Cell-cell interactions in the gastrointestinal tumour-microenvironment

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Chapter 5

Interaction between colon cancer cells and fibroblasts generates myofibroblasts via activation of Transforming Growth Factor-β1

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Submitted

Abstract

In colonic cancer myofibroblasts are present in high abundance. However, their origin and function remains unknown. We observed that the TGF-β/Smad signalling pathway is strongly activated in cancer-associated myofibroblasts (CAFs). Since TGF-β1 can induce trans-differentiation of myofibroblasts *in vitro,* we analysed how TGF-β influences the colon tumour-microenvironment. Both HCT116 colon cancer cells and CAFs responded to exogenous TGF-β with strongly increased expression of plasminogen activator inhibitor-1 and matrix metalloproteinases (MMPs). Moreover, we observed an 18-fold induction of TGF-β1 production in TGF-β stimulated CAFs, indicating an autocrine feedback loop. Analysis of the TGF-β activation mechanism revealed that neither CAFs, nor cancer cells were able to activate exogenous small latent TGF-β1 complexes. However, culture of CAFs with HCT116 derived medium, containing high amounts large latent TGF-β1, strongly increased TGF-β signalling and smooth muscle actin expression, indicating release from latent complexes and subsequent myofibroblast trans-differentiation. Experiments using various protease inhibitors suggested that this co-culture induced TGF-β activation was not dependent on proteolytic activity.

In summary, we showed that the interplay between colonic cancer cells and CAFs enhanced TGF-β activation and subsequent myofibroblast trans-differentiation. This in turn led to cumulative production of TGF-β and proteinases within the tumour-microenvironment, creating a cancer-promoting feedback loop.

Introduction

The interaction between carcinoma cells and fibroblasts within the tumour-microenvironment contributes to cancer initiation, progression and eventually metastasis in many cancer types[1-4]. Cancer-associated myofibroblasts are a heterogeneous group of fibroblasts expressing vimentin, fibroblast-activating protein (FAP) and α-smooth-muscle actin (SMA)[5-9]. Compared to normal mucosa the number of myofibroblasts is strongly increased in colorectal cancer[8,10], where they influence the immune response and show increased synthesis of chemokines, cytokines, proteolytic enzymes and several components of the extracellular matrix (ECM)[9,11]. The origin of this distinct cell-population has not fully been clarified yet[9], but based on primarily *in vitro* studies, the most commonly accepted hypothesis is that cancer-associated myofibroblasts are generated by Transforming Growth Factor-β (TGF-β) induced trans-differentiation of resident fibroblasts[1,12].

Next to its contribution to the hyperactivation of resident fibroblasts, TGF-β is involved in carcinogenesis via tumour angiogenesis, increased production of ECM and proteolytic enzymes and immune suppression[13-15]. TGF-β is synthesized as a latent precursor consisting of the TGF-β homodimer non-covalently linked to the Latency Associated Protein (LAP) forming the small latent complex (SLC) and the latent TGF-β binding molecule (LTBP), which connects the complex to the ECM as the large latent complex (LLC)[16]. Removal of both LAP and LTBP is the crucial regulation mechanism for TGF-β bio-activity. Activation can occur through proteolytic processing (e.g. plasmin[17] and matrix metalloproteinases (MMPs)[18-21]) or non-proteolytically via conformational changes (e.g. integrins[22]). The activating mechanism seems to be strongly dependent on the cell/tissue type or the experimental conditions. Active TGF-β is able to bind to the widely expressed TGF-β type II Receptor (TβRII), which subsequently recruits and transphosphorylates the TGF-β type I receptor (TβRI), activin receptor-like kinase (ALK)-5. This results in signal transduction via phosphorylation of the Smad2/Smad3 complex, which recruits Smad4 and translocates to the nucleus where transcription is initiated.

In this study we evaluated whether the enhanced numbers of myofibroblasts in colonic cancer could be derived from epithelial-cell-mesenchymal cell interaction involving TGF-β1 activation. First SMA expression and TGF-β1 activity were immunohistochemically studied in human colonic cancer specimens. Next we assessed TGF-β1 response of both tumour cells and cancer-associated fibroblasts (CAFs) for the expression of target genes and proteases implicated in TGF-β activation. Co-culture experiments revealed that the interaction between

tumour cells and CAFs led to TGF-β1 activation and subsequent differentiation of fibroblasts into myofibroblasts. In turn, these myofibroblasts showed strongly increased expression of proteases and TGF-β1 creating a cancer-progressing feedback loop.

Materials and methods

Patient material

Tissue specimens from patients undergoing surgical resection for colonic carcinoma at the Department of Oncologic Surgery, Leiden University Medical Centre, were collected. Tissue was fixated, dehydrated through graded alcohol and xylene and embedded in paraffin. Furthermore tissues from the same patients were used for isolation of primary cancer associated fibroblasts (CAFs). Human samples were used according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Centre.

Immunohistochemistry

Immunohistochemical staining was performed as described before[23]. In short, sequential sections were deparaffinized, rehydrated and endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in methanol. Antigen retrieval was performed by boiling in 0.01 M sodium citrate, pH 6.0 for 10 minutes followed by overnight incubation at room temperature (RT) with primary antibodies described in table 1. Sections were incubated with appropriate biotinylated secondary antibodies, streptavidin biotin complex (all Dako, Denmark) and staining was visualized using diaminobenzidine and H_2O_2. Representative photomicrographs were taken with a Nikon Eclipse E800 microscope equipped with a Nikon DXM1200 digital camera.

Table 1 Antibodies used for immunohistochemistry.

Antibody	Clone	Manufacturer	Dilution	Antigen retrieval
Pan-Cytokeratin	C11	Santa Cruz, USA	1:1000	Citrate[1]
Vimentin	V9	Santa Cruz	1:400 paraffin 1:1000 cytospins	Citrate[1]
SMA	ASM-1	Progen, Heidelberg, Germany	1:400 paraffin 1:1500 cytospins	Citrate[1]
pSmad2	Rabbit polyclonal	Provided by P. ten Dijke[59]	1:500	Citrate[1]
TGF-β	TB21	Anogen	1:1000	Citrate[1]

[1] *Boiling in 0.01 M Sodium Citrate buffer pH 6.0, 10 minutes*

Cell culture, isolation of fibroblasts and preparation of conditioned media (CM)

Colon carcinoma cell lines HT29, HCT116, LS180, Lovo, SW480, SW948, and CaCo-2 were cultured in DMEM/F12 + GlutaMAX-1 (Invitrogen, the Netherlands), 10 mM HEPES, 50 µg/ml gentamycin, 100 U/ml penicillin and 100 µg/ml streptomycin (all Invitrogen), supplemented with 10% heat inactivated Fetal Calf Serum (FCS, Perbio Science, Belgium) or 20% in case of CaCo-2. Human cancer-associated fibroblasts were isolated from the non-necrotic part of the tumour. The tissue was washed with PBS, cut into 5 mm pieces and incubated in 75 cm^2 tissue culture flasks (Corning, the Netherlands). After 7-10 days fibroblast-like cell outgrowth was observed. The fibroblast origin of these cells was confirmed by positive staining for vimentin and negative staining for pan-cytokeratin. The cells were maintained in complete DMEM/F12 containing 10% FCS and used at passage 5-11.

Conditioned media (CM) from colon cancer cell lines and cancer-associated fibroblasts were prepared by seeding the cells in culture flasks and growing them to sub-confluence. Then medium was changed to serum free (SF)-DMEM/F12, containing HEPES and antibiotics as described above, and incubated for 4 days. CM used for stimulation was diluted 2 fold with fresh SF-DMEM/F12. To obtain myofibroblast CM, fibroblasts were stimulated with 5 ng/ml TGF-β1 (recombinant human, Peprotech, United Kingdom) for 24h. After three washes with SF-DMEM/F12, cells were incubated for 4 days with SF-DMEM/F12. Medium was analysed for MMP-2 and MMP-9 levels by zymography as described before[24] and for TGF-β1 levels by ELISA as described below.

MTS proliferation assay

HT29 and HCT116 colon cancer cells were seeded in 96-well plates (Greiner Bio-one, 2,000 or 10,000 cells/well) and allowed to attach overnight. Cells were stimulated with 5 ng/ml TGF-β1 for 6h or 72h and subsequently the medium was changed to 100 µl complete DMEM + 20 µl MTS substrate (Promega, USA). The metabolic activity of the cells was analyzed by absorbance change at 490 nm after 2h.

TGF-β response assay

TGF-β response in tumour cells was determined using a $(CAGA)_{12}$-MLP-Luciferase promoter reporter construct[25]. This construct contains 12 repeats of the Smad3/4 binding element derived from the PAI-1 promoter and was shown to be highly specific and sensitive to TGF-β. Tumour cells were seeded in 24 well plates (Corning) and allowed to attach overnight.

Subconfluent cells were transfected using Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocol. 0.1 μg β-galactosidase plasmid was co-transfected to correct for transfection efficiency. After 6 hours medium was changed to complete DMEM/F12 and the cells were incubated for 24h and serum starved overnight. Cells were stimulated with 0-5 ng/ml TGF-β1 or under indicated conditions. After stimulation the cells were washed, lysed and luciferase activity was determined according to the manufacturer's protocol (Promega, USA). β-galactosidase activity in the lysates was determined using β-gal substrate (0.2M H_2PO_4, 2 mM $MgCl_2$, 4 mM ONPG, 0.25% β-mercaptoethanol,) and measuring absorbance change at 405 nm. For all samples the luciferase count was corrected for β-galactosidase activity and the relative increase in luciferase activity was calculated versus controls.

Cancer-associated fibroblasts were infected using an adenoviral Ad-$(CAGA)_9$-MLP-Luc promoter reporter construct[26]. Cells (18000/well) were infected with 1E+6 pfu virus. After infection medium was changed to complete DMEM/F12 for 24 hours and the fibroblasts were serum starved overnight, stimulated, lysed and the luciferase activity was determined as described for the colonic cells.

Western blotting

The expression of TGF-β signalling molecule Smad4 was analyzed by western blotting. Cell lysates of all colon cancer cell lines were prepared using a 50 mM Tris/HCl buffer, pH 7.6 containing 1% Triton X-100 and sonification for 30 seconds. Protein concentrations were determined using the BCA assay (Pierce, USA). Equal protein amounts were separated on 10% SDS-PAGE under reducing conditions. Proteins were transferred to a nitrocellulose membrane (Whattman, Germany) and aspecific binding was blocked with 2.5% milk powder (Biorad laboratories, USA) in PBS containing 0.05% Tween-20 (PBST, Merck, Germany) for 2 hours at RT. Blots were incubated overnight with mouse monoclonal anti-Smad4 antibodies (BD Biosciences, Belgium) in 0.5% bovine serum albumin (BSA)/PBST. Detection was performed by incubation with goat-anti-mouse HRP labelled secondary antibodies (Dako) and chemoluminescence by Super Signal West according to the manufacturers' protocol (Pico Chemoluminescent substrate, Pierce). Blots were stripped using restore stripping buffer (Pierce) and reprobed with mouse β-actin as a loading control.

RNA isolation and real-time PCR analysis

Expression of TGF-β target genes and MMPs were analyzed in HT29, HCT116, SW948 and CaCo-2 cells. Cells were grown to subconfluence, harvested and RNA was isolated using RNeasy minipreps according to the manufacturers' instructions (Qiagen, USA). RNA concentration and purity was determined by A260/A280. cDNA synthesis was performed from 1 μg RNA using random primers. cDNA samples were subjected to 40 cycles Real-Time PCR analysis using primers as previously described[27,28], except for MMP-7, MMP-13 and MMP-28 primer sets which were purchased from Applied Biosystems, the Netherlands. All values were normalized for cDNA content by GAPDH expression. To study TGF-β mediated upregulation of these genes HT29, HCT116 and cancer- associated fibroblasts were grown to subconfluence and stimulated with 5 ng/ml TGF-β1 for 24 hours. RNA was isolated as described above. TGF-β does not influence GAPDH expression[29].

TGF-β1 ELISA

TGF-β levels in cell culture supernatants were determined by an ELISA for active TGF-β1 (R&D Systems). Total TGF-β levels were determined in parallel after acid activation of the latent TGF-β1 in the same samples as described before[23].

HT29 invasion assay

HT29 spheroids were grown by the liquid overlay technique as described before[30]. Spheroids were collected and 10 spheroids were embedded in collagen gels in 48-well plates (Corning), consisting of 10% DMEM/F12 with 0-10 ng/ml TGF-β1 and 1 mg/ml collagen type 1 (Vitrogen, Nutacon, USA). Spheroids were analyzed at 4-14 days for invasiveness and the formation of distant metastasis like cell clusters. Representative photomicrographs were taken using a Zeiss Axiovert microscope using the 10x objective.

TGF-β1 activation experiments

To analyze TGF-β1 activation by tumour cells and cancer-associated fibroblasts these cells were transfected as described above. Cells were stimulated with 10 ng/ml rh-small latent TGF-β1 complex (R&D systems) or CM from tumour cells or cancer-associated fibroblasts.

To examine the contribution of proteolytic enzymes in TGF-β1 activation, fibroblast were stimulated with HCT116 CM in the presence or absence of several inhibitors: 10 μM ALK-5 inhibitor SB421543 (Sigma-Aldrich, Germany), 1 μM GM6001 (broad spectrum MMP inhibitor), 1 μM specific MMP-2/9 or specific MMP-13 inhibitor, 10 μM specific MMP-3

inhibitor (all Calbiochem, USA), 1 μM Marimastat (broad spectrum MMP inhibitor, kindly provided by British Biotech Pharmaceuticals, UK), 10 μg/ml aprotinin (serine protease inhibitor including plasmin), 20 μM E64 (cystein protease inhibitor) or 100 nM α-2 macroglobulin (all Sigma-Aldrich). Combinations of inhibitors were also tested. Incubations were performed for 24h and luciferase activity was determined as described above. For fibroblast experiments parallel incubations were performed, medium was harvested for the TGF-β1 ELISA and cells were harvested by trypsinisation. Cytospin preparations were made and cells were analyzed for SMA content by immunohistochemistry. SMA content was scored in a double blinded manner by one individual.

Results

Immunohistochemistry

In normal mucosa vimentin positive cells included fibroblasts, endothelial cells and inflammatory cells, whereas epithelial cells stained for pan-cytokeratin (not shown). A single layer of vimentin positive pericryptal cells co-stained for SMA indicating the myofibroblast phenotype (approximately 5%, Figure 1A). Staining for total TGF-β was low in normal mucosa, but epithelial cells stained for pSmad2 in the nucleus, indicating TGF-β signalling.

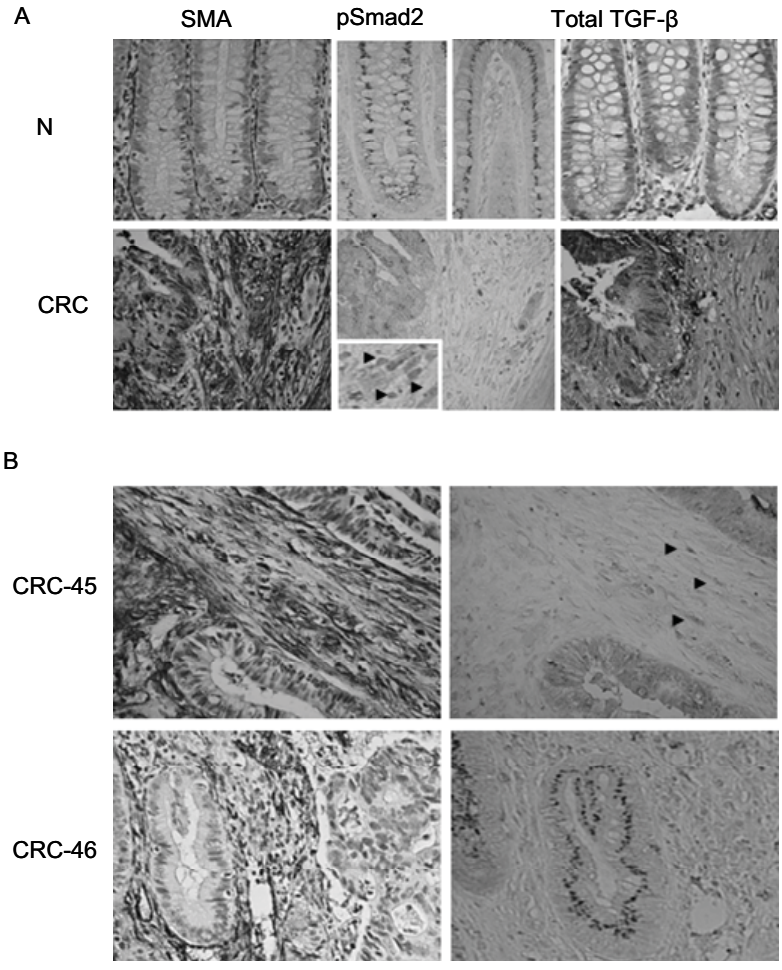

Figure 1. Immunohistochemistry on CRC tissue samples. Normal colonic mucosa displays a single layer of SMA positive myofibroblasts along the crypt axis, which show no nuclear staining for pSmad2, in contrast to epithelial cells, which show a gradient in staining from bottom to top of the crypt. Total TGF-β is present in low levels in normal mucosa, whereas it is strongly increased in CRC (A). Strongly increased SMA expression and a shift from epithelial to mesenchymal nuclear accumulation of pSmad2 occurs in CRC, although some samples, like CRC-46, still show nuclear accumulation of pSmad2 in malignant cells next to myofibroblasts (B). Full-colour illustration at page 205.

pSmad2 nuclear staining intensity varied from the bottom of the crypts, where proliferation takes place, to the top (Figure 1A) consistent with the anti-proliferative action of TGF-β in normal tissue. pSmad2 was barely detectable in fibroblasts or myofibroblasts. In colonic cancer total TGF-β staining was strongly enhanced in malignant epithelial cells and the tumour ECM. The majority of the fibroblasts (up to 90%) was of the SMA-positive myofibroblast phenotype and strongly showed nuclear pSmad2 accumulation (Figure 1A,

lower panel). The upper panel in Figure 1B shows the reduced epithelial nuclear localization of pSmad2 which is observed in the majority of the CRCs, indicating a shift from primarily epithelial TGF-β signalling in normal tissue to mesenchymal signalling in adjacent tumours. In contrast, the lower panel in Figure 1B shows another colonic tumour in which, besides surrounding fibroblasts, also the malignant epithelial cells still displayed nuclear pSmad2 localization.

*Characterization of (myo)fibroblas*ts

To examine the mechanism of fibroblast trans-differentiation, we isolated fibroblasts from colonic cancer tissue. The number of SMA positive myofibroblasts was evaluated by immunohistochemistry on cytospin preparations and varied between 50 and 90%. All tumour-derived fibroblasts showed a dose-dependent induction of TGF-β response (3-10 fold) as shown in figure 2A.

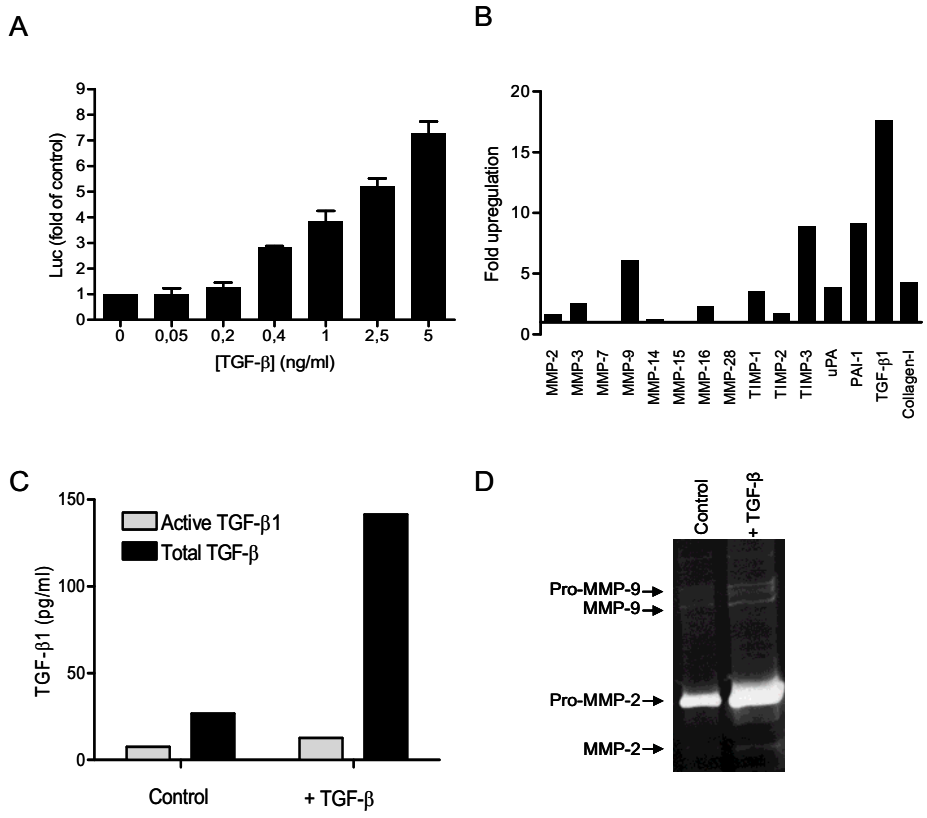

Figure 2. TGF-β regulated expression of target genes in cancer-associated fibroblasts.
Fibroblasts respond dose-dependently to exogenous TGF-β1 (A, n=3-7 independent experiments) and real-time PCR analysis showed upregulation of various proteinases and especially TGF-β mRNA (B). Secretion of TGF-β is also strongly enhanced into the medium of TGF-β stimulated fibroblasts (C). MMP-2 and MMP-9 protein secretion is increased in TGF-β stimulated fibroblasts versus their non stimulated counterparts (D).

Real-Time PCR analysis revealed high basal expression of collagen type-1 and plasminogen activator inhibitor (PAI)-1 in these cells, confirming the predominant myofibroblast phenotype, but also the expression of MMPs was generally high compared with colon cancer cells (Table 2). TGF-β1 treatment increased the expression of collagen-1, PAI-1, uPA and various MMPs and TIMPs (Figure 2B). Interestingly, the expression of TGF-β1 was induced 18 fold under TGF-β1 stimulation, indicating a strong autocrine regulatory loop. The protein expression of a selection of the up-regulated genes was verified using various techniques. TGF-β stimulation of fibroblasts indeed led to a strong increase of total TGF-β1 protein levels combined with a low increase in TGF-β1 activity (Figure 2C). Zymogram analysis revealed strongly increased MMP-2 and MMP-9 levels in the medium of stimulated fibroblasts compared to non-stimulated cells (Figure 2D).

Table 2. Expression MMPs and TGF-β target genes by colon cancer cell lines and cancer-associated fibroblasts.

Target	HT29	HCT116	SW948	CaCo-2	CAF
MMP-2	++	- (*)	++	+	+++
MMP-3	+	- (*)	++	+/-	+++
MMP-7	+++	+	+	+	++
MMP-9	++	+	++	+	+
MMP-13	+	+/-	++	+	+++
MMP-14	+	+	++	++	+++
MMP-15	++	+	+	++	-
MMP-16	++	++	++	++	++
MMP-17	-	++	++	+	++
MMP-28	-	-	+	-	-
TIMP-1	++	++	++	+++	+++
TIMP-2	++	++	++	++	+++
TIMP-3	+	+	++	++	+++
uPA	++	+	++	+/-	-
PAI-1	++	++	++	+	+++
TGF-β1	+++	+++	+++	+++	+++
Collagen-1	-	-	-	-	+++

Expression of MMPs and TGF-β target genes was determined in non-stimulated cells.

– No expression (dCt >20), +/- very low (dCt 15-20), + moderate (dCt 10-15), ++ high (dCt 5-10, +++ very high (dCt <5). All dCt values are expressed versus GAPDH as housekeeping gene. 40 cycles of Real-Time PCR were used. CAF = Cancer-associated fibroblast, MMP = Matrix Metalloproteinase, TIMP = Tissue Inhibitor of Matrix Metalloproteinases, uPA = urokinase type Plasminogen Activator, PAI-1 = Plasminogen Activator Inhibitor. (*) = expressed upon TGF-β stimulation.

Characterization of colonic cancer cell lines

To evaluate the variation in TGF-β1 response in colonic cancer cells we used a TGF-β promoter reporter construct in a panel of colon carcinoma cell lines. LS180, LOVO, SW480, SW948 and CaCo-2 cells showed no induction of luciferase activity after TGF-β treatment as shown by a representative graph in figure 3A.

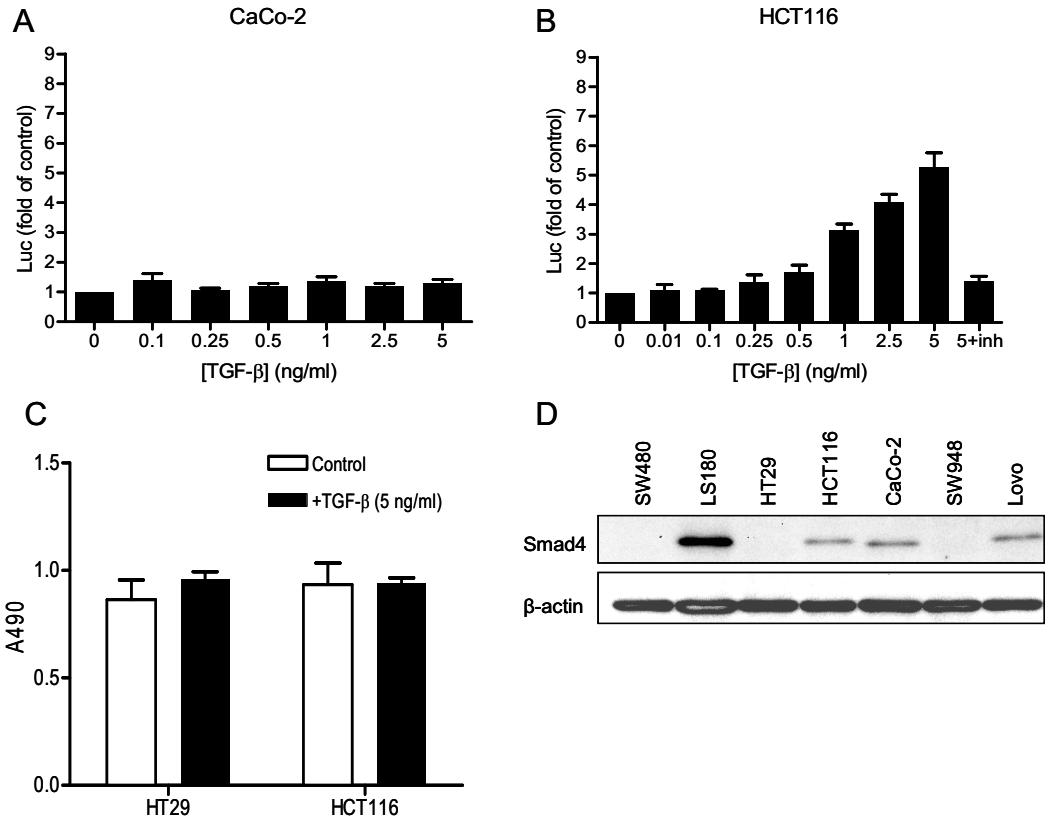

Figure 3. TGF-β response of colonic tumour cells. The majority of the cell lines is not responsive to exogenously added TGF-β (A, represented by CaCo-2, n=3 independent experiments,), whereas HCT116 cells dose-dependently respond to TGF-β (B). Proliferation is not inhibited by TGF-β in HT29 and HCT116 cells as determined by MTS proliferation assay after 72 hours stimulation (C). Smad4 western blot analysis revealed that the most of the non-responsive cell lines do not express Smad4 protein (D). Data represent 3-7 independent experiments performed in triplicate (mean ± SEM)

HT29 cells were partly responsive; only stimulation with concentrations of more than 5 ng/ml TGF-β1 increased luciferase activity (not shown). In contrast HCT116 cells showed dose-dependent induction of luciferase activity up to 5-fold when treated with TGF-β1 (Figure 3B). Addition of ALK-5 inhibitor abolished the TGF-β1 induced response completely. Cell-proliferation was not inhibited by 6h or even 72 hours of TGF-β1 treatment as shown by a

MTS proliferation assay in Figure 3C. Analysis of Smad4 protein, important in downstream TGF-β signalling, revealed no detectable expression in SW480, SW948 and HT29, whereas expression was observed in CaCo-2, LS180 and HCT116 cells (Figure 3D). Next we analyzed the basal expression levels of TGF-β1, PAI-1 and invasion related proteinases in HCT116, HT29, SW948 and CaCo-2 cells by Real-Time PCR. The data revealed high expression of TGF-β1 in all cell lines, intermediate levels of PAI-1, and variable expression of uPA and MMPs (Table 2). Collagen-1 was not expressed, as expected. To analyze TGF-β1 mediated regulation of expression of these genes, only partly responsive HT29 cells and responsive HCT116 cells were treated with 5 ng/ml active TGF-β1. Figure 4A shows that TGF-β stimulation of HT29 cells resulted in up-regulation of uPA and PAI-1, but expression of several MMPs and TIMPs was even stronger induced. TGF-β mRNA itself showed only a 2-fold up-regulation. In HCT116 cells a similar pattern was observed (Figure 4B), but the induction of MMP-2, MMP-13, PAI-1 and uPA was much stronger. However, TGF-β1 was not up-regulated in HCT116 cells after TGF-β stimulation. Increased expression of proteinases in cancer is associated with invasiveness of tumour cells via the degradation of ECM and by the processing/release of cytokines and chemokines. Figure 4C illustrates the effect of 0-10 ng/ml TGF- β1 in an invasion assay using HT29 spheroids embedded in a 3-dimensional collagen matrix. TGF-β1 treatment clearly induced migration of spheroids of the only partly responsive HT29 cells into the collagen matrix, eventually leading to distant metastasis-like cell clusters. Unfortunately the effect on high responding HCT116 cells could not be evaluated due to poor spheroid formation of these cells.

Latent TGF-β1 activation

Tumour cells secrete the majority of TGF-β as a latent complex, needing processing to become biologically active. Therefore we evaluated whether *in vivo* interactions within the tumour-microenvironment could enable activation of the latent TGF-β complex. TGF-β responsive HCT116 cells, transfected with the CAGA construct and incubated with 10 ng/ml small latent TGF-β complex (SLC), did not show increased luciferase activity, implying no activation of the latent complex (Figure 4D). Stimulation of HCT116 cells with CM from HT29, containing high levels (1-2 ng/ml) of large latent TGF-β complex (LLC) led to a minor increase in activity. HCT116 cells stimulated with cancer-associated fibroblast derived CM, resulted in a 4-fold increased luciferase activity (Figure 4D), indicating that the interaction of tumour cells with fibroblast-derived factors mediated activation of TGF-β1 and could therefore be responsible for fibroblast trans-differentiation *in vivo*.

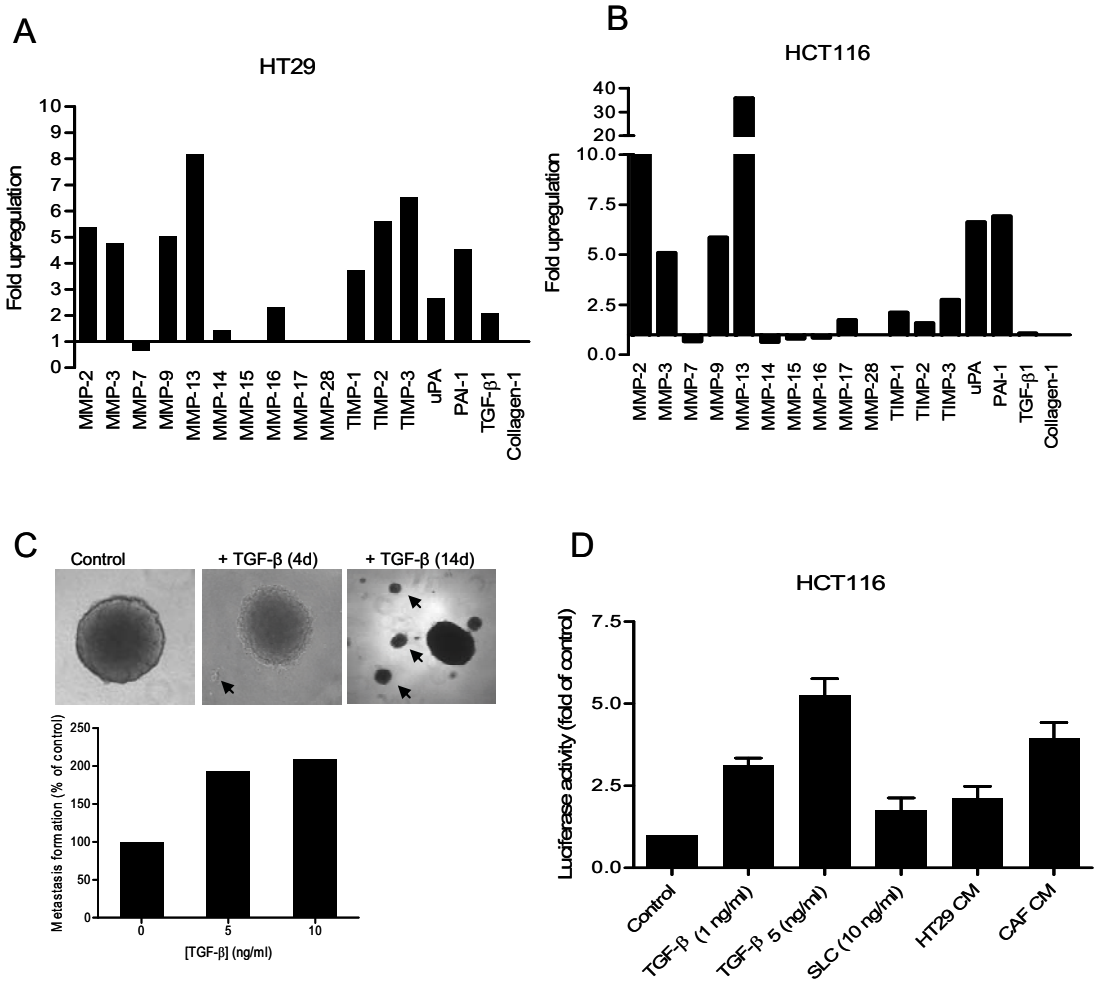

Figure 4. Regulation of proteinases and TGF-β targets genes in tumour cells by TGF-β. Real-time PCR analysis revealed that HT29 (A) and HCT116 (B) cells show upregulation of proteinases and TGF-β target genes upon stimulation with 5 ng/ml TGF-β. 3-dimensionally cultured, collagen embedded HT29 spheroids show increased TGF-β1-induced invasive properties and the formation of distant metastasis like cell clusters (C). Experiments using HCT116 cells revealed that these cells are not capable of activating recombinant SLC or HT29 derived LLC. However, strongly increased signalling is observed when combined with cancer associated fibroblast CM (D, 3-7 experiments, mean + SEM).

TGF-β induced fibroblast stimulation

Compared to treatment with recombinant TGF-β, incubation of fibroblasts with CM from HT29 revealed only a minor increase in luciferase activity (Figure 5A). Treatment with HCT116 CM containing high levels (2-3 ng/ml) of LLC, and only minor amounts of active TGF-β1 (25-80 pg/ml), however led to a 5-fold increase compared to control levels. Addition of an ALK-5 inhibitor completely abolished the response, confirming that the induction was

TGF-β mediated (Figure 5A). Analysis of the fibroblast medium after incubation with HCT116 showed enhanced active TGF-β1 levels (figure 5B). Furthermore, fibroblasts treated with HCT116 CM showed increased SMA expression until 95% comparable to addition of 5 ng/ml TGF-β1, whereas parallel incubations with SLC or HT29 CM showed no change in SMA expression (Figure 5C). These data indicated that the interaction of HCT116 medium with fibroblasts lead to increased activation of TGF-β1 complex and subsequent myofibroblast trans-differentiation.

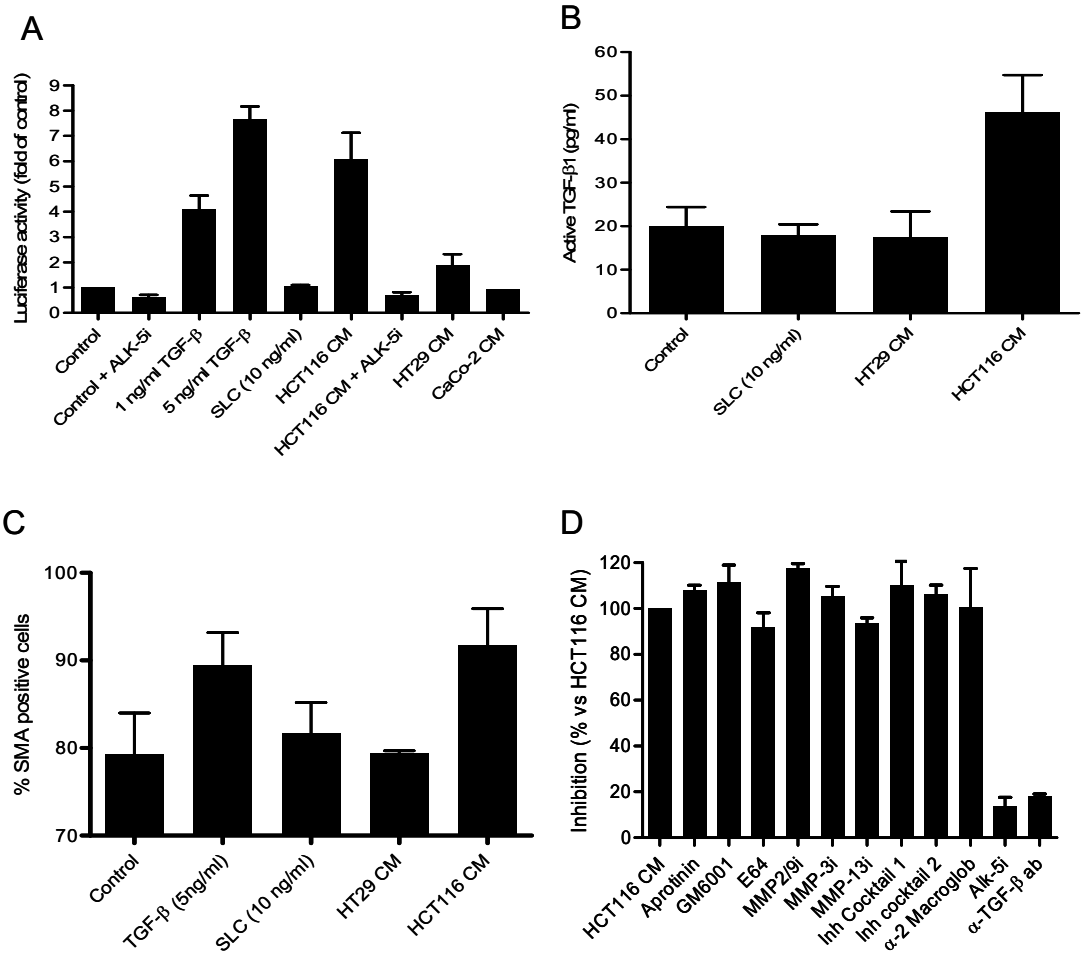

Figure 5. Myofibroblast generation in CRC. Co-culture of fibroblasts with HCT116 medium leads to strongly increased TGF-β signalling (A), increased levels active TGF-β in the medium after stimulation (B) and trans-differentiation into myofibroblasts as shown by increased SMA levels on cytospins (C). Induction of TGF-β response cannot be inhibited by addition of separate proteinases inhibitors or cocktails, but is inhibited by ALK-5 inhibitor or neutralising TGF-β antibody (D). Data represent 3-7 experiments, mean + SEM.

Because several proteolytic enzymes have been associated with the release of TGF-β *in vitro*, we analyzed the effect of various protease inhibitors on the HCT116 CM induced activation of fibroblasts. None of the used inhibitors, i.e. Aprotinin (plasmin and serine protease inhibitor), GM6001 (broad range MMP-inhibitor), E64 (broad range cystein protease inhibitor), specific inhibitors of MMP-2/9, MMP-3 and MMP-13 and α2-macroglobulin (BMP-1 and MMP inhibitor), were able to inhibit the HCT116 induced response (Figure 5D). To exclude the possible necessity of mutual activating proteolytic cascades, different cocktails of inhibitors were tested, also resulting in no inhibiting effect, whereas the controls ALK-5 inhibitor and neutralizing TGF-β antibody inhibited the signal by more than 90 %.

Discussion

Although most cancers derive from epithelial cells, tumour stroma is thought to be a key player in the initiation, and progression of carcinomas, clearly illustrated by its prognostic relevance for the survival of colorectal cancer patients[31,32]. Within the tumour-microenvironment especially cancer-associated myofibroblasts are associated with auto- and paracrine signalling[1,9,33,34]. Myofibroblast have been shown to be capable of influencing epithelial cell proliferation and enhance their invasive and metastatic potential[35]. Interactions between cancer cells and myofibroblasts can occur through direct cell-cell contact via for instance extracellular matrix metalloproteinases inducer (EMMPRIN), a potent inducer of the myofibroblast phenotype[36], but the majority of interactions is mediated by soluble factors. Important fibroblast-derived soluble factors influencing the epithelial cells are scatter factor/hepatocyte growth factor (SF/HGF), insulin like growth factor and TGF- β1[33]. TGF-β1 has also been shown to be a key inducer of myofibroblast trans-differentiation *in vitro*. Several studies suggested that the majority of the cancer-associated myofibroblasts *in vivo* are derived from TGF-β1 mediated conversion of resident fibroblasts[34,37,38]. In our *in vitro* experiments colonic fibroblasts responded indeed strongly to active TGF-β1, showing up-regulation of SMA, PAI-1, collagen-1, MMPs and in particular TGF-β1, concomitant with trans-differentiation into myofibroblasts.

Our immunohistochemistry data showed a clear shift in TGF-β signalling in colonic fibroblasts. Normal mucosa fibroblasts and SMA positive myofibroblasts showed hardly nuclear staining for pSmad2, indicating low TGF-β signalling in the normal stroma. In colonic cancers, however, the enhanced population of myofibroblasts showed strong nuclear accumulation of pSmad2, indicating TGF-β signalling in these cells, probably by increased

availability of active TGF-β1 in the tumour-microenvironment. Up-regulation of growth factors is a common phenomenon in many cancer types, but because most of these factors are secreted as inactive precursors, the subsequent activation seems to be even more crucial for the regulation of their bio-activity than over-expression[23,39]. For TGF-β1 we have recently shown that although the protein levels are already up-regulated in pre-malignant colorectal adenomas, increased TGF-β activity was only observed in carcinomas (Hawinkels *et al*, submitted), suggesting that over-activation and not over-expression is characteristic of malignant progression. In the present study we showed that cancer-associated fibroblasts were not efficient in releasing TGF-β1 from the SLC or HT29-derived LLC. However, combining fibroblasts with HCT116 CM led to activation of TGF-β1 levels resulting in enhanced TGF-β response, and subsequently leading to myofibroblast trans-differentiation. Although previous studies showed that the co-culture of dermal fibroblasts with keratinocytes increased myofibroblast levels[38] and that breast cancer cells in co-culture with fibroblasts can also induce the myofibroblast phenotype via direct cell-cell contact[40], our data indicate that TGF-β1 could also be efficiently activated by soluble factors secreted by colonic cancer cells in combination with cancer-associated fibroblasts.

Proteolytic cleavage or release by proteinases is often implicated in the processing of latent growth factors within the tumour-microenvironment[24]. Especially plasminogen activators and MMPs seem to be important in the regulation of growth factor bio-activity [41]. Several *in vitro* studies described that latent forms of TGF-β can be proteolytically activated via MMP-1[42], MMP-2[43], MMP-3[20,44], MMP-9[21], MMP-14[45], MMP-28[46], BMP-1[18] and plasmin[17, 47]. Because *in vivo* many of these proteinases are produced as inactive zymogens, proteolytic activation cascades involving several cell types are often required for the local presence of active enzymes. For instance the activation mechanism of pro-MMP-2 via respectively EMMPRIN, MMP-14 and TIMP-2 led to active MMP-2 solely at the interface between cancer cells and myofibroblasts, despite the presence of pro-MMP-2 throughout the tumours[48,49]. As a consequence, the activation of latent TGF-β activation by one specific MMP could only be efficient if the for this MMP specific activating (e.g. other proteinases), docking (e.g. MMP-14) and inhibiting proteins (e.g. TIMPs) are locally balanced. Our analysis using various proteinase inhibitors revealed that in our model, the proteinases secreted by the tumour cells in combination with the fibroblasts were not sufficient to generate TGF-β activation. The HCT116 medium contained minor amounts of active TGF-β, which might be generated via proteolytic processing of latent TGF-β, presumably at the tumour cell surface. Other factors secreted by tumour cells might also influence the effects of TGF-β on myofibroblast

differentiation, like has been shown for interleukin-1, which can inhibit TGF-β induced myofibroblast differentiation[38]. However, these soluble factors do not seem to be major contributors to myofibroblast trans-differentiation in our experiments, because 90% of the HCT116 CM induced response could be inhibited by a neutralising TGF-β antibody or an ALK-5 inhibitor. Besides proteolytic release several non-proteolytic mechanisms of TGF-β activation have been described, mainly involving release from the SLC, rather than the LLC, e.g. thrombospondin1[50,51] and αvβ6 integrin[22]). In addition, Wipff *et al.* showed that myofibroblasts can activate LLC TGF-β by mechanical contraction involving myofibroblast αvβ5 integrin releasing the active TGF-β molecule from the LLC[52]. In our model it might be that the minor amounts of active TGF-β1 in the HCT116 CM mediate the first trans-differentiation into myofibroblasts, leading to the subsequent non-proteolytic activation of more TGF-β1 via binding of tumour cell- or fibroblast-derived LLC to myofibroblast αvβ5 integrin. The small amount of active TGF-β in the HCT116 CM, which would not be sufficient to generate the high induction of response in the CAFs, might trigger this process and explain the difference observed between HT29 CM and HCT116, which both contain large amounts LLC, but differ in the amount of active of TGF-β1.

We showed that TGF-β activation did not only lead to production of increased levels of TGF-β and proteinases in fibroblasts, but also to increased production of MMPs in tumour cells, reflecting a double paracrine mechanism, efficiently enhancing itself, once started. Many studies have indicated that TGF-β plays a tumour-suppressive role in normal tissue and pre-malignant cancer stages, as it inhibits epithelial cell proliferation. Only in later stages epithelial cells become refractory to growth inhibitory properties[13,15,53,54], apparently leading to the presence of higher tumour-cell derived active TGF-β levels, and culminating in the overproduction of TGF-β by myofibroblasts. Immunohistochemistry on colorectal cancer specimen indeed revealed decreased nuclear accumulation of pSmad2 in tumour epithelial cells in the majority of the tumours, reflecting decreased TGF-β responsiveness. This was confirmed *in vitro*, where we show that some colon cancer cell lines are not TGF-β1 responsive probably by mutations in the TGF-β receptors, Smad4 or other mutations in the signalling pathway as have been reported in colorectal cancer[55,56]. Although HT29 cells do not express Smad4 protein these cells are still partly TGF-β responsive, as has been noticed before[57], indicating the contribution of other, non-Smad dependent signalling pathways. Apparently these pathways also lead to invasive processes as we have shown for HT29 spheroids embedded in a collagen matrix. Moreover, over-production of TGF-β by these cancer cells could still contribute to the tumour-promoting effect, via interaction with

surrounding fibroblasts, leading to activation of TGF-β and subsequent differentiation of fibroblasts. In turn myofibroblasts are able to express other tumour cell growth-promoting chemokines and cytokines like SF/HGF, Stromal Derived Factor or VEGF which can promote tumour angiogenesis.

In conclusion we have shown that HT29 and HCT116 cells and cancer-associated fibroblasts respond to TGF-β1 by upregulation of MMP, TIMP and TGF-β expression. Both tumour cells and fibroblast themselves are not capable of activation of latent TGF-β whereas co-culture of conditioned media of these cells leads to increased TGF-β activation via a non-proteolytic mechanism and myofibroblast differentiation. We propose that tumour cells produce latent TGF-β, which is activated via interaction with surrounding normal fibroblasts via a non-proteolytic mechanism. In turn, fibroblasts are trans-differentiated into myofibroblasts, which increase production of TGF-β, and invasion related proteases, resulting in a cancer progressing feedback loop (Figure 6). These data further emphasize the role of tumour-stroma interactions and myofibroblasts and validate further to explore the tumour stroma as potential therapeutic target[5,58].

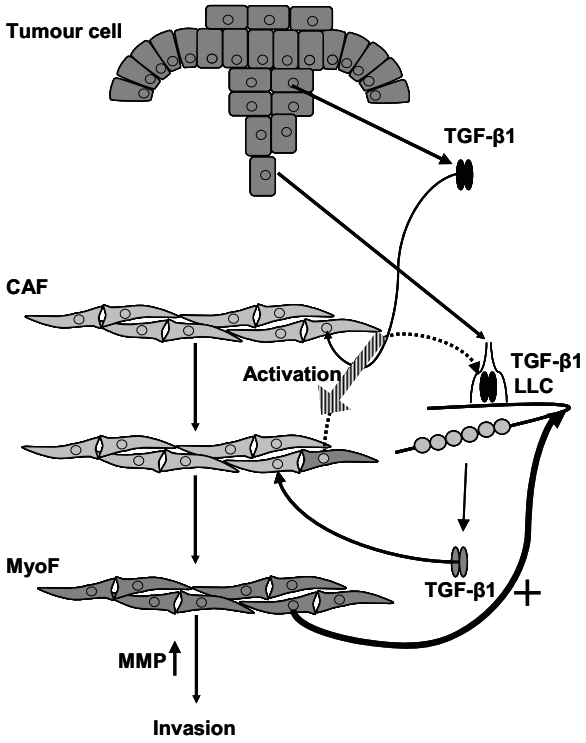

Figure 6. Proposed mode lfor tumour myofibroblast differentiation. Initially tumour cells secrete minor amounts of TGF-β, which initiates the trans-differentiation of myofibroblasts. The myofibroblasts are in turn capable of binding tumour cell derived-LLC and releasing active TGF-β via SMA mediated contractile properties in a non-proteolytical manner. This results in more trans-differentiation, increased synthesis of latent TGF-β and proteinases by both the myofibroblasts and tumour cells, facilitating invasion. Together these interactions create a cancer enhancing feedback loop in the tumour microenvironment.

Acknowledgements

We thank Dr R. Hanemaaijer (TNO Quality of Life BioSciences, Leiden, the Netherlands) for helpful suggestions and critical reviewing the manuscript. Eveline de Jonge-Muller (Dept. of Gastroenterology-Hepatology, LUMC) and Adri Mulder-Stapel (TNO Quality of Life BioSciences) are acknowledged for excellent technical support. This work was supported by the EC Tumor-Host Genomic project and the Centre for Biomedical Genetics (E.W., P.t.D.).

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