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Inhibition of integrin $\alpha_v\beta_6$ changes fibril thickness of stromal collagen in experimental carcinomas

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Abstract

Background: Chemotherapeutic efficacy can be improved by targeting the structure and function of the extracellular matrix (ECM) in the carcinomal stroma. This can be accomplished by e.g. inhibiting TGF- β 1 and - β 3 or treating with Imatinib, which results in scarcer collagen fibril structure in xenografted human KAT-4/HT29 (KAT-4) colon adenocarcinoma.

Methods: The potential role of $\alpha_v\beta_6$ integrin-mediated activation of latent TGF- β was studied in cultured KAT-4 and Capan-2 human ductal pancreatic carcinoma cells as well as in xenograft carcinoma generated by these cells. The monoclonal $\alpha_v\beta_6$ integrin-specific monoclonal antibody 3G9 was used to inhibit the $\alpha_v\beta_6$ integrin activity.

Results: Both KAT-4 and Capan-2 cells expressed the $\alpha_v\beta_6$ integrin but only KAT-4 cells could utilize this integrin to activate latent TGF- β in vitro. Only when Capan-2 cells were co-cultured with human F99 fibroblasts was the integrin activation mechanism triggered, suggesting a more complex, fibroblast-dependent, activation pathway. In nude mice, a 10-day treatment with 3G9 reduced collagen fibril thickness and interstitial fluid pressure in KAT-4 but not in the more desmoplastic Capan-2 tumors that, to achieve a similar effect, required a prolonged 3G9 treatment. In contrast, a 10-day direct inhibition of TGF- β 1 and - β 3 reduced collagen fibril thickness in both tumor models.

Conclusion: Our data demonstrate that the $\alpha_v\beta_6$ -directed activation of latent TGF- β plays a pivotal role in modulating the stromal collagen network in carcinoma, but that the sensitivity to $\alpha_v\beta_6$ inhibition depends on the simultaneous presence of alternative paths for latent TGF- β activation and the extent of desmoplasia.

Background

Carcinomal stroma shares many characteristics with a healing wound or with a chronic inflammatory lesion. Commonly observed stromal features are leukocyte infiltration, fibrin deposition, increased blood vessel permeability for plasma proteins, and increased production and metabolism of ECM proteins [1, 2]. Malignant and normal stromal cells, including infiltrating inflammatory cells, participate in reciprocal signaling that results in the buildup of the carcinomal stroma [2, 3]. Also, both human and experimental carcinomas have a pathologically elevated intratumoral

interstitial fluid pressure (P_{IF}) [4]. Several P_{IF} -lowering agents increase the uptake and efficacy of small molecular weight chemotherapeutic agents in experimental carcinomas [4] suggesting that an elevated P_{IF} reflects impediment of solute transport into carcinoma tissue.

Carcinoma-associated fibroblasts (CAFs) originate from phenotypic switches of infiltrated or resident mesenchymal cells and may also originate from epithelial-to-mesenchymal transition of carcinoma cells. This process can be triggered by soluble mediators secreted by malignant cells or by infiltrating leukocytes [3, 5]. CAFs or a CAF subpopulation is the major producer of the stromal collagen fiber network and the other ECM components. In many aspects CAFs resemble “activated” fibroblasts in healing wounds and in fibrotic lesions. The formation of CAFs is modulated by TGF- β [6] that also stimulates collagen production

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and constitutes a key factor in the development of fibrosis [7, 8]. The three TGF- β isoforms, TGF- β 1, - β 2 and - β 3, are secreted as small latent complexes (SLC) in which the active growth factors are non-covalently bound to latency associated peptides (LAPs) (for a recent review see: [9]). During maturation in the endoplasmic reticulum most of the nascent SLC becomes covalently bound via disulfide bonds to latent TGF- β binding proteins (LTBP), forming the large latent complex (LLC) that covalently associates with the ECM [9]. Since latent TGF- β complexes are inactive, a TGF- β release mechanism is necessary in order for the cytokine to bind and stimulate its cognate receptors. The known release mechanisms include the action of reactive oxygen species, extreme acidification, proteolytic cleavage, interaction with thrombospondin, or conformational change of the latent complex, [9]. Among the conformational change release mechanisms the integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ have been extensively described [9]. Data from studies with genetically modified mice suggest that integrin-directed activation of latent TGF- β complexes plays a major role in TGF- β dependent processes in vivo [10, 11]. The integrin $\alpha_v\beta_6$ is implicated in development [10] and in cancer [12, 13], its expression is restricted to cells of epithelial origin, and it is upregulated in many cancer types and can be used as a prognostic factor [14]. $\alpha_v\beta_6$ promotes carcinoma growth and invasion [13] and can act pro-migratory in pancreatic cancer cells [15] in addition to its TGF- β activating function that is important also for matrix metalloproteinase expression [16].

Previously, we have shown that human KAT-4 carcinoma cells transfer TGF- β to fibroblasts via intercellular contacts, resulting in an increased collagen type I protein synthesis [17]. In vivo, treatment with the soluble TGF- β type II receptor-murine Fc:IgG_{2A} fusion protein (Fc:T β RII), a specific inhibitor of TGF- β 1 and - β 3, reduces intratumoral collagen density and influences the collagen network architecture in xenograft human KAT-4 cell-derived carcinoma [18]. In addition, Fc:T β RII lowers intratumoral P_{IFN}, dampens the expression of inflammation-related genes, reduces vascular protein leakage, and increases the treatment efficacy of doxorubicin [19]. Here we investigated the importance of $\alpha_v\beta_6$ integrin for the collagen network ultrastructure in the previously characterized model of colorectal carcinoma, KAT-4, and in a model of desmoplastic pancreatic cancer, Capan-2 [20].

Methods

Reagents

The function-blocking anti- $\alpha_v\beta_6$ antibody 6.3G9 (henceforth referred to as 3G9) has been described previously [21]. The TGF- β receptor type II-murine Fc:IgG_{2A} chimeric protein (Fc:T β RII) that functionally inhibits TGF- β 1 and - β 3, as well as control IgG₁ clone 1E6, were all prepared sterile and pyrogen-free at Biogen and used as solutions of the purified

proteins in PBS and stored at -80°C until use. The c-RGD peptides cyclo-(Arg-Gly-Asp-D-Phe-Val), a selective inhibitor of the $\alpha_v\beta_3$ integrin [22], was from Bachem (Bubendorf, Switzerland) (H-2574) and Cilengitide, a selective inhibitor of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [23] was from Selleckchem (Munich, Germany). The ROCK inhibitor Y-27632 was from Sigma-Aldrich. Human fibronectin and vitronectin were purified from fresh human plasma as described [24, 25].

TGF- β assay

The quantitative PAI-1/luciferase bioassay for TGF- β [21, 26] was used to investigate the production of TGF- β by carcinoma cells in vitro. TGF- β induces the expression of plasminogen-activator inhibitor-1 (PAI-1) in Mink Lung Epithelial cells (MLEC, clone C32) transfected with a truncated PAI-1 promoter fused to the firefly luciferase reporter gene. For co-culture assays 96- or 24-well plates for transwell assays (Corning® HTS Transwell®; Sigma Aldrich Sweden AB, Stockholm, Sweden) were pre-coated 3 h or overnight with DMEM containing 10% FBS. MLEC were seeded in DMEM supplemented with 0.1% BSA (Sigma Aldrich) and allowed to attach for 3 h. KAT-4 and Capan-2 cells were then added to the wells or into the transwell inserts. KAT-4 and Capan-2 carcinoma cells were incubated with 3G9, Fc:T β RII or control IgG₁ for 30 min on ice before they were added to the MLEC. Plates were incubated at 37°C for 20 h. Cells and the conditioned medium were harvested and analyzed according to the manufacturer's protocol (Luciferase Assay System; Promega Biotech AB, Nacka, Sweden). Capan-2 cells were pre-acclimatized to growth in DMEM when to be co-cultured with fibroblasts. F99 normal human fibroblasts were isolated from explant cultures of human skin from patients who underwent breast reduction surgery. MLEC (0.25×10^4 cells) and a combined 0.25×10^4 of malignant cells or fibroblasts were seeded per well in 96 well plates.

Animals

Athymic C57BL nu/nu female mice were purchased from Taconic Europe (Ry, Denmark) and were between 6 and 10 weeks of age. Prior to the inoculation of carcinoma cells mice were acclimatized at the local animal facility. Animals were individually identified by ear markings. All animal experiments were performed at the animal facilities of the National Veterinary Institute and Lund University, Sweden, in accordance with approval by the ethical committees for animal experiments in Uppsala and Malmö/Lund, Sweden. The number of animals was minimized to comply with guidelines from the Ethical Committee and EU legislation on the use of laboratory animals.

Cells/xenograft models

KAT-4 cells were originally described as originating from a thyroid tumor [27], but a thyroid origin of the KAT-4 carcinoma has been questioned and the cells have been identified as part of the human colorectal adenocarcinoma cell line HT-29 [28]. Human pancreatic carcinoma 139/Capan-2/JE cells were from ATCC. The identities of the two cell lines were ascertained by Short Tandem Repeat loci analyses (IdentiCell, Aarhus, Denmark). Capan-2 showed a 100% match, whereas KAT-4, as expected [28], matched with HT-29 although alleles D13S317:12 and TH01:9 were absent. Normal human dermal F99 fibroblasts that had been isolated as described [29] were kindly donated by prof. Bengt Gerdin (Uppsala University). KAT-4 cells and F99 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Stockholm, Sweden) and Capan-2 cells were grown in McCoy's 5A medium with GlutaMax (Gibco), both supplemented with 10% FBS (Gibco) and antibiotics. In the protocol used in Uppsala 50 μ L of cell suspensions containing either 2×10^6 KAT-4 cells or 5×10^6 Capan-2 cells were injected *s.c.* in the left flank of the mice. Mice that developed carcinomas were inspected every other day and the external sizes (length x width x height) of the carcinomas were measured using a vernier caliper.

Treatment with 3G9 and Fc:T β RII

Treatments were initiated when KAT-4 and Capan-2 tumors reached a size of approximately 100 mm³. Tumor treatment groups were size-matched and animals were treated with PBS or control IgG₁, 3G9 IgG, or Fc:T β RII via intra-peritoneal injection in a maximal volume of 100 μ L. 3G9 IgG was administered at doses of 3, 10 and 30 mg/kg and Fc:T β RII at a dose of 10 mg/kg at days 1 and 7 in experiments with KAT-4 carcinomas. Capan-2 carcinomas were treated with 10 mg/kg of 3G9 IgG or Fc:T β RII at days 1, 3 and 7. Animals were sacrificed and carcinomas collected at day 11. For some experiments Capan-2 carcinomas were treated according to an extended protocol with 10 mg/kg 3G9 IgG administered at days 1, 3, 7, 10 and 14 and carcinomas collected at day 19 (extended protocol).

Measurement of interstitial fluid pressure

Tumor P_{IF} was measured by the "wick-in needle" technique as we have previously described [30]. For data recording and collection the Microsoft Excel, ADI PowerLab (Software Chart v. 4.1 and Scope v. 3.6.8 for Macintosh) was used.

Immunohistochemistry and histology

Tumors were dissected, embedded in OCT (Sakura) and frozen at -80 °C. Cryosections (6–8 μ m) were fixed in acetone at 8 °C, blocked in 20% goat serum (Serotec,

Oxford, UK), incubated with monoclonal rat anti-mouse PECAM-1 (CD31) clones Mec 13.3 and 390 (BD, San Diego, CA, USA) and incubated with goat anti-rat biotinylated IgG (Vector laboratories, Burlingame, CA, USA). To visualize $\alpha_v\beta_6$ in tissue sections purified 3G9 was biotinylated with N-hydroxysuccinimide biotin ester overnight at 4 °C, followed by a single desalting step with PD-10 columns in PBS with 0.1% BSA and 0.02% azide as preservative. Sections were blocked in 5% swine serum (Sigma) and 2% BSA (Sigma), incubated with biotinylated 3G9 antibody and then with Fluorescein Avidin D (Vector Labs). Slides were mounted with Vectamount containing DAPI (Vector Labs), and the staining was analyzed using a Nikon Eclipse 80i microscope (Nikon Instruments, Japan).

For histological assessment, Masson's trichrome and Sirius red staining was performed using the Accustain Masson Trichrome stain (Sigma-Aldrich) and 0.1% Picrosirius Red solution (Solveco, Sweden) according to the manufacturer's instructions. Hematoxylin and eosin staining was performed according to standard protocols.

Determination of vessel density

CD31 immunostaining was performed on KAT4 and Capan-2 carcinomas treated with 3G9 or PBS. Sections were then analyzed after imaging. CD31- positive structures, having an area greater or equal to that of the smallest discernible vessel (29 μ m²), were counted and plotted per mm². All comparisons were done from sections stained simultaneously, with the same exposure for image capture, and a defined threshold for image analysis. Images were analyzed in ImageJ software (NIH, Bethesda, Maryland, USA).

Hydroxyproline measurement

KAT4 carcinomas were hydrolyzed in 6 M HCl for 4 h at 120 °C at a pressure of 2 atm. Hydroxyproline content in the hydrolysates was determined essentially as previously described [31].

Electron microscopy

For transmission electron microscopy analyses were fixed in 0.15 M sodium cacodylate-buffered 2% glutaraldehyde, post-fixed in 0.15 M sodium cacodylate-buffered 1% osmium tetroxide, dehydrated in graded ethanol series, impregnated in acetone and embedded in epoxy resin. Ultrathin sections were examined in a Philips CM-10 electron microscope (Philips, Amsterdam, Netherlands) [32]. Collagen fibril diameter was measured using ImageJ software (NIH, Bethesda, Maryland, USA).

Flow cytometry

Cells (5×10^6) were incubated with the anti- $\alpha_v\beta_6$ antibody 3G9 or mouse IgG as a negative control in PBS for

1 h, washed and incubated with FITC-conjugated rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) secondary antibody for 45 min, washed and suspended in FACS buffer. A titration was done with both primary and secondary antibodies to determine the appropriate amount, which was 0.12 mg/mL 3G9 and 1:100 dilution of the secondary antibody. The flow cytometry analysis was performed using a FACSAria (Becton Dickinson).

RNA extraction and Illumina gene expression analysis

Total RNA from 3-dimensional free floating collagen gels was extracted with the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA) according to the manufacturers' instructions. RNA was quantified with Nanodrop (ThermoFisher), integrity-validated on Bioanalyzer, and run on an Illumina platform (SCIBLU, LTH, Lunds University). Signal intensities were first filtered using internal standards, then by normalization to the observed background level. Collagen gels were made in 48-well plates, coated for 2 h with 2% BSA (Sigma) at 37 °C. Carcinoma cells were re-suspended to a concentration of 1×10^6 cells/ml and mixed 1:9 with a freshly prepared collagen gel (50% 2xDMEM, 10% 200 mM HEPES pH 8 and 40% 3.1 mg/ml type I bovine collagen (Biomatrix)). Gels were allowed to solidify at 37 °C for 2 h before being floated with serum free DMEM media and incubated at 37 °C 5% CO₂ for 48 h. Gels were dissolved and stored in Trizol (ThermoFisher) for later total RNA extraction.

Microarray data analysis

Illumina Microarray (SCIBLU) data was initially pre-processed and normalized using the Quantile Normalization method [33]. Analyses were performed using GenomeStudio software V2011.1. The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE85255 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85255>). Non-annotated probe sets and probe sets with signal intensities below the median level of negative control intensities, in 20% of the samples, not belong to one condition, were excluded. Differential expression analysis was performed using DESeq2 script from Bioconductor [34].

Statistical methods

Unpaired, two-tailed Student's *t*-test and the Mann-Whitney *U* test were used and $p < 0.05$ was considered statistically significant. The standard deviations of data points are indicated in the figures. All statistics were performed in GraphPad Prism 6 (La Jolla, CA, USA). All in vitro data was collected from a minimum of three or more independent experiments.

Results

KAT-4 and Capan-2 human carcinoma cells produce and secrete latent TGF- β

Conditioned media (CM) from cultures of KAT-4 and Capan-2 cells were collected and analyzed to measure total TGF- β levels using luciferase-based MLEC assay. Cell culture medium from MLEC was used as a negative control. Heat-activated CM from KAT-4 and Capan-2 cell cultures elicited PAI-1 activity (TGF- β signaling) in the reporter cells, whereas the corresponding non-activated CM had no effect (Fig. 1a). Heat-activated CM from Capan-2 and KAT-4 cell cultures contained similar amounts of TGF- β . The results show that both KAT-4 and Capan-2 carcinoma cells produced and secreted latent TGF- β .

Next, we elucidated whether KAT-4 and Capan-2 cells were able to activate latent TGF- β when co-cultured with MLEC allowing for the formation of direct cell-cell contacts. Active TGF- β was transferred from KAT-4 to the adjacent MLEC when the two cell types were co-cultured (Fig. 1b). When KAT-4 cells were separated from the MLEC cells by a porous membrane that allowed free passage of growth factors (Transwell system) no TGF- β signaling was detected in the MLEC. Capan-2 cells lacked the ability to activate the growth factor also when co-cultured with the reporter MLEC (Fig. 1b).

Cultured carcinoma cells activate latent TGF- β by an integrin $\alpha_v\beta_6$ -dependent mechanism

To validate that KAT-4 and Capan-2 cells express similar amounts of cell surface integrin $\alpha_v\beta_6$ we used flow cytometry and the anti-integrin $\alpha_v\beta_6$ specific monoclonal antibody 3G9 (Additional file 1). 3G9 IgG effectively inhibited TGF- β elicited signaling in MLEC co-cultured with KAT-4 cells over a wide range of IgG concentrations (0.004 to 10 μ g/mL) demonstrating the involvement of integrin $\alpha_v\beta_6$ in KAT-4 cell-driven activation of latent TGF- β (Fig. 1c). Furthermore, the specific TGF- β 1 and - β 3 inhibitor Fc:T β R2 (10 μ g/mL) inhibited TGF- β elicited signaling in MLEC co-cultured with KAT-4 cells, demonstrating that KAT-4 cells produce and elicit a TGF- β signaling in the target MLEC cells through TGF- β 1 and/or - β 3 (Fig. 1d). Addition of 3G9 or Fc:T β R2 to co-cultures of MLEC and Capan-2 cells had no effect on TGF- β signaling (Fig. 1e). Normal human dermal F99 fibroblasts co-cultured with MLEC were unable to activate latent TGF- β but active TGF- β was generated when F99 fibroblasts, Capan-2 cells and MLEC were co-cultured; this activation was inhibited by 3G9 (Fig. 1e). To further control for integrin specificity we investigated the effects of cilengitide, an Arg-Gly-Asp containing peptide that inhibits the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in nM ranges, and the $\alpha_v\beta_3$ selective antagonist peptide cRGD. Neither of the peptides inhibited the activation of latent TGF- β by

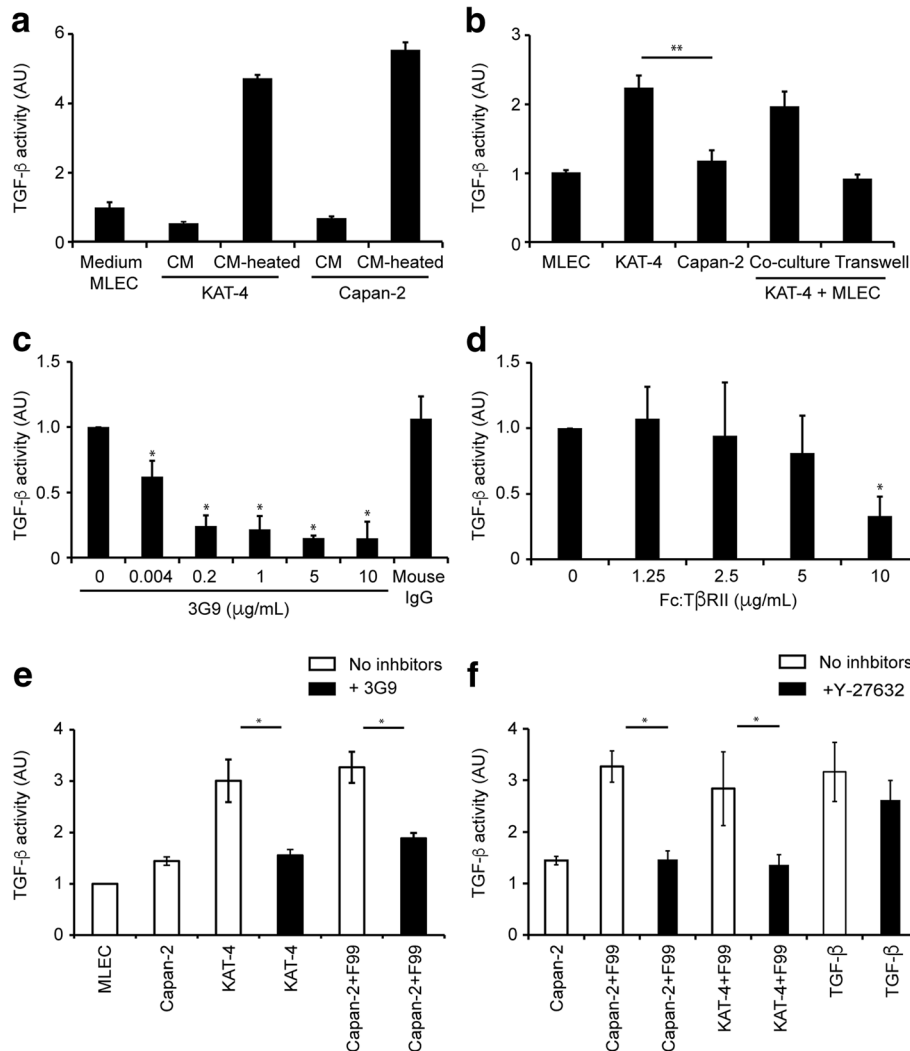


Fig. 1 Luciferase-based assay showing TGF- β activity in mink lung epithelial cells (MLEC). **a** Effects by TGF- β elicited signaling in MLEC by heat-activated (CM heated) or non-activated (CM) conditioned medium from KAT-4 and Capan-2 cell cultures. **b** TGF- β activity in cultures of MLEC, KAT-4, Capan-2, and co-cultures of KAT-4 and MLEC ($p = 0.0014$). **c** Effect of 3G9 antibody on TGF- β activity in co-cultures of MLEC and KAT-4 cells. **d** Effect of Fc:T β RII on TGF- β activity in co-cultures of MLEC with KAT-4 cells. **e** Effect of 3G9 on TGF- β activity in KAT-4 and MLEC co-cultured with F99 fibroblasts and Capan-2 cells. **f** Effects of the ROCK inhibitor Y-27632 (10 μ M) on TGF- β activity in fibroblasts co-cultured with tumor cells, as well as effects of TGF- β supplementation (10 nM). All data points were compiled from a minimum of three independent experiments performed in triplicates. Error bars are standard deviations. Asterisks indicate $p < 0.05$ as analyzed with Student's t-test

Capan-2/F99 cells over a wide concentration range, suggesting that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins were dispensable for the activation process (Additional file 2). Finally, to investigate whether Capan-2 cells alone are unable to activate latent TGF- β due to insufficient amounts of auxiliary proteins, such as fibronectin [35], we cultured Capan-2 cells on dishes pre-coated with human plasma fibronectin or vitronectin. Neither condition rendered Capan-2 cells capable of activating latent TGF- β (Additional file 2).

Rho kinases and the down-stream effector ROCK play a pivotal role in $\alpha_v\beta_6$ -mediated activation of latent TGF- β by epithelial cells [36]. We therefore investigated the effects of the selective ROCK I and II inhibitor Y-27632

on carcinoma cell-driven activation of latent TGF- β . Supplementing the cell cultures with 10 μ M Y-27632 effectively inhibited the activation of latent TGF- β by both KAT-4 and Capan-2 cells co-cultured with F99 fibroblasts (Fig. 1f). Y-27632 had, however, no effect on signaling elicited by active TGF- β resulting in increased transcription of PAI-1 in MLEC (Fig. 1f).

Gene expression profiles of KAT-4 and Capan-2 cells cultured in three-dimensional collagen gels

To elucidate the differences that influence the dependence on the $\alpha_v\beta_6$ -mediated latent TGF- β activation we performed comparisons of gene expression profiles in KAT-4 and

Capan-2 cells grown in free-floating collagen gels. Illumina microarray platform analysis revealed that neither of the cell lines expressed transcripts characteristic of epithelial-to-mesenchymal transition, including genes encoding a set of ECM, ECM-modifying enzymes, cytoskeletal proteins, and PDGFs and PDGF receptors (Fig. 2). The expression signals of the glucagon gene (*GCG*), which is specifically expressed by α -cells of the islets of Langerhans, are shown to indicate background expression levels (Fig. 2). Thirty-four transcripts were differentially expressed in KAT-4 vs. Capan-2 cells, at cut-offs of \log_2 4-fold difference, significance level < 0.05 and Q-value (Additional file 3). Among the keratin genes *KRT20* was highly expressed in KAT-4 cells, and *KRT7* was expressed in Capan-2 cells, which is in line with a colonic origin of KAT-4 cells and a ductal lining origin of Capan-2 cells. Among the extracellular space-related transcripts Capan-2 cells had upregulated tissue plasminogen activator (*PLAT*) and urokinase-type plasminogen activator (*PLAU*), and also transcripts encoding ECM protein cross-linking enzymes (*TGM2* and *LOXL4*). KAT-4 cells expressed higher levels of cell adhesion-related *TSPAN8* and *BSG*. Both cell lines had upregulated number of different metabolism- and immune response-related transcripts.

Comparison of KAT-4 and Capan-2 carcinomas

Experimental carcinomas generated after xenografting KAT-4 or Capan-2 cells into nude mice were analyzed for morphology and collagen content. The respective carcinomas, grown to similar sizes, differed both in carcinoma cell density and in ECM structure (Fig. 3a). Capan-2 carcinomas were substantially richer in collagenous ECM and had lower cell density (Fig. 3a, b). Biotinylated 3G9 bound to epithelial cells both in KAT-4 and Capan-2 carcinomas,

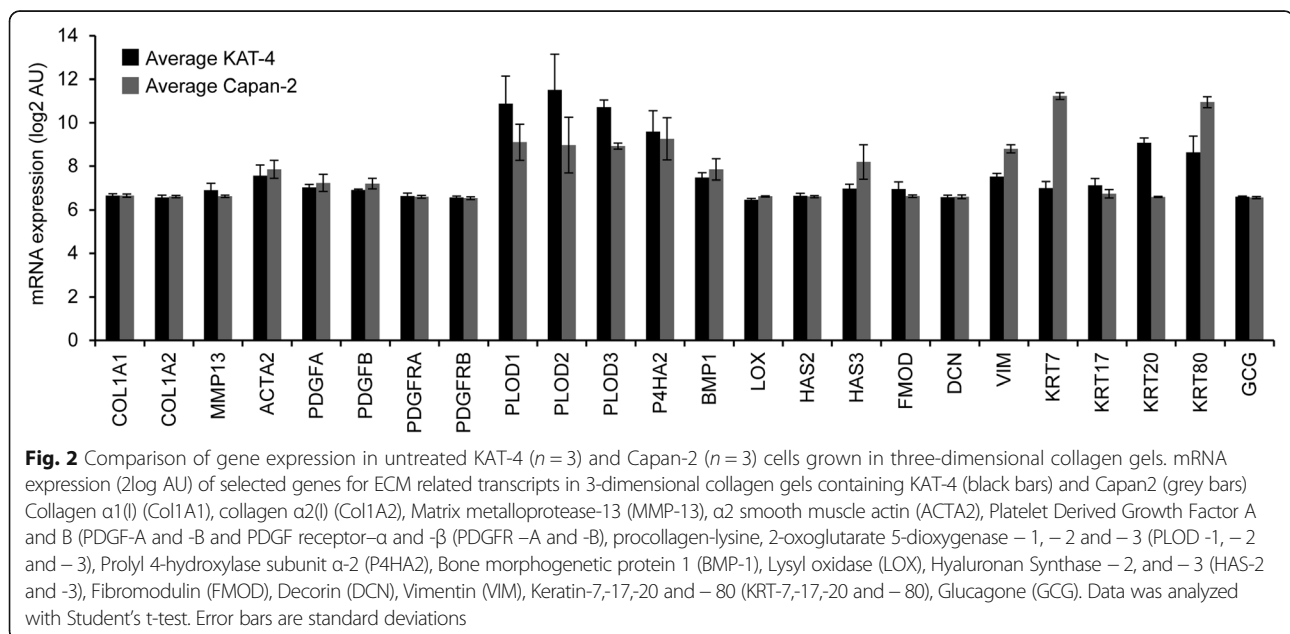
suggesting maintained integrin $\alpha_v\beta_6$ -expression during in vivo growth (Fig. 3a). Finally, in vitro-cultured KAT-4 cells grew at a faster rate than Capan-2 cells (data not shown) and this difference was reflected in tumor growth in vivo, i.e. KAT-4 carcinomas grew faster than Capan-2 carcinomas (Fig. 3c).

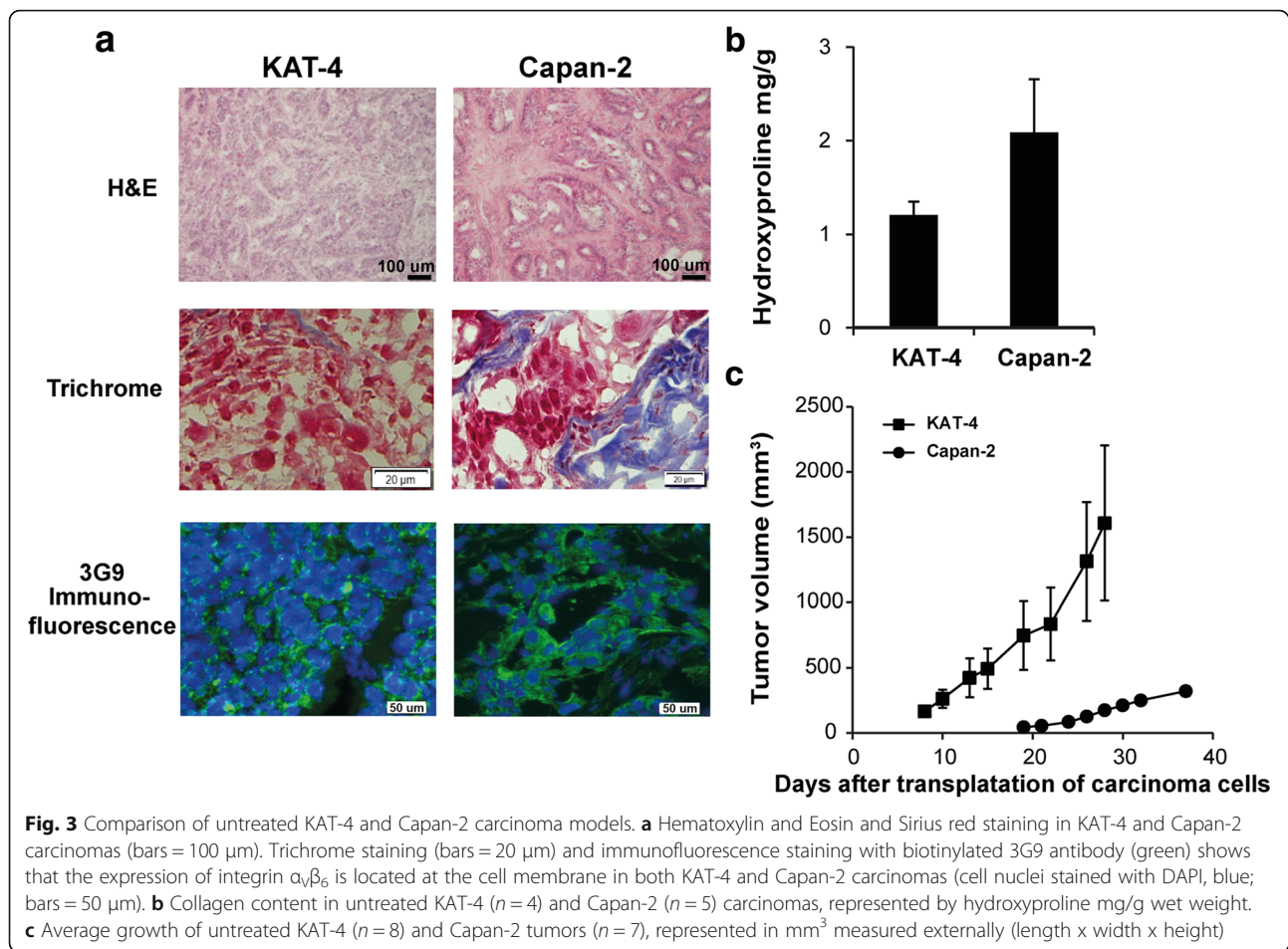
Blockage of integrin $\alpha_v\beta_6$ reduces collagen fibril diameter in KAT-4 and Capan-2 carcinomas

Transmission electron microscopy analysis revealed that collagen fibrils in Capan-2 were on average 4 nm thicker than in KAT-4 carcinomas; they were also skewed towards thicker fibrils (compare untreated samples (black bars) in e.g. Fig. 4b, d). Treatment of Capan-2 carcinomas with Fc:T β R2 at 10 mg/kg at days 1, 3 and 7 significantly reduced collagen fibril thickness (Fig. 4a). These data demonstrate a dependence of TGF- β signaling for stromal collagen network architecture in this tumor model. By contrast, fibril thickness was not affected after a 10-day treatment with 3G9 at 10 mg/kg (Fig. 4b); fibril thickness did, however, decrease significantly following an extended treatment protocol (injections at days 1, 3, 7, 10 and 14) (Fig. 4c). In contrast, a 10-day treatment of KAT-4 carcinomas with 3G9 was sufficient to significantly reduce the mean collagen fibril thickness (Fig. 4d).

Blockage of integrin $\alpha_v\beta_6$ reduces interstitial fluid pressure

We next investigated the role of $\alpha_v\beta_6$ -mediated activation of latent TGF- β in maintaining a high P_{IF} in experimental carcinoma. KAT-4 carcinoma-bearing mice were treated with 3G9 at doses of 3, 10 and 30 mg/kg of body weight for 10 days. P_{IF} measured at day 11, was significantly lower in all treatment groups ($p < 0.008$ for all three





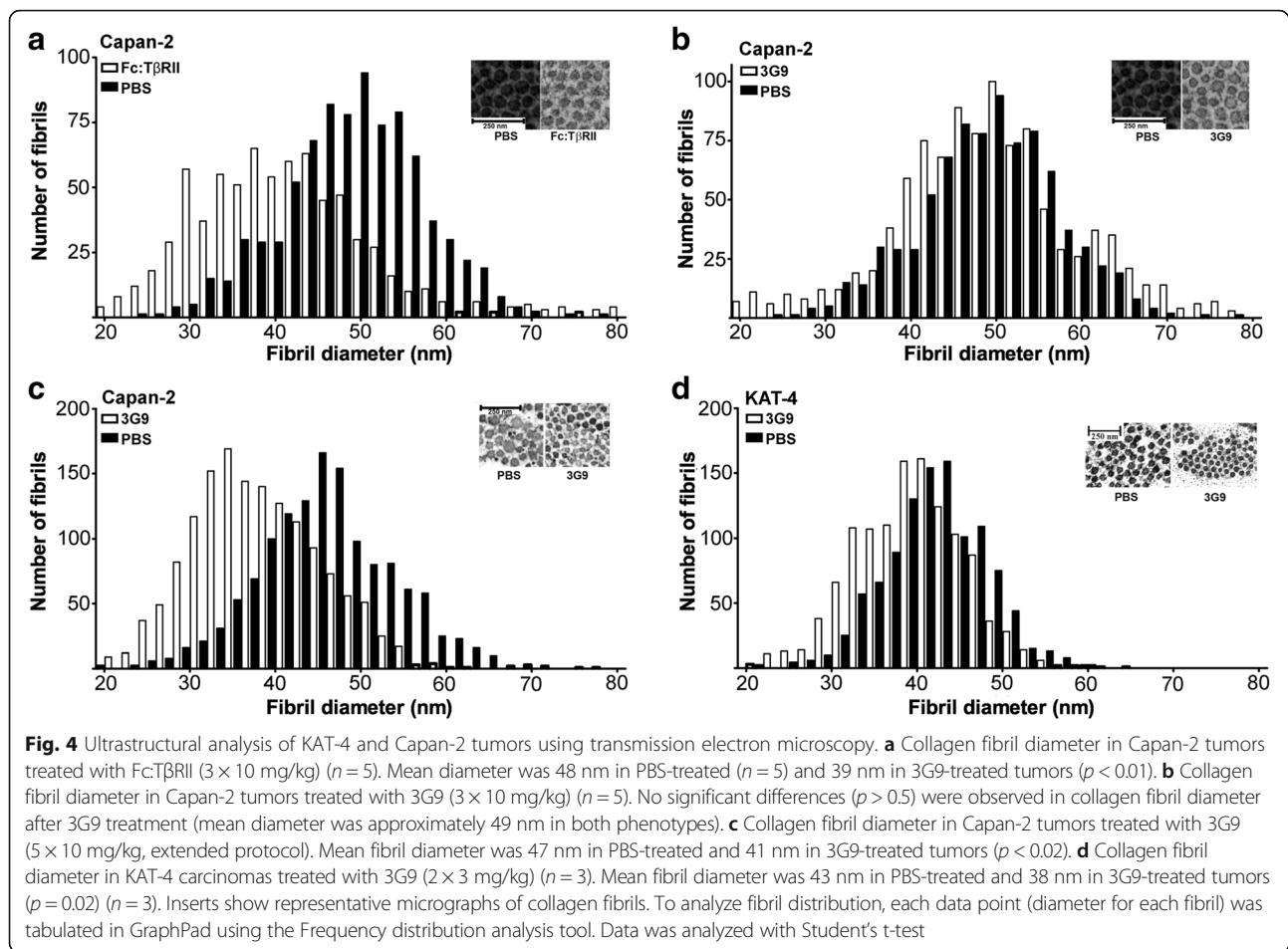
groups, $n = 10$) (Fig. 5a). Administration of 10 mg/kg of Fc:T β RII at days 1 and 7 also reduced P_{IF} in accordance with previous reports [37] ($p < 0.005$) (Fig. 5a). In Capan-2 carcinomas P_{IF} values were generally higher than in KAT-4 carcinomas and had a much larger inter-tumor variation. Treatment of Capan-2 carcinomas with 3G9 administered at a dose of 10 mg/kg had no significant effect on P_{IF} (Fig. 5b). Treatment of Capan-2 carcinomas with 3G9 according to the extended protocol with injections at days 1, 3, 7, 10 and 14 resulted, however, in a trend for a lowered P_{IF} (measured on day 19, Fig. 5b), although this effect did not reach significance due to the large inter-tumoral variation (Fig. 5b). No significant correlation between tumor size and P_{IF} was observed in any of the two carcinoma models (Additional file 4). Staining with the endothelial cell marker CD31 did not show any significant effect of 3G9 on blood vessel density in either Capan-2 or KAT-4 carcinomas (Fig. 5c, d).

Discussion

We hypothesized that $\alpha_v\beta_6$ integrin plays an essential role in the activation of latent TGF- β in carcinoma and thereby participates in processes dependent on TGF- β

signaling. To test this hypothesis we utilized the $\alpha_v\beta_6$ integrin-blocking antibody 3G9 in two experimental models of carcinoma - KAT-4 (HT-29) colorectal adenocarcinoma and Capan-2 ductal pancreatic carcinoma. Our data indicate that $\alpha_v\beta_6$ integrin is involved in latent TGF- β activation, but also that the dependency on this pathway may differ between different carcinoma types.

The pivotal role of $\alpha_v\beta_6$ integrin in latent TGF- β activation in carcinoma is concluded from several lines of evidence: firstly, KAT-4 cells activated latent TGF- β and transferred the active growth factor to target cells by an $\alpha_v\beta_6$ -dependent process; Capan-2 cells, however, were unable to activate latent TGF- β unless they were co-cultured with human F99 fibroblasts. The latter effect was dependent on $\alpha_v\beta_6$ integrin since it was blocked by 3G9 IgG and not by peptides that inhibit the function of $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins. Secondly, the blocking of either $\alpha_v\beta_6$ integrin or TGF- β 1 and - β 3 resulted in thinner collagen fibril structure. This phenotypic response was evident in KAT-4 carcinomas after a 10-day treatment period with 3G9 IgG (akin to the effects of the specific TGF- β 1 and - β 3 inhibitor Fc:T β RII [18, 37]), the same effect was observed in the Capan-2



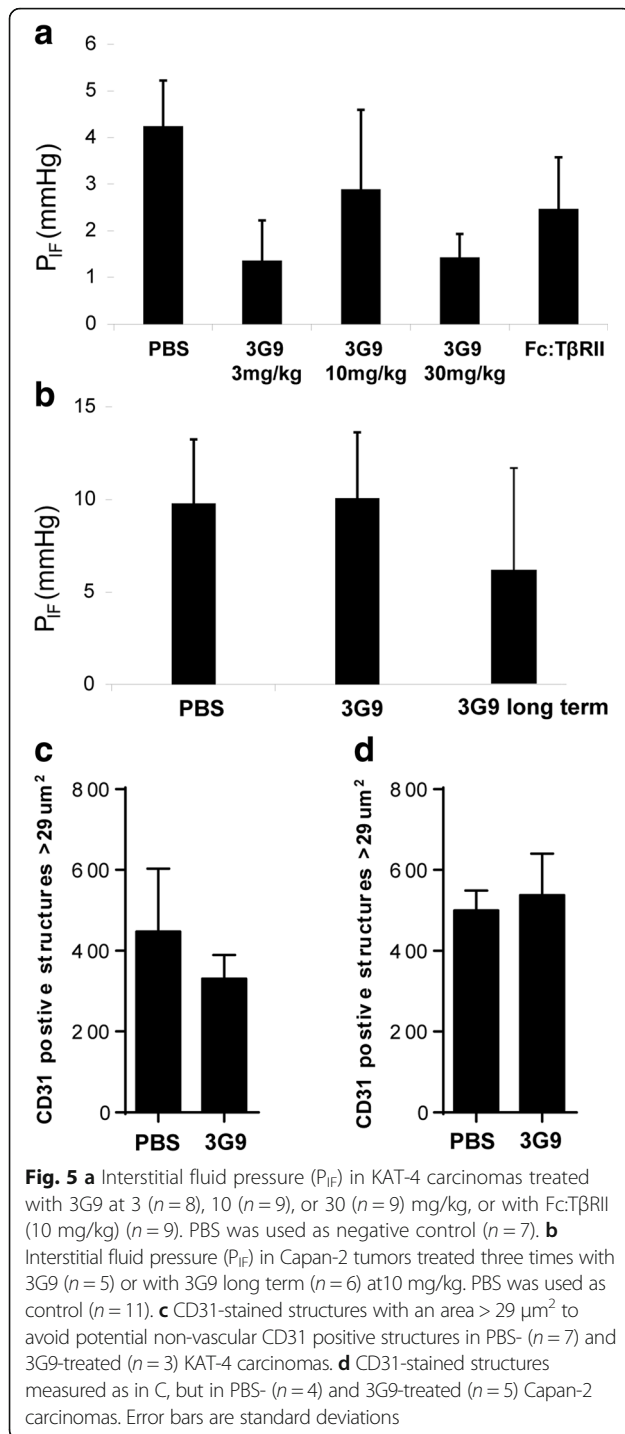
model albeit after a longer treatment period with 3G9 IgG.

Whereas blockage of TGF-β1 and -β3 in the Capan-2 carcinoma model resulted in thinner collagen fibrils after a 10-day treatment period a prolonged 19-day treatment period was needed to achieve the same effect by 3G9 IgG. The reason for this discrepancy is not clear. 3G9 IgG and Fc:TβRII have similar molecular weights and structures and it is unlikely that the difference in sensitivity to 3G9 IgG and Fc:TβRII can be attributed to disparities in the uptake and distribution in the carcinoma tissue. In our microarray study we observed that Capan-2 cells overexpressed *LOXL4* and *TGM2*, both functioning in cross-linking of extracellular matrix proteins [38, 39], which could affect the mechanical forces generated by integrin pulling on the matrix-embedded latent TGF-β complex. Capan-2, or indeed even other desmoplastic carcinomas, may therefore propagate a distinct ECM structure that enhances the $\alpha_V\beta_6$ -dependent latent TGF-β activation pathway [40] thereby requiring a prolonged treatment period with repeated injection of 3G9 IgG. We also observed an increased abundance of several ECM-related transcripts in Capan-2 cells, transcripts that

could contribute to the extent of $\alpha_V\beta_6$ -dependent TGF-β activation. A higher expression of *PLAT* and *PLAU* genes, encoding tissue plasminogen activator and urokinase-type plasminogen activator, could lead to a more pronounced conversion of plasminogen to plasmin, a process that has been implicated in enhanced latent TGF-β activation [9].

Activation of latent TGF-β by both KAT-4 and Capan-2/F99 were dependent on the downstream effector of Rho GTPase, ROCK. This is in agreement with published reports [36] and also with the notion that generation of tension is important for integrin $\alpha_V\beta_6$ -directed activation of latent TGF-β [9, 40]. It is thus possible that the different abilities of KAT-4 and Capan-2 cells to activate latent TGF-β stem from differences in capacities to generate cellular tension. For example, we observed that the latent TGF-β was activated when Capan-2 cells were co-cultured with F99 fibroblasts, which could be explained by the F99 fibroblasts supplying tensile forces to adjacent Capan-2 cells.

With regards to cultured KAT-4 cells' ability to autonomously activate latent TGF-β using the $\alpha_V\beta_6$ integrin we observed that the single-cell KAT-4 cultures express higher levels of *TSPAN8* - a member of tetraspanin proteins that co-localize with integrins and influence cellular



motility [41]. It is conceivable that this protein may interact with $\alpha_V\beta_6$ integrin and thus influence the activation of latent TGF- β .

Previous studies of experimental carcinomas have revealed a correlation between P_{IF} and specific collagen network properties like collagen fibril density, fibril structure or network architecture [18, 42, 43]. Treatment of experimental carcinoma with imatinib (STI571, Glivec) significantly

lowers P_{IF} , increases the extracellular fluid volume and enhances the dynamic exchange of solutes between tumor interstitium and blood [44, 45]. These effects are paralleled by a decrease in average collagen fibril diameter and an increase in collagen turnover in stroma ECM [45]. The effects of imatinib resemble those of inhibitors of the $\alpha_V\beta_6$ /TGF- β signaling axis. Imatinib selectively inhibits the tyrosine kinase c-Abl [46] and c-Abl is stimulated by TGF- β in a non-Smad dependent pathway [47]. It is possible that inhibition of c-Abl constitutes a convergence point for the effects on carcinoma stroma exerted by inhibitors of the $\alpha_V\beta_6$ /TGF- β signaling axis and imatinib. We have observed that imatinib abolished TGF β -elicited activation of the PAI-1 promoter in the mink-lung epithelial cells (Additional file 5). These data show that imatinib can inhibit at least some aspects of TGF- β -elicited signaling, aspects that may be of importance for the build-up and maintenance of pathologic stroma in carcinoma.

In the ECM, collagen together with hyaluronan and other glycosaminoglycans (GAG) form a functional and integrated matrix that determines fluid transport properties through tissues [48]. Interference either by alterations or depletions of the collagen or GAG components will change the physiological properties, including P_{IF} . The data reported herein, emphasizing the importance of the collagen network, are clearly in accordance with this model, as collagen provides the framework for GAGs. In the less collagenous KAT-4 model P_{IF} was reduced as collagen fibril diameter decreased. Capan-2 tumors had a trend towards decreased P_{IF} ; these tumors, however, are more collagenous, which may reduce the hydration of GAGs in spite of a reduced fibril diameter, even after an extended treatment period.

Conclusion

TGF- β s have dual roles during tumor development. In early malignant lesions they inhibit cell proliferation while during later stages they promote carcinoma progression by stimulating epithelial-to-mesenchymal transition, suppressing immune surveillance and aiding in the formation of a permissive microenvironment [6]. Notwithstanding the complexities of TGF- β pathway modulation our study shows that in selected carcinomas an inhibition of $\alpha_V\beta_6$ /TGF- β signaling axis alters the tumor stroma towards an alteration of the collagen network structure that previously has been shown to be associated with improved drug efficacy; the present study could open new venues in the research to improve chemotherapeutic efficacy.

Additional files

Additional file 1: FACS analysis showing that both KAT-4 and Capan-2 tumor cells expressed $\alpha_V\beta_6$ integrin on their cell surfaces (black peaks). IgG is negative control (grey peaks). (TIF 144 kb)

Additional file 2: TGF- β activation and inhibition in fibroblast and Capan-2 co-cultured with MLEC. Data was analyzed with Student's t-test. Error bars are standard deviations. **A)** TGF- β activation inhibition with Cilengitide (0 to 10 μ M). **B)** TGF- β activation inhibition with cRGD peptide (0 to 10 μ M). **C)** Effects on TGF- β activation by co-culturing KAT-4 cells (Kat4, K) or Capan-2 (Capan2, C) together with MLEC reporter cells (M) on plastic dishes coated with human plasma fibronectin (HFN) or human plasma vitronectin (Vn). (TIF 8227 kb)

Additional file 3: Differential gene expression in KAT-4 vs. Capan-2 single-cell cultures. Transcripts with log₂ fold change > 4 are shown and annotated using GO terms or experimental evidence reported in Uniprot. Positive fold change means higher transcript levels in Capan-2, negative fold change means higher transcript levels in KAT-4. (PDF 44 kb)

Additional file 4: Relationships between carcinoma size (weight) and interstitial fluid pressure (P_{IF}). P_{IF} values were recorded in PBS-treated (control) KAT-4 ($n = 9$) and Capan-2 ($n = 14$) carcinomas, the carcinomas were excised and finally weighed. The data are partly collected from carcinomas showed in in Fig. 5. (PDF 40 kb)

Additional file 5: Effects of the tyrosine kinase inhibitor imatinib (STI571) on TGF β -induced PAI-1 expression in mink lung epithelial cells (MLEC) was recorded as detailed in Material and Methods. The signal recorded after stimulation with 10 nM TGF- β was normalized to 1 ($n = 6$). The effect on TGF- β -elicited signaling by 10 μ M STI571 is shown ($p < 0.0001$, $n = 3$). Error bars \pm SD. All reagents were added simultaneously to the MLEC cultures at the start of the incubations. (TIF 393 kb)

Abbreviations

CM: Conditioned media; ECM: Extracellular matrix; Fc: β RII: soluble TGF- β receptor type II IgG_{2A} fusion protein; GAG: Glycosaminoglycans; MLEC: Mink Lung Epithelial cells; PAI-1: Plasminogen-activator inhibitor-1; P_{IF} : Interstitial fluid pressure; ROCK: Rho-associated coiled coil forming protein serine/threonine kinase; TEM: Transmission electron microscopy; TGF- β : Transforming growth factor- β ; tissue plasminogen activator; uPA: urokinase-type plasminogen activator

Funding

This study was supported by funds from the Swedish Cancer Society (to KR), the Swedish Research Council (to KR).

Availability of data and materials

The Illumina micro array data have been deposited in NCBI's Gene Expression Omnibus [49] and are accessible through GEO Series accession number GSE85255 (Olsson PO, Rubin K. Tumor cell to fibroblast expression analysis. NCBI's Gene Expression Omnibus August 30 ed2016). <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85255>

Authors' contributions

AVS, SMV, LAK, NEH and KR conceived and designed the study. NEH, BB, RG and KR supervised the study. MG, TF, ASV, RG, POO and KSZ performed the experiments. All authors participated in the analyses of the data. KR drafted and supervised the writing of the manuscript and all authors contributed to the final version. All authors read and approved the final manuscript.

Ethics approval

All animal experiments were performed at the animal facilities of the National Veterinary Institute and Lund University, Sweden, in accordance with approval by the ethical committees for animal experiments in Uppsala and Malmö/Lund, Sweden. The number of animals was minimized to comply with guidelines from the Ethical Committee and EU legislation on the use of laboratory animals.

Consent for publication

All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Competing interests

Three authors (LAK, PHW, SMV) were employees of Biogen at the time the work was completed. One of the authors (PHW) still is an employee at Biogen. None of the other authors report any potential competing interests.

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Received: 5 April 2018 Accepted: 19 June 2018

Published online: 02 July 2018

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