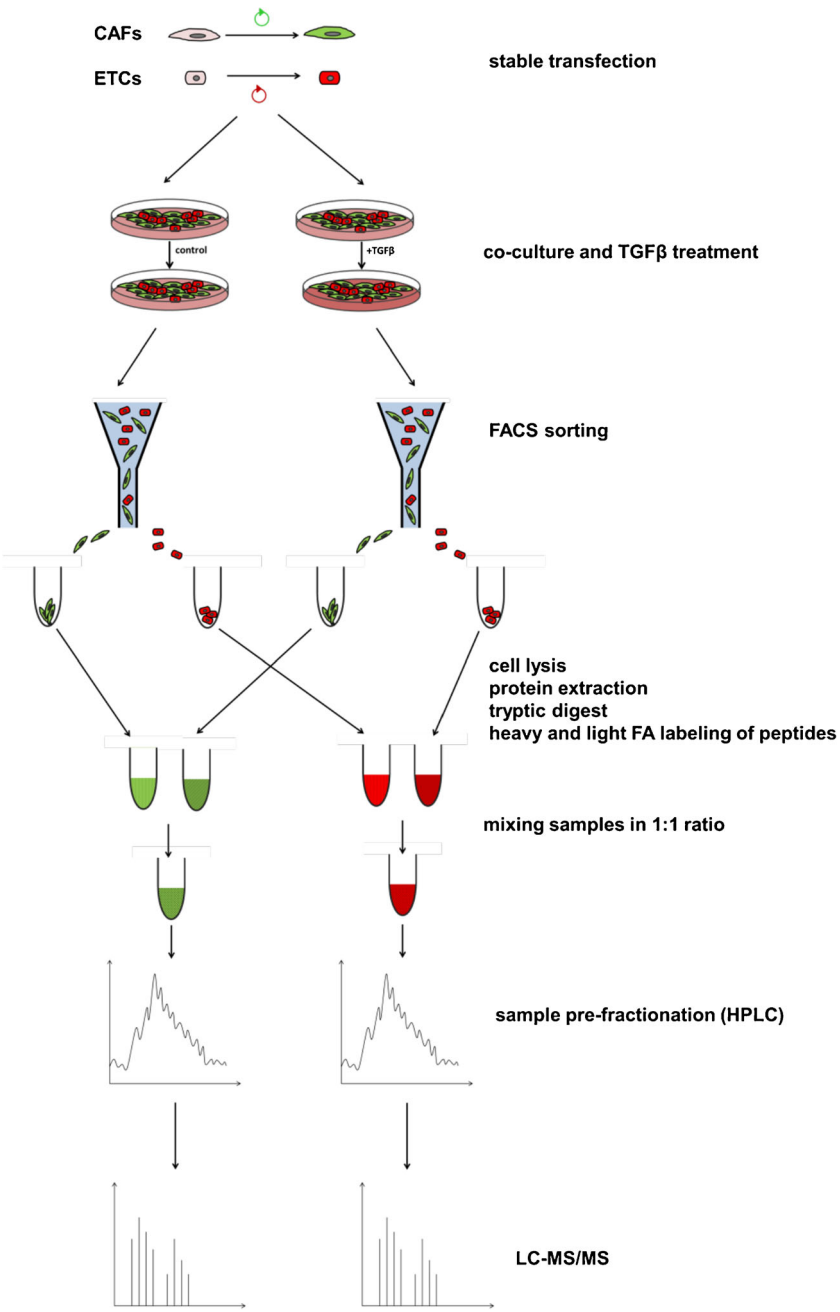

Fig. 1 Proteome profiling workflow of CAFs and CECs grown in co-culture. CAFs and ETCs were stably transfected with GFP and mCherry, respectively. Labeled cells were grown in co-culture and treated for 48 h with TGFβ. Untreated cells were used as controls. Cells were FACS-sorted according to expression of the respective fluorescent labels. After lysis and tryptic digestion the peptides were labeled with light or heavy FA. Samples of treated and untreated cells of the same cell type were mixed at a 1:1 ratio. Peptides were purified, pre-fractionated on HPLC and analyzed by LC-MS/MS.

patients [14, 15]. Previously, we have shown that TGFβ treatment of FAPα-deprived CAFs can rescue their fibroblastic phenotype [9]. A strong responsiveness of CAFs to TGFβ is in line with previous reports on differential cellular responses to external signals. Calon et al. [2] showed that TGFβ-stimulated ETCs were not tumorigenic upon subcutaneous injection into mice, whereas co-injection of fibroblasts did lead to tumor formation, which highlights a unique role of CAFs in tumorigenesis. To investigate the effect of TGFβ on individual tumor cellular components, we performed a proteomic analysis of co-cultured CAF (CT5.3shFAPGFP) and ETC (HCT116mCherry) cells treated with TGFβ. The applied workflow is schematically depicted in Fig. 1. FACS separation of cells expressing different fluorescent proteins (FPs) allowed for a quantitative proteome profiling of individual cell types.

Single-cell suspensions of CAFs and ETCs expressing different FPs were seeded in dishes to form 2D co-cultures. By doing so, we found that TGFβ treated co-cultures exhibited differential morphologies compared to control cells (Fig. 2a) and a shift in CAF/ETC ratio towards a higher CAF abundance. The decreased ETC abundance in TGFβ-treated co-cultures might suggest a TGFβ-driven growth suppression

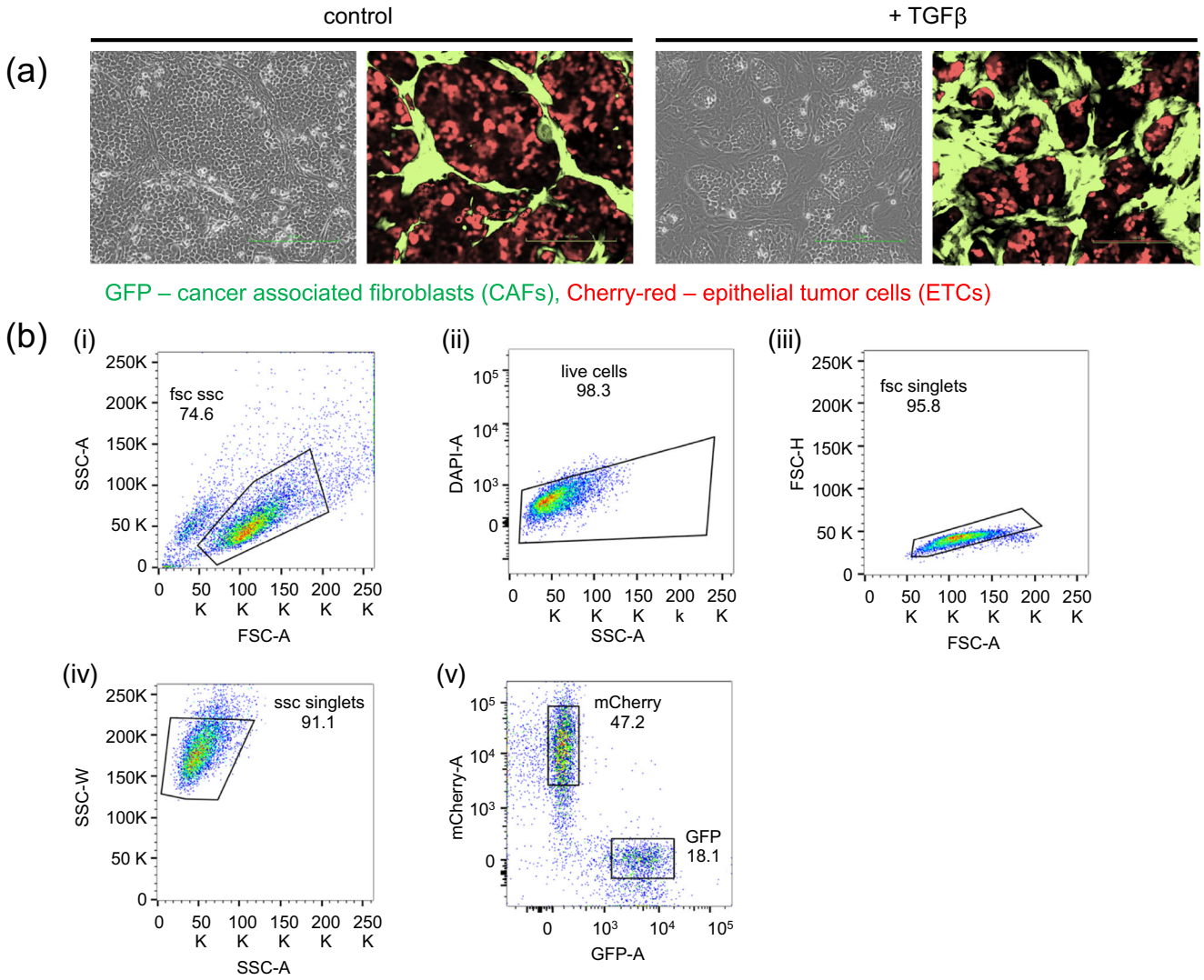

Fig. 2 **a** Co-culture of CAFs and ETCs after treatment with TGFβ compared to the untreated control. The picture was created by overlaying the green and red channels; *red* and *green* colors were added manually. **b** FACS separation of cells expressing GFP and mCherry (representative bivariate plots are shown). SSC – side scatter, FSC – forward scatter, pos – positive cells. (*i*) Gating on forward and side scatter to remove cell debris, (*ii*) DAPI staining for dead cell exclusion, (*iii*) and (*iv*) gating for single cells using FSC and SSC, respectively, in order to eliminate possible doublets and cell clusters, (*v*) Separation of two distinct cell populations based on the expression of either GFP or mCherry

of epithelial cells [16]. We found, however, that TGFβ stimulation had no detectable effect on HCT116 cell proliferation in mono-culture (data not shown).

To further elucidate the differential effect of TGFβ on CAFs and ETCs in a direct co-culture system, we used FPs as unique CAF (GFP) and ETC (mCherry) markers for FACS sorting. In doing so, TGFβ treated and control cultures were harvested and subjected to FACS sorting, after which 5×10^4 to 2×10^6 separated cells (both CAFs and ETCs) were isolated (Fig. 2b). These cells were used for quantitative global proteomic profiling, in which TGFβ treated CAFs and ETCs were compared to their untreated counterparts, respectively (Fig. 1), in two biological replicates. Peptides were isotopically labeled using either light or heavy formaldehyde. To exclude label bias, we applied a label swap for each replicate. Through LC-MS/MS analysis 2909 and 3366 proteins (false discovery rate (FDR) < 1%) were identified and quantified for the CAFs in replicates 1 and 2, respectively, with 2349 proteins consistently identified and quantified in both replicates. For the ETCs, 3253 proteins were identified in replicate 1 and 4018 proteins in replicate 2 (FDR < 1%), with 2748 proteins consistently identified and quantified in both replicates. In all experiments we found an overlap of 1881 identified and quantified proteins (Fig. 3a). Relative protein abundances were measured for TGFβ treated and untreated samples and protein ratios were expressed as fold change (Fc) values ($\log_2$ of TGFβ treated/untreated). The fold change distribution in all experiments was close to normal (Fig. 3b), with a narrower

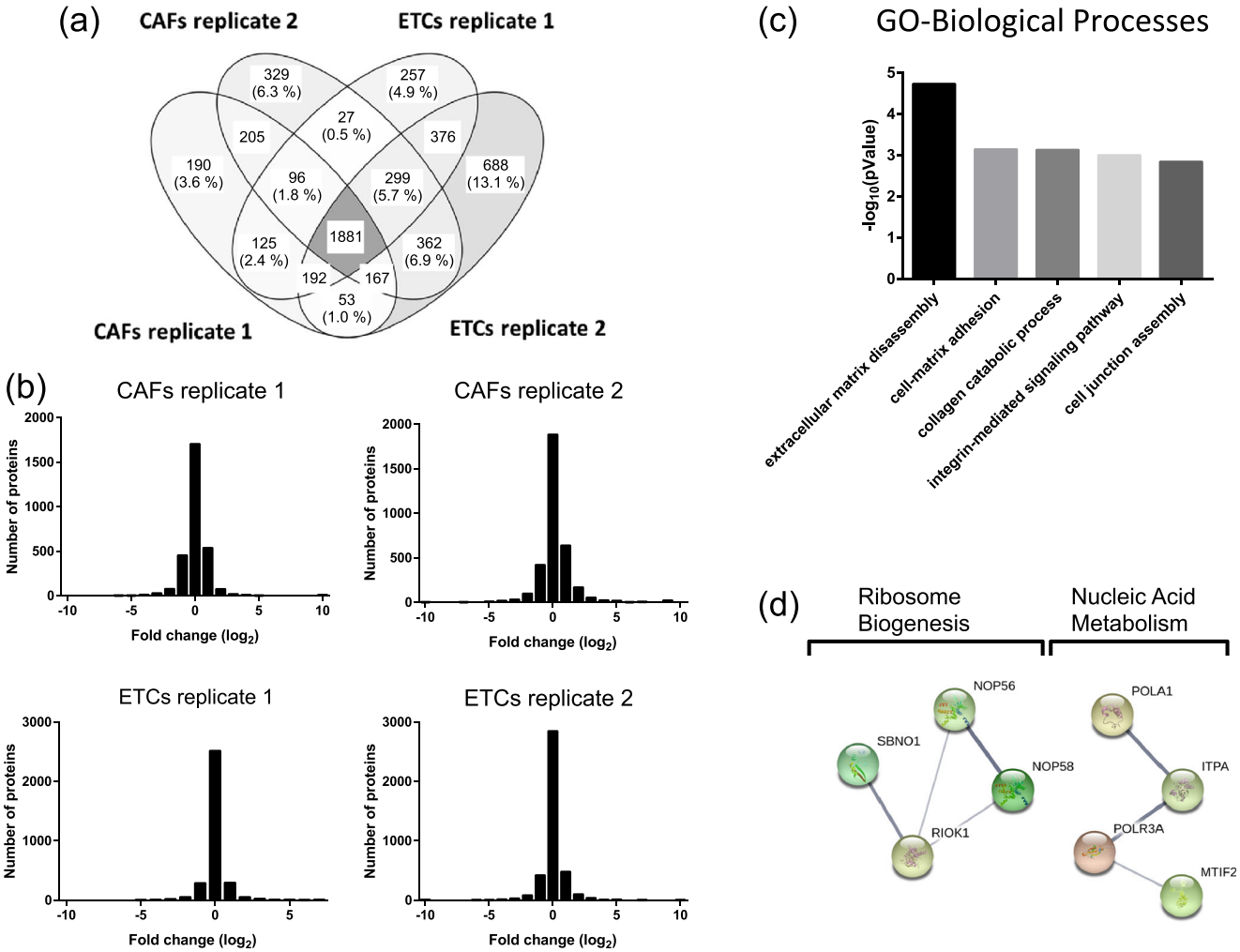

Fig. 3 **a** Overlap of proteins consistently identified and quantified. 1881 proteins were found in all four experiments. The Venn Diagam (created by software Venny 2.1 http://bioinfogp.cnb.csic.es/tools/venny/) shows higher numbers of shared proteins between replicates of ETCs or CAFs compared to the overlap between different cell lines. **b** Distribution of fold-change values (log_2 of light/heavy or heavy/light ratios) in four independent experiments. Histograms show a broader distribution and, thus, a higher number of altered proteins (Fc > 0.58 or Fc < −0.58) in CAFs than in ETCs. **c** Biological themes emerging from proteins up-regulated in CAFs co-cultured with ETCs upon TGFβ treatment, according to GO annotations performed using a R studio-based script. GO-Biological Processes annotations indicate enrichment in proteins clustering around extracellular processes. The full output data are listed in Supplementary Table 2. **d** STRING v10 (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis of proteins altered in ETCs upon TGFβ treatment. Connections show confidence (line thickness indicates the summed-up strength of data support). Connections are based on the categories "Neighborhood", "Gene Fusion", "Co-occurrence", "Co-expression Experiments", "Databases", and "Textmining" at a medium confidence level (0.4)

range in ETCs than in CAFs, indicating less alterations in protein abundances in the ETCs.

To determine the impact of TGFβ on the proteome composition of CAFs and ETCs, we defined the following criteria: (a) the protein was identified and quantified in both experiments, (b) protein abundance was consistently increased or decreased > 50% in both experiments, (c) a favorable manual inspection of the extracted ion chromatograms. Similar criteria have successfully been used in previous studies to quantitatively delineate proteins affected by treatment [17]. By using these criteria, 118 up-regulated and 71 down-regulated proteins were highlighted in CAFs upon TGFβ treatment, whereas only 27 proteins showed increased and 25 proteins decreased abundances in TGFβ treated ETCs (Tables 1 and 2, Supplementary Table 1 a-d). This finding suggests that exposure to TGFβ causes a substantial perturbation in proteome composition in CAFs but not in ETCs, underscoring a higher responsiveness of CAFs to TGFβ compared to ETCs. Lack of overlap between proteins affected in ETCs and CAFs indicates a differential effect of TGFβ on cells of epithelial versus mesenchymal origin.

To elucidate biological "themes" within the proteins affected by TGFβ treatment in CAFs, we performed a non-redundant GO-enrichment analysis using TopGO. By doing so, a notable enrichment was noted for proteins involved in extracellular matrix (ECM) and cell-matrix adhesion, such as

Table 1 List of proteins significantly and consistently affected in both replicates 1 and 2 (r1 and r2) of CAFs by TGFβ treatment - Uniprot ID and recommended name according to Uniprot database (Uniprot Consortium 2013). Fc- values (log_2 of TGFβ treated / control cells ratios for replicates 1 and 2) indicate the fold change of protein in each experiment as well as average Fc

Uniprot ID	Protein name	Fc (log_2) TGFβ/ctr r1	Fc (log_2) TGFβ/ctr r2	Average Fc
Q9BV57	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	0.82	0.80	0.81
Q9NRX2	39S ribosomal protein L17, mitochondrial	0.77	1.52	1.14
Q9H2W6	39S ribosomal protein L46, mitochondrial	0.62	1.30	0.96
Q8N5N7	39S ribosomal protein L50, mitochondrial	0.67	1.98	1.32
P25325	3-mercaptopyruvate sulfurtransferase	2.07	1.27	1.67
P35573	4-alpha-glucanotransferase;Amylo-alpha-1,6-glucosidase	0.59	1.08	0.84
O95870	Abhydrolase domain-containing protein 16A	1.07	1.22	1.14
P00813	Adenosine deaminase	4.54	0.75	2.65
Q9BTE6	Alanyl-tRNA editing protein Aarsd1	0.69	1.13	0.91
Q9H553	Alpha-1,3/1,6-mannosyltransferase ALG2	1.67	1.16	1.41
P03928	ATP synthase protein 8	1.13	1.75	1.44
P07738	Bisphosphoglycerate mutase	1.88	2.16	2.02
Q13895	Bystin	1.72	1.91	1.81
P19022	Cadherin-2	1.32	2.81	2.06
P51911	Calponin-1	1.40	1.69	1.54
O60716	Catenin delta-1	0.91	0.75	0.83
Q9UBR2	Cathepsin Z	0.85	3.23	2.04
O43310	CBP80/20-dependent translation initiation factor	1.17	1.62	1.39
Q5SW79	Centrosomal protein of 170 kDa	0.88	0.86	0.87
Q969X6	Cirhin	0.88	1.36	1.12
P02452	Collagen alpha-1(I) chain	1.52	1.92	1.72
P20908	Collagen alpha-1(V) chain	1.62	2.64	2.13
Q02388	Collagen alpha-1(VII) chain	1.10	2.19	1.65
P08123	Collagen alpha-2(I) chain	1.36	1.50	1.43
P05997	Collagen alpha-2(V) chain	1.24	2.05	1.64
Q9UBG0	C-type mannose receptor 2	0.74	0.92	0.83
Q00535	Cyclin-dependent kinase 5	0.64	0.63	0.64
Q96BY6	Dedicator of cytokinesis protein 10	1.32	1.34	1.33
Q86TI2	Dipeptidyl peptidase 9	0.64	0.92	0.78
Q9UBS4	DnaJ homolog subfamily B member 11	1.17	2.83	2.00
P39656	Dolichyl-diphosphooligosaccharide	0.62	0.65	0.63
P43897	Elongation factor Ts, mitochondrial	0.67	1.22	0.94
P29323	Ephrin type-B receptor 2	1.52	1.12	1.32
Q15006	ER membrane protein complex subunit 2	0.69	1.97	1.33
Q8WVX9	Fatty acyl-CoA reductase 1	0.69	1.24	0.97
Q96AC1	Fermitin family homolog 2	0.72	0.78	0.75
P35555	Fibrillin-1	1.20	1.43	1.31
P09038	Fibroblast growth factor 2	1.03	1.66	1.34
P02751	Fibronectin	3.20	2.91	3.06
Q53EP0	Fibronectin type III domain-containing protein 3B	0.85	2.23	1.54
Q14315	Filamin-C	0.64	0.74	0.69
Q12841	Follistatin-related protein 1	2.07	1.31	1.69
Q13643	Four and a half LIM domains protein 3	0.67	1.15	0.91
O94925	Glutaminase kidney isoform, mitochondrial	0.77	0.98	0.88
P06737	Glycogen phosphorylase, liver form	1.72	0.62	1.17
P62993	Growth factor receptor-bound protein 2	0.64	0.62	0.63
P04792	Heat shock protein beta-1	0.62	0.80	0.71

Table 1 (continued)

Uniprot ID	Protein name	Fc (log_2) TGFβ/ctr r1	Fc (log_2) TGFβ/ctr r2	Average Fc
Q12824	hSNF5	1.13	0.81	0.97
Q9Y5U9	Immediate early response 3-interacting protein 1	1.52	0.83	1.18
P29218	Inositol monophosphatase 1	1.62	0.74	1.18
Q16270	Insulin-like growth factor-binding protein 7	0.77	1.72	1.25
P56199	Integrin alpha-1	3.06	1.59	2.32
Q9UKX5	Integrin alpha-11	0.64	1.71	1.17
P06756	Integrin alpha-V	0.97	1.10	1.04
P11047	Laminin subunit gamma-1	1.77	1.01	1.39
P46379	Large proline-rich protein BAG6	1.62	0.67	1.14
Q8N6Y2	Leucine-rich repeat-containing protein 17	2.94	2.01	2.48
Q9NZU5	LIM and cysteine-rich domains protein 1	0.64	0.78	0.71
P14174	Macrophage migration inhibitory factor	1.82	0.71	1.27
Q9Y5P6	Mannose-1-phosphate guanyltransferase beta	0.94	0.61	0.77
Q9UNF1	Melanoma-associated antigen D2	0.72	0.73	0.72
P01033	Metalloproteinase inhibitor 1	2.27	3.54	2.91
Q8NF91	Nesprin-1	1.32	3.29	2.30
P23497	Nuclear autoantigen Sp-100	1.13	2.29	1.71
P49790	Nuclear pore complex protein Nup153	0.82	1.90	1.36
P37198	Nuclear pore glycoprotein p62	1.52	4.75	3.14
Q8WX93	Palladin	1.17	1.85	1.51
Q96JY6	PDZ and LIM domain protein 2	1.77	1.78	1.78
P50479	PDZ and LIM domain protein 4	0.94	0.91	0.92
Q9NR12	PDZ and LIM domain protein 7	1.07	1.43	1.25
O14908	PDZ domain-containing protein GIPC1	0.67	1.06	0.87
Q92626	Peroxidasin homolog	0.85	0.74	0.79
O15254	Peroxisomal acyl-coenzyme A oxidase 3	0.94	0.69	0.81
Q16822	Phosphoenolpyruvate carboxykinase [GTP]	1.32	0.65	0.98
O60256	Phosphoribosyl pyrophosphate synthase-associated protein 2	0.64	0.88	0.76
P78330	Phosphoserine phosphatase	0.91	1.39	1.15
P13796	Plastin-2	0.62	0.74	0.68
Q86SR1	Polypeptide N-acetylgalactosaminyltransferase 10	1.00	0.89	0.95
Q9UI14	Prenylated Rab acceptor protein 1	1.52	0.63	1.08
Q15113	Procollagen C-endopeptidase enhancer 1	1.82	0.60	1.21
O15460	Prolyl 4-hydroxylase subunit alpha-2	0.69	1.08	0.89
Q5VYK3	Proteasome-associated protein ECM29 homolog	1.36	0.72	1.04
Q8N8S7	Protein enabled homolog	1.40	0.95	1.17
Q9BRX2	Protein pelota homolog	0.88	2.59	1.73
O95487	Protein transport protein Sec24B	0.82	1.86	1.34
Q969M3	Protein YIPF5	0.74	0.65	0.70
P28300	Protein-lysine 6-oxidase	1.10	9.32	5.21
Q15276	Rab GTPase-binding effector protein 1	1.67	2.24	1.95
Q96QB1	Rho GTPase-activating protein 7	3.35	3.70	3.53
Q8TDP1	Ribonuclease H2 subunit C	0.67	2.87	1.77
P60891	Ribose-phosphate pyrophosphokinase 1	0.97	1.10	1.03
O75326	Semaphorin-7A	0.62	7.46	4.04
Q15257	Serine/threonine-protein phosphatase 2A activator	0.85	0.62	0.73
P62140	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	0.69	0.87	0.78

Table 1 (continued)

Uniprot ID	Protein name	Fc (log_2) TGFβ/ctr r1	Fc (log_2) TGFβ/ctr r2	Average Fc
P53814	Smoothelin	1.52	1.44	1.48
P55011	Solute carrier family 12 member 2	1.77	1.09	1.43
Q96L92	Sorting nexin-27	0.72	0.62	0.67
P09486	SPARC	0.74	1.06	0.90
Q9NUQ6	SPATS2-like protein	0.91	1.22	1.06
Q86XZ4	Spermatogenesis-associated serine-rich protein 2	0.82	4.30	2.56
Q9UH99	SUN domain-containing protein 2	0.69	0.60	0.65
P23381	T1-TrpRS	0.72	0.69	0.70
Q9HBL0	Tensin-1	1.94	1.07	1.50
Q8NG11	Tetraspanin-14	1.10	1.17	1.13
P07996	Thrombospondin-1	2.62	1.09	1.86
O43294	Transforming growth factor beta-1-induced transcript 1 protein	2.07	1.71	1.89
Q15582	Transforming growth factor-beta-induced protein ig-h3	2.13	1.44	1.78
Q01995	Transgelin	1.24	2.26	1.75
Q6NUQ4	Transmembrane protein 214	0.69	1.27	0.98
Q5BJD5	Transmembrane protein 41B	0.72	0.78	0.75
O75962	Triple functional domain protein	0.77	0.73	0.75
P09493	Tropomyosin alpha-1 chain	0.97	1.03	1.00
P49815	Tuberin	0.62	5.05	2.83
P41240	Tyrosine-protein kinase CSK	0.74	1.29	1.02
Q15678	Tyrosine-protein phosphatase non-receptor type 14	0.74	0.74	0.74
Q13404	Ubiquitin-conjugating enzyme E2 variant 1	0.72	2.34	1.53
P61960	Ubiquitin-fold modifier 1	1.48	0.65	1.07
P50552	Vasodilator-stimulated phosphoprotein	0.59	0.78	0.68
P82675	28S ribosomal protein S5, mitochondrial	−0.72	−1.70	−1.21
P17050	Alpha-N-acetylgalactosaminidase	−1.96	−1.67	−1.81
Q5VW32	BRO1 domain-containing protein BROX	−1.57	−1.18	−1.37
Q8IWX8	Calcium homeostasis endoplasmic reticulum protein	−0.73	−0.76	−0.74
Q9UDT6	CAP-Gly domain-containing linker protein 2	−0.80	−0.78	−0.79
Q96DG6	Carboxymethylenebutenolidase homolog	−2.45	−0.72	−1.58
P11717	Cation-independent mannose-6-phosphate receptor	−0.75	−1.29	−1.02
O76074	cGMP-specific 3′,5′-cyclic phosphodiesterase	−0.77	−1.97	−1.37
P45973	Chromobox protein homolog 5	−1.41	−1.26	−1.34
Q8N3U4	Cohesin subunit SA-2	−1.35	−0.76	−1.06
Q99715	Collagen alpha-1(XII) chain	−0.58	−2.20	−1.39
Q9UEE9	Craniofacial development protein 1	−0.71	−1.64	−1.17
P14927	Cytochrome b-c1 complex subunit 7	−0.91	−1.04	−0.97
Q16643	Drebrin	−1.69	−1.11	−1.40
Q6XZF7	Dynamin-binding protein	−2.64	−0.78	−1.71
Q96C19	EF-hand domain-containing protein D2	−1.64	−2.89	−2.26
Q6P2E9	Enhancer of mRNA-decapping protein 4	−0.66	−0.83	−0.74
P02792	Ferritin light chain	−0.95	−1.26	−1.11
Q9H4A6	Golgi phosphoprotein 3	−1.57	−0.88	−1.22
P29992	Guanine nucleotide-binding protein subunit alpha-11	−0.85	−3.47	−2.16
P26583	High mobility group protein B2	−1.38	−1.31	−1.34
P17096	High mobility group protein HMG-I/HMG-Y	−0.75	−0.60	−0.68
Q13325	Interferon-induced protein with tetratricopeptide repeats 5	−1.58	−0.86	−1.22
P51553	Isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial	−0.92	−1.56	−1.24

Table 1 (continued)

Uniprot ID	Protein name	Fc (log_2) TGFβ/ctr r1	Fc (log_2) TGFβ/ctr r2	Average Fc
Q07666	KH domain-containing, RNA-binding, signal transduction-associated protein 1	−0.60	−0.61	−0.60
Q16363	Laminin subunit alpha-4	−0.87	−1.64	−1.25
Q96IJ6	Mannose-1-phosphate guanyltransferase alpha	−0.98	−0.70	−0.84
Q96GX9	Methylthioribulose-1-phosphate dehydratase	−1.16	−5.27	−3.21
Q99797	Mitochondrial intermediate peptidase	−0.79	−1.06	−0.92
O15439	Multidrug resistance-associated protein 4	−1.30	−1.04	−1.17
Q9NUJ1	Mycophenolic acid acyl-glucuronide esterase, mitochondrial	−1.35	−0.66	−1.00
P14649	Myosin light chain 6B	−1.19	−0.91	−1.05
P15559	NAD(P)H dehydrogenase [quinone] 1	−0.74	−0.70	−0.72
P41227	N-alpha-acetyltransferase 10	−0.75	−0.95	−0.85
Q8NEY1	Neuron navigator 1	−0.74	−0.75	−0.75
Q12769	Nuclear pore complex protein Nup160	−1.87	−3.12	−2.50
Q9NVE7	Pantothenate kinase 4	−1.04	−0.96	−1.00
Q9H300	P-beta;Presenilins-associated rhomboid-like protein	−0.77	−0.91	−0.84
P32119	Peroxiredoxin-2	−0.82	−0.75	−0.78
Q96G03	Phosphoglucomutase-2	−1.44	−1.22	−1.33
O00625	Pirin	−1.53	−1.22	−1.37
P05120	Plasminogen activator inhibitor 2	−0.96	−0.61	−0.79
P61758	Prefoldin subunit 3	−0.58	−0.93	−0.76
O95571	Protein ETHE1, mitochondrial	−1.34	−1.56	−1.45
Q96C01	Protein FAM136A	−0.79	−0.70	−0.74
O94992	Protein HEXIM1	−0.67	−0.66	−0.66
Q08AM6	Protein VAC14 homolog	−1.69	−1.59	−1.64
Q6GMV3	Putative peptidyl-tRNA hydrolase PTRHD1	−2.10	−0.58	−1.34
Q96T37	Putative RNA-binding protein 15	−0.62	−1.16	−0.89
Q9H6Z4	Ran-binding protein 3	−0.88	−0.67	−0.77
P20340	Ras-related protein Rab-6A	−1.01	−0.67	−0.84
P18754	Regulator of chromosome condensation	−1.00	−0.63	−0.81
Q86TG7	Retrotransposon-derived protein PEG10	−6.27	−1.11	−3.69
Q92541	RNA polymerase-associated protein RTF1 homolog	−0.75	−0.96	−0.86
P82979	SAP domain-containing ribonucleoprotein	−0.86	−1.64	−1.25
Q15424	Scaffold attachment factor B1	−1.00	−0.86	−0.93
P50452	Serpin B8	−0.66	−0.72	−0.69
P02768	Serum albumin	−0.76	−2.77	−1.76
O95810	Serum deprivation-response protein	−1.32	−1.67	−1.49
Q29RF7	Sister chromatid cohesion protein PDS5 homolog A	−1.05	−0.67	−0.86
P16949	Stathmin	−1.02	−0.78	−0.90
Q68CZ2	Tensin-3	−1.45	−1.14	−1.29
P51580	Thiopurine S-methyltransferase	−0.98	−1.59	−1.28
Q9UDY2	Tight junction protein ZO-2	−0.92	−2.29	−1.60
Q15370	Transcription elongation factor B polypeptide 2	−0.69	−0.66	−0.67
Q12788	Transducin beta-like protein 3	−0.71	−3.47	−2.09
Q9H2D6	TRIO and F-actin-binding protein	−0.67	−1.24	−0.95
Q9NYL9	Tropomodulin-3	−0.60	−0.63	−0.61
Q53GS9	U4/U6.U5 tri-snRNP-associated protein 2	−0.67	−1.33	−1.00
Q5SNT6	WASH complex subunit FAM21B	−1.39	−0.88	−1.13
O75717	WD repeat and HMG-box DNA-binding protein 1	−1.28	−1.22	−1.25

Table 2 List of proteins significantly and consistently affected in both replicates 1 and 2 (r1 and r2) of CECs by TGFβ treatment - Uniprot ID and recommended name according to Uniprot database (Uniprot Consortium 2013). Fc- values (log^2 of TGFβ treated / control cells ratios for replicates 1 and 2) indicate the fold change of protein in each experiment as well as average Fc

Uniprot ID	Protein name	Fc (log_2) TGFβ/ctr r1	Fc (log_2) TGFβ/ctr r2	Average Fc
Q8IV48	3′-5′ exoribonuclease 1	0.70	0.81	0.76
Q8N983	39S ribosomal protein L43, mitochondrial	1.00	3.17	2.08
P10253	70 kDa lysosomal alpha-glucosidase	3.00	0.63	1.81
Q9UHB7	AF4/FMR2 family member 4	0.83	1.80	1.32
Q12802	A-kinase anchor protein 13	1.76	0.84	1.30
P27338	Amine oxidase [flavin-containing] B	0.92	1.10	1.01
Q01433	AMP deaminase 2	0.90	5.03	2.97
Q9P2R3	Ankyrin repeat and FYVE domain-containing protein 1	1.90	2.81	2.36
Q9BY43	Charged multivesicular body protein 4a	1.32	1.01	1.16
Q2NKX8	DNA excision repair protein ERCC-6-like	0.72	0.65	0.69
Q8IXB1	DnaJ homolog subfamily C member 10	3.64	2.81	3.23
O94905	Erlin-2	3.99	0.70	2.34
Q99447	Ethanolamine-phosphate cytidylyltransferase	0.81	3.16	1.98
O75223	Gamma-glutamylcyclotransferase	0.70	1.01	0.86
P35527	Keratin, type I cytoskeletal 9	3.49	1.87	2.68
Q6P1M3	Lethal(2) giant larvae protein homolog 2	0.98	0.76	0.87
Q969J3	Loss of heterozygosity 12 chromosomal region 1 protein	2.48	5.50	3.99
P01130	Low-density lipoprotein receptor	0.66	0.75	0.70
Q9Y5J7	Mitochondrial import inner membrane translocase subunit Tim9	1.00	0.96	0.98
O15439	Multidrug resistance-associated protein 4	1.42	2.48	1.95
Q9UL63	Muskelin	1.19	1.48	1.34
Q8NFW8	N-acylneuraminate cytidylyltransferase	1.11	0.64	0.88
Q8TBC4	NEDD8-activating enzyme E1 catalytic subunit	1.11	0.98	1.04
Q12830	Nucleosome-remodeling factor subunit BPTF	3.22	1.62	2.42
Q96G03	Phosphoglucomutase-2	1.56	1.94	1.75
P40763	Signal transducer and activator of transcription 3	0.64	0.66	0.65
Q9UBQ0	Vacuolar protein sorting-associated protein 29	0.64	0.77	0.71
Q9UJX3	Anaphase-promoting complex subunit 7	−0.67	−1.33	−1.00
P53365	Arfaptin-2	−0.73	−1.64	−1.18
Q9NXV2	BTB/POZ domain-containing protein KCTD5	−0.71	−0.66	−0.69
Q13616	Cullin-1	−0.58	−1.16	−0.87
P27707	Deoxycytidine kinase	−3.38	−0.66	−2.02
P53634	Dipeptidyl peptidase 1	−1.76	−0.73	−1.24
P09884	DNA polymerase alpha catalytic subunit	−0.85	−1.16	−1.01
O14802	DNA-directed RNA polymerase III subunit RPC1	−1.29	−1.48	−1.38
Q8N766	ER membrane protein complex subunit 1	−0.72	−0.73	−0.72
Q3B7T1	Erythroid differentiation-related factor 1	−0.62	−0.66	−0.64
Q3SXM5	Inactive hydroxysteroid dehydrogenase-like protein 1	−0.79	−1.01	−0.90
Q9BY32	Inosine triphosphate pyrophosphatase	−1.13	−1.07	−1.10
Q9Y2K7	Lysine-specific demethylase 2A	−3.11	−1.83	−2.47
Q9BV36	Melanophilin	−0.90	−0.78	−0.84
Q9NPA3	Mid1-interacting protein 1	−2.52	−1.93	−2.23
O00567	Nucleolar protein 56	−2.83	−0.96	−1.89
Q9Y2X3	Nucleolar protein 58	−0.79	−1.88	−1.33
A3KN83	Protein strawberry notch homolog 1	−0.87	−2.37	−1.62
Q96DB5	Regulator of microtubule dynamics protein 1	−1.14	−0.88	−1.01
Q92599	Septin-8	−1.04	−0.66	−0.85
Q9BRS2	Serine/threonine-protein kinase RIO1	−0.67	−1.60	−1.13
P82094	TATA element modulatory factor	−3.37	−0.88	−2.12
P13726	Tissue factor	−0.96	−1.33	−1.14
P46199	Translation initiation factor IF-2	−1.39	−0.78	−1.08
Q86U90	YrdC domain-containing protein	−0.77	−2.10	−1.44

fibronectin and different collagens (Fig. 3c and Supplementary Tables 2 a and b). The impact of TGFβ on mechanical properties of the ECM and on cellular interactions with extracellular proteins is well-documented [18] and several proteins that we found to be up-regulated in CAFs upon TGFβ stimulation in co-culture have been previously reported to be TGFβ targets [19]. TGFβ exhibits a dual role in cancer, i.e., as an anti-proliferative factor and as an inducer of apoptosis [20, 21]. In the proteome analysis of co-cultured ETCs upon TGFβ treatment, we observed a down-regulation of proteins involved in nucleic acid synthesis and in ribosome biosynthesis (Fig. 3d). This finding is indicative of an impaired

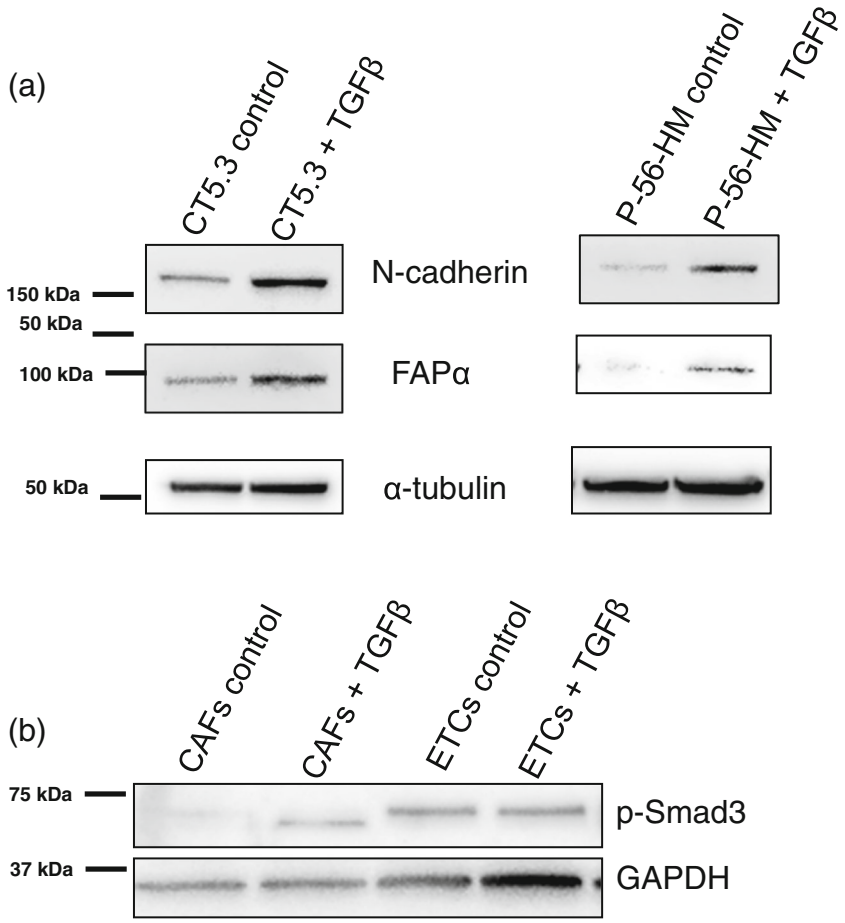

Fig. 4 **a** Expression of EMT markers in TGFβ treated CAFs. CT5.3 and P-56-HM cells were treated with TGFβ after which cell lysates were analyzed by Western blotting for the expression of N-cadherin and FAPα. None of these proteins was detected in ETCs, either in the presence or absence of TGFβ (data not shown). **b** Smad3 phosphorylation analysis in TGFβ treated CAFs and ETCs. CT5.3 and HCT116 cells were treated with TGFβ for 48 h in monocultures. Smad3 phosphorylation was detected by Western blotting. Smad3 phosphorylation was stimulated in CAFs, but not in ETCs

cellular growth and proliferation and is in agreement with the decreased number of ETCs observed in the TGFβ treated co-culture compared to the corresponding untreated control (Fig. 2a).

One of the most prominent modes of action of TGFβ is the induction of an EMT-like cellular behavior [7, 21]. We found that the EMT marker N-cadherin [22] was consistently up-regulated in CAFs (Fc values in replicates 1 and 2: 1.32 and 2.81, respectively), whereas in ETCs its expression was not detectable (Table 2, Supplementary Table 1 c and d). Next, we independently validated the impact of TGFβ on N-Cadherin expression in mono-cultured CAFs. In both CT5.3 CAFs (derived from colorectal cancer) and P-56-HM CAFs (derived from distal bile duct adenocarcinoma) we found that TGFβ treatment resulted in increased N-cadherin levels (Fig. 4a). As expected, N-cadherin expression was not detected in ETCs, nor in any of the three colon cancer-derived cell lines tested: HCT116, SW480 and SW620. We also validated the impact of TGFβ on the CAF marker protein FAPα in both CAF lines (CT5.3 and P-56-HM) and found increased levels upon TGFβ treatment, which is of interest since earlier studies have indicated that FAPα depletion results in reduced TGFβ levels, thus pointing at a mutual feedback system. Lastly, we assessed the phosphorylated Smad3 (pSmad3) levels upon TGFβ treatment in monocultured CAFs and ETCs. In agreement with the strong effect of TGFβ on co-cultured CAFs, and a limited effect on co-cultured ETCs, we found that TGFβ increased the phosphorylation of Smad3 in monocultured CAFs, but not in monocultured ETCs (Fig. 4b). This finding further strengthens a differential responsiveness of CAFs and ETCs to TGFβ.

Previously, it has been shown that different cellular components within tumors may exhibit differential responses to external stimuli [2]. Our current study nicely illustrates how cell type-specific effects of growth factors can be assessed. We performed proteome profiling of two cell types, CAFs and ETCs, grown in a 2D co-culture and stimulated with TGFβ. We observed different architectures in TGFβ-treated co-cultures compared to untreated control cultures. Quantitative proteomic profiling of the two different cell types indicated a strong response to TGFβ by CAFs but not by ETCs. Our findings are in agreement with previous results indicating that TGFβ may lead to up-regulation of

structural ECM proteins in CAFs and may stimulate N-cadherin expression. The differential responsiveness of stromal and tumor cells may be exploited for novel therapeutic avenues targeting stromal cells within the tumor microenvironment.

Acknowledgements We thank Franz Jehle for technical support in mass spectrometry and Patrick Heisterkamp for technical support in Western blot analysis. OS is supported by the Deutsche Forschungsgemeinschaft (SCHI 871/5, SCHI 871/8, SCHI 871/9, SCHI 871/11, INST 39/900-1 and SFB850-Project B8), the European Research Council (ERC-2011- StG 282111-ProteaSys) and the Excellence Initiative of the German Federal and State Governments (EXC 294, BIOSS).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. M.R. Junttila, F.J. de Sauvage, Influence of tumour microenvironment heterogeneity on therapeutic response. Nature **501**, 346–354 (2013)
2. A. Calon, E. Lonardo, A. Berenguer-Llergo, E. Espinet, X. Hernando-Momblona, M. Iglesias, M. Sevillano, S. Palomo-Ponce, D.V. Tauriello, D. Byrom, C. Cortina, C. Morral, C. Barcelo, S. Tosi, A. Riera, C.S. Attolini, D. Rossell, E. Sancho, E. Batlle, Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. Nat Genet **47**, 320–329 (2015)
3. M. Augsten, Cancer-associated fibroblasts as another polarized cell type of the tumor microenvironment. Front Oncol **4**, 62 (2014)
4. Y. Han, Y. Zhang, T. Jia, Y. Sun, Molecular mechanism underlying the tumor-promoting functions of carcinoma-associated fibroblasts. Tumour Biol **36**, 1385–1394 (2015)
5. S. Kakarla, X.T. Song, S. Gottschalk, Cancer-associated fibroblasts as targets for immunotherapy. Immunotherapy **4**, 1129–1138 (2012)
6. P. Brunker, K. Wartha, T. Friess, S. Grau-Richards, I. Waldhauer, C. Ferrara Koller, B. Weiser, M. Majety, V. Runza, H. Niu, K. Packman, N. Feng, S. Daouti, R.J. Hosse, E. Mossner, T.G. Weber, F. Herting, W. Scheuer, H. Sade, C. Shao, B. Liu, P. Wang, G. Xu, S. Vega-Harring, C. Klein, K. Bosslet, P. Umana, RG7386, a novel tetravalent FAP-DR5 antibody, effectively triggers FAP-dependent, avidity-driven DR5 hyperclustering and tumor cell apoptosis. Mol Cancer Ther **15**, 946–957 (2016)
7. J.P. Thiery, H. Acloque, R.Y. Huang, M.A. Nieto, Epithelial-mesenchymal transitions in development and disease. Cell **139**, 871–890 (2009)
8. J.J. Dore Jr., M. Edens, N. Garamszegi, E.B. Leof, Heteromeric and homomeric transforming growth factor-beta receptors show distinct signaling and endocytic responses in epithelial cells. J Biol Chem **273**, 31770–31777 (1998)
9. M.M. Koczorowska, S. Tholen, F. Bucher, L. Lutz, J.N. Kizhakkedathu, O. De Wever, U.F. Wellner, M.L. Biniossek, A. Stahl, S. Lassmann, O. Schilling, Fibroblast activation protein-alpha, a stromal cell surface protease, shapes key features of cancer associated fibroblasts through proteome and degradome alterations. Mol Oncol **10**, 40–58 (2016)
10. J.D. Knopf, S. Tholen, M.M. Koczorowska, O. De Wever, M.L. Biniossek, O. Schilling, The stromal cell-surface protease fibroblast activation protein-alpha localizes to lipid rafts and is recruited to invadopodia. Biochim Biophys Acta **1853**, 2515–2525 (2015)
11. L. Van Hoorde, K. Braet, M. Mareel, The N-cadherin/catenin complex in colon fibroblasts and myofibroblasts. Cell Adhes Commun **7**, 139–150 (1999)
12. M.G. Bachem, E. Schneider, H. Gross, H. Weidenbach, R.M. Schmid, A. Menke, M. Siech, H. Beger, A. Grunert, G. Adler, Identification, culture, and characterization of pancreatic stellate cells in rats and humans. Gastroenterology **115**, 421–432 (1998)
13. A. Franceschini, D. Szklarczyk, S. Frankild, M. Kuhn, M. Simonovic, A. Roth, J. Lin, P. Minguez, P. Bork, C. von Mering, L.J. Jensen, STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Res **41**, D808–D815 (2013)
14. E.M.F. De Sousa, X. Wang, M. Jansen, E. Fessler, A. Trinh, L.P. de Rooij, J.H. de Jong, O.J. de Boer, R. van Leersum, M.F. Bijlsma, H. Rodermond, M. van der Heijden, C.J. van Noesel, J.B. Tuynman, E. Dekker, F. Markowetz, J.P. Medema, L. Vermeulen, Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. Nat Med **19**, 614–618 (2013)
15. A. Calon, E. Espinet, S. Palomo-Ponce, D.V. Tauriello, M. Iglesias, M.V. Cespedes, M. Sevillano, C. Nadal, P. Jung, X.H. Zhang, D. Byrom, A. Riera, D. Rossell, R. Mangues, J. Massague, E. Sancho, E. Batlle, Dependency of colorectal cancer on a TGF-beta-driven program in stromal cells for metastasis initiation. Cancer Cell **22**, 571–584 (2012)
16. S.S. Huang, J.S. Huang, TGF-beta control of cell proliferation. J Cell Biochem **96**, 447–462 (2005)
17. S. Tholen, C. Wolf, B. Mayer, J.D. Knopf, S. Loffek, Y. Qian, J.N. Kizhakkedathu, M.L. Biniossek, C.W. Franzke, O. Schilling, Skin barrier defects caused by keratinocyte-specific deletion of ADAM17 or EGFR are based on highly similar proteome and degradome alterations. J Proteome Res **15**, 1402–1417 (2016)
18. B. Hinz, The extracellular matrix and transforming growth factor-beta1: tale of a strained relationship. Matrix Biol **47**, 54–65 (2015)
19. P. Ranganathan, A. Agrawal, R. Bhushan, A.K. Chavalmane, R.K. Kalathur, T. Takahashi, P. Kondaiah, Expression profiling of genes regulated by TGF-beta: differential regulation in normal and tumour cells. BMC Genomics **8**, 98 (2007)
20. P.M. Siegel, J. Massague, Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer **3**, 807–821 (2003)
21. H. Ikushima, K. Miyazono, TGFbeta signalling: a complex web in cancer progression. Nat Rev Cancer **10**, 415–424 (2010)
22. A. Gheldof, G. Berx, Cadherins and epithelial-to-mesenchymal transition. Prog Mol Biol Transl Sci **116**, 317–336 (2013)