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Transforming Growth Factor- β modulates Pancreatic Cancer Associated Fibroblasts cell shape, stiffness and invasion

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Abstract

Background—Tumor microenvironment consists of the extracellular matrix (ECM), stromal cells, such as fibroblasts-FBs and cancer associated fibroblasts-CAFs, and a myriad of soluble factors. In many tumor types, including pancreatic tumors, the interplay between stromal cells and the other tumor microenvironment components leads to desmoplasia, a cancer-specific type of fibrosis that hinders treatment. Transforming growth factor beta (TGF- β) and CAFs are thought to play a crucial role in this tumor desmoplastic reaction, although the involved mechanisms are unknown.

Methods—Optical/fluorescence microscopy, Atomic Force Microscopy, image processing techniques, invasion assay in 3D collagen I gels and real-time PCR were employed to investigate the effect of TGF- β on normal pancreatic FBs and CAFs with regard to crucial cellular morphodynamic characteristics and relevant gene expression involved in tumor progression and metastasis.

Results—CAFs present specific myofibroblast-like characteristics, such as α -smooth muscle actin expression and cell elongation, they also form more lamellipodia and are softer than FBs. TGF- β treatment increases cell stiffness (Young's modulus) of both FBs and CAFs and increases CAF's (but not FB's) elongation, cell spreading, lamellipodia formation and spheroid invasion. Gene expression analysis shows that these morphodynamic characteristics are mediated by Rac, RhoA and ROCK expression in CAFs treated with TGF- β .

Conclusions—TGF- β modulates CAFs', but not FBs', cell shape, stiffness and invasion.

General Significance—Our findings show for the first time the effect of TGF- β on CAFs' behavior and stiffness providing new insights into the mechanisms involved.

Keywords

Atomic Force Microscopy; desmoplasia; cell mechanics; collagen gels; cell invasion; stress fibers; lamellipodia

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Introduction

The tumor microenvironment consists of the extracellular matrix (ECM), stromal cells and countless soluble factors in the extracellular milieu, whose importance in cancer progression and metastasis is indisputable (1, 2). Stromal cells, such as fibroblasts (FBs) are in close interaction with cancer cells (3) and are responsible for a desmoplastic reaction that results in extensive production and remodeling of tumor ECM. Pancreatic tumors are highly desmoplastic, being characterized by the intra-tumoral accumulation of excess amount of ECM, such as collagen type I and hyaluronan (4) that hinders effective treatment and it is responsible in large part for the low survival rates observed in these cancers.

FBs are mesenchymal-derived cells with a characteristic spindle-like morphology, and are usually present in the interstitial space of normal tissues, emedded within fibrillar ECM (5, 6). They were originally identified as cells of the connective tissue that synthesize collagen and are now thought to be crucially involved in many vital cellular processes such as ECM deposition, secretion of ECM-degrading proteases (i.e. matrix metalloproteinases), regulation of epithelial cell differentiation, regulation of inflammation, and wound healing (5–7).

Interestingly, FBs can acquire an “*activated*” phenotype which can be induced by various stimuli, such as Transforming Growth Factor-beta (TGF- β) and altered ECM composition (6, 7). Activated fibroblasts, also known as “*myofibroblasts*”, express alpha-smooth-muscle actin (α -SMA), a cytoskeletal protein associated with smooth muscle cells, which is the most common marker of activated FBs (8), and are characterized by increased capacity to secrete ECM components, remodel the ECM and form contractile bundles composed of actin and myosin (5, 8–10). Although myofibroblasts were originally identified in wound healing, they are abundant in cancer (1). Notably, in the case of cancer, myofibroblasts are also known as cancer associated fibroblasts (CAFs) (7), while terms such as carcinoma-associated fibroblasts, tumor-associated fibroblasts, peritumoral (myo-)fibroblasts, reactive stroma fibroblasts, may also be found in the literature (5, 11). It has been suggested that CAFs are recruited to the tumor microenvironment by cancer cells through various growth factors and cytokines in order to form a myofibroblastic microenviroment that supports malignancy, cell invasion and metastasis (1, 12). CAFs are also thought to exert mechanical forces on surrounding ECM (5, 12). It has also been demonstrated that CAFs enhance cancer cell proliferation and angiogenesis (1) and increase the invasiveness of originally non-invasive cancer cells (11). Additionally, CAFs have the tendency to aggregate peritumorally and encircle carcinoma cells, being the first to invade through adjacent normal tissues (1).

Although, FBs, CAFs and TGF- β are generally thought as key players in desmoplasia development in cancer, the exact mechanism of their action as well as their interplay is still not well-defined. Also the effect of TGF- β on the morphodynamic characteristics of CAFs as well as their invasive phenotype are not yet fully clarified. Furthermore, as desmoplasia has been proposed to inhibit drug delivery and enhance tumor progression and metastasis (13), defining the mechanistic interactions between CAFs and TGF- β can provide a new basis for the development of an improved treatment (14–17). In the present work, optical and fluorescence microscopy, Atomic Force Microscopy (AFM), image processing techniques,

three dimensional (3D) *in vitro* invasion assays and real-time polymerase chain reaction (PCR) were employed to investigate the effect of TGF- β on normal pancreatic FBs and CAFs with regard to several cellular morphodynamic characteristics and relevant gene expression.

Materials and Methods

Cell culture

Native human FBs derived from pancreas (Cat.# SC00A5) and pancreatic CAF (Cat.# CAF08) cell lines were purchased from Neuromics (Edina, MN) and cultured in MSC-GRO (VtroPlus III, low serum, complete) medium. The experiments were performed in cells without any treatment (parental cells), cells treated with 5 ng/ml TGF- β (18, 19) for 2 days (TGF- β treated cells) (18, 20) and cells treated with the same amount of solvent (4mM HCL 1mg/ml with 1mg/ml Bovine Serum Albumin) as the one in which TGF- β was dissolved (control cells).

Assessment of lamellipodia formation

For quantifying the number of cells forming lamellipodia, cells were stained with phalloidin (see Supplement), and observed under an Olympus BX53 fluorescent microscope. Multiple pictures were taken and the number of cells forming lamellipodia compared to the total number of phalloidin-stained cells was counted manually. At least 250-300 cells were used for quantification per condition and the mean ratio of lamellipodia-forming cells compared to total in each condition was assessed from 3 independent experiments.

α -SMA quantification

To quantify α -SMA levels in cells, a previously described method was used (21, 22). Briefly, after obtaining images from α -SMA stained cells (see Supplement) using the fluorescent microscope, an outline was drawn by hand around each cell using ImageJ (NIH, Bethesda, MD) software and circularity, area, and mean fluorescence were measured. Also three background areas per cell were measured in order to calculate the mean fluorescence of the background. The total corrected cellular fluorescence = integrated density – (area of selected cell $\times$ mean fluorescence of background readings), was calculated.

Cell Elongation

Cell elongation was assessed using optical microscopy images from live cells. Pictures of individual cells (not forming clusters) were taken using a Nikon Eclipse TS100 inverted microscope equipped with a digital camera (Olympus XC50 Color CCD camera, 5 megapixel) and a Nikon Ph1 DL 10x 0.25 phase microscope objective lens. ImageJ software was used to automatically measure factor E from cells (23). Factor E equals to the long axis divided by the short axis minus one. The *elongation factor* E describes the extent in which the equimomental ellipse is lengthened or stretched out (24). Thus, E is zero for a circle, and one for an ellipse with an axis ratio 1:2. The cells that presented E values 0–0.5 were considered as spherical, 0.5–1 as ellipsoid, and E values higher than 1 as elongated (25).

Stress fibers

For the characterization of the actin stress fibers, the *FilamentSensor* tool was used (26). After taking images with an Olympus BX53 fluorescent microscope from cells stained with phalloidin, the actin filament structure of TGF- β -treated and untreated FBs and CAFs was reconstructed using the filament sensor tool. In the reconstructed images each color corresponds to a different fiber orientation.

Spreading

Cells were plated on 24-well culture plates and incubated at 37 °C for 30 and 40 min for FBs and CAFs, respectively. Cells were then fixed in 4% paraformaldehyde (PFA, Sigma P6148) and the cell morphology was observed under optical microscope. Unspread cells were defined as round cells, while spread cells were defined as cells with extended processes (27, 28). The percentage of spread cells was quantified by analyzing at least 300 cells from 5 randomly selected fields. Three independent experiments were performed and results represent mean values from all three of them.

Atomic Force Microscopy (AFM)

For AFM characterization of cells and collagen I gel (see Supplement for gel formation methodology), a Molecular Imaging-Agilent PicoPlus AFM system was used. Imaging of fixed cells was performed in contact mode in air and under phosphate buffered saline (PBS) with silicon and V-shaped silicon nitride probes (SICON-Applied Nanostructures and PNP-TR-Nanoword). Force spectroscopy on live cells was performed with V-shaped soft silicon nitride probes (MLCT, Bruker) and the collected force curves were analyzed by AtomicJ (29) so as to calculate the sample's Young's modulus using the Hertz model. Collagen I gels were characterized with V-shaped PNP-TR probes in contact mode. The AFM image processing from all the samples was performed by using the PicoView software (Agilent) and the freeware scanning probe microscopy software WSxM 5.0 dev.2.1 (30). A detail representation of the AFM methods is presented in the Supplement.

Invasion assay

The “hanging drop” technique was used for the formation of FB and CAF cell spheroids (see Supplement), as described previously (31–34). Immediately after transferring the spheroids into wells of a 96-well plate containing collagen I gel, images were taken (time zero) with an optical microscope. Spheroids were then incubated at 37°C for 6h and new images were taken. Cell invasion through surrounding collagen was assessed using ImageJ software and spheroids' size (average of the major and minor axis length) after 6h was compared to the initial size at time zero (33–35).

Real Time PCR

The quantification of gene expression was performed by real-time PCR using CFX96 Real Time PCR (BioRad). Quantification of relative gene expression was performed using the Ct method (see Supplement).

Statistical analysis

For the statistical analysis comparison of means with standard errors was used. Each experimental group was repeated at least three times. Statistical analysis was performed using Student's t-test. $P < 0.05$ was regarded as statistically significant.

Results

Cell Characterization

In order, to investigate the effect of TGF- β on normal human pancreatic FBs and CAFs with regard to several cellular morphodynamic characteristics and relevant gene expression, we first set out to characterize the two cell lines. In that context, optical microscopy imaging was initially used to study the morphological characteristics of the cells. As seen in Figure 1A, CAFs are significantly more elongated than FBs, while quantitative analysis of the acquired images using elongation factor E (*i.e.*, the long axis divided by the short axis minus one) demonstrated that this difference is statistically significant (Figure 1B). Additionally, by considering the cells that presented E values of 0.0–0.5 as spherical, 0.5–1.0 as ellipsoid, and > 1.0 as elongated (23, 36), a higher percentage of elongated cells was seen in CAFs compared to FBs (Figure 1C).

In order to better characterize the cells with regard to morphology, the high-resolution AFM imaging was recruited (Figure 1D) as a supportive technique to optical microscopy. AFM analysis verified that both FBs and CAFs have an elongated spindle-like shape characteristic of fibroblasts. Furthermore, the presence of a large oval nucleus with one or two nucleoli in the case of CAFs and a flatter nucleus in FBs (7), was confirmed by AFM.

To confirm that CAFs are indeed “activated” FBs (myofibroblasts), we assessed the expression of α -SMA, the most common marker for activated fibroblasts (1, 6, 7, 11). As shown in Figure 1E, CAFs express higher levels of α -SMA compared to FBs. Moreover, analysis of the cells by immunofluorescent staining confirmed that CAFs express higher levels of α -SMA (Figure 1F), which is in accordance with mRNA expression of α -SMA analyzed by real time PCR (Figure 1G).

Subsequently, we stained cells with phalloidin, a natural fungal toxin known to bind and stabilize filamentous actin (F-actin). As shown in Figure 2A, both FBs and CAFs exhibited intense stress fibers. Strikingly however, CAFs had increased formation of lamellipodia, the thin, sheet-like membrane protrusions that are normally found at the leading edge of moving cells. We, thus, quantified the cells that formed lamellipodia in each cell line compared to the total number of cells, and found that a larger number of CAFs form lamellipodia than FBs (Figure 2B), suggesting a more migratory phenotype. Furthermore, since stress fiber orientation has been associated with increased migration, we analyzed the images from the phalloidin-stained cells using the *FilamentSensor* tool software to visualize stress fiber orientation. This software enabled us to view the direction of each fiber, which is represented by a different color. As shown in Figure 2C stress fibers in FBs were randomly organized (as seen by the many different colors), while stress fibers in CAFs were nicely oriented towards one direction (only one color seen). The rich actin filaments present in CAFs is also shown in Figure 2D, where AFM imaging was employed to obtain images of

stress fibers. Interestingly, since nanomechanical assessment has been gaining ground in cancer diagnostics, showing that more metastatic cells are usually “softer” or less stiff cells (37–41), we extended our AFM studies to include force spectroscopy in live cells. In that regard, we measured cell stiffness by means of Young’s modulus measurements. As shown in Figure 2E, the Young’s modulus of FBs and CAFs was compared in percentage differences (a.u.) (42) and it was found that CAFs are “softer” (i.e., lower Young’s modulus) compared to FBs, with a modulus ratio (FBs/CAFs) (40) of ~ 1.3 . This further confirms that CAFs exhibit a more aggressive phenotype. In order to investigate the relationship between actin filaments and cell’s elasticity measurements, we used Cytochalasin D a known inhibitor of actin polymerization. Fluorescence and AFM imaging of live cells demonstrated that Cytochalasin D indeed depolymerized actin both in FBs and CAFs (Supplementary Figure 1A and B). Also, a significant reduction of Young’s modulus was measured in both cell lines following cytochalasin D treatment (Supplementary Figure 1C) in agreement with pertinent studies using other cell types, confirming that the elastic properties of living cells are closely related to actin filaments (40, 43–45).

Taking all the above into account, we concluded that the selected CAFs present specific characteristics of the myofibroblast phenotype (α -SMA expression), as well as properties that enhance migration (elongated cell shape, presence of lamellipodia, oriented stress fibers and low stiffness).

Effect of TGF- β on cell morphology and stiffness

As TGF- β is an important determinant in desmoplasia, we wanted to investigate the effect of TGF- β treatment on the main cellular characteristics of pancreatic FBs and CAFs such as α -SMA expression, cell elongation, stiffness and lamellipodia formation.

Firstly, from the α -SMA immunofluorescence staining and real-time PCR analysis (Figure 3 and Supplementary Figure 2), it was revealed that FBs α -SMA mRNA expression is increased following TGF- β treatment, while CAFs had reduced α -SMA expression. This result was also verified by western blot (Supplementary Figure 3 A and B). The increased α -SMA expression of FBs is a marker of the activation of the FBs into the myofibroblast phenotype.

In order to quantitatively evaluate the changes in cell shape induced by TGF- β , we measured the elongation factor E . Our results demonstrated that TGF- β didn’t affect cell elongation (Figure 4C) in any of the cell lines tested. This might be due to the fact that the original cell shape of FBs and CAFs is already elongated and spindle-like. However, when we investigated whether TGF- β -induced changes in the percentage of cells that were spherical, ellipsoid or elongated, we found that the percentage of elongated cells was significantly higher in CAFs compared to FBs (Figure 4D). Consequently, these results indicate that although TGF- β treatment did not affect the elongation factor E for FBs and CAFs, it did increase the population of CAFs having elongated shape ($E > 1$).

Interestingly, it has been demonstrated that cell deformability is altered during cancer progression, putting biomechanics of living cells in the focus of cancer research. Hence, we employed AFM indentation experiments, where cells’ elasticity in terms of Young’s

modulus of live cells was measured. AFM measurements on live cells showed that TGF- β affected cell stiffness in both FBs and CAFs (Figure 4E) with cells becoming stiffer (in terms of Young's modulus values).

We then tested lamellipodia formation following TGF- β treatment in both cell lines. As shown in Figure 4F and G, TGF- β treatment promoted lamellipodia formation only in CAFs, while no statistically significant difference was observed in FBs, suggesting that CAFs are more responsive to TGF- β with many morphological characteristics being affected by treatment.

These results suggest that in the case of FBs, TGF- β treatment was sufficient to initiate their "activation" as shown by α -SMA expression (staining and real-time PCR), although other differences in terms of cell shape elongation or lamellipodia formation was not observed. With regard to CAFs, which are already activated FBs, TGF- β treatment increased the population of elongated cells as well as the population of cells forming lamellipodia, both of which are significant characteristics contributing to CAFs' aggressive phenotype. Moreover, the increased stiffness which was recorded in both cell lines implies a possible alteration in the stress fibers, which could be mediated by the Rho GTPases pathway (i.e. RhoA, Rac) that is critically involved in the organization of the cytoskeleton and relevant cytoskeleton-dependent processes such as stress fiber and lamellipodia formation, cell spreading, and cell migration and invasion (46).

TGF- β promotes cell spreading in CAFs but not FBs through upregulation of Rac

We next investigated the effect of TGF- β on cell shape modulation by assessing cell spreading. In Figure 6A and B, fluorescent images of spread cells stained with phalloidin are presented showing that actin stress fibers are randomly oriented in both cell lines tested during cell spreading. Figure 6C and D show high resolution AFM deflection images for FBs and CAFs during the spreading phase, providing a more realistic representation of the cell morphology.

Interestingly, although both CAFs and FBs spread well, optical microscope images of untreated and TGF- β -treated FBs and CAFs (Figure 6 A, B and C) demonstrated that TGF- β had a negligible effect on FBs, whereas it increased the spreading of CAFs. These results are in accordance with gene expression analysis experiments which demonstrate that TGF- β treatment on CAFs, upregulates Rac, which is required for actin polymerization and is a known mediator of cell spreading (Figure 6D) (46).

Effect of TGF- β on cell invasion

Since TGF- β promoted lamellipodia formation (Figure 4G) and cell spreading (Figure 6 A-D) in CAFs, we then sought to find out how the metastatic potential of FBs and CAFs is affected by TGF- β . The effect of TGF- β on cell invasion was investigated by generating cell spheroids and embedding them in 3D collagen gels.

In that regard, we first set out to establish a 3D culture system that would allow cells to grow in 3D in an attempt to better approximate real tumor setting and the cell-ECM interactions in place. Thus, we generated collagen I gels containing 1.0 mg/ml collagen I. Collagen I was

selected for the generation of the 3D matrix environment as the most abundant protein found in tissues' ECM (47), making the system more physiologically relevant. We selected 1 mg/ml collagen concentration as it has been demonstrated that cells have an optimum cell invasion in this intermediate stiffness condition (i.e. 1.0 mg/ml collagen) (33). In order to characterize the generated gels in terms of structure, we employed AFM, which can be used for imaging and characterizing of collagen samples without destroying the fibrillar structure of collagen (36, 42, 48, 49). As shown in Figure 7A collagen gels consisted of fibers with 3D random orientations, confirming that the formed gels mimic collagen-rich tissues. Furthermore, AFM imaging from histological sections from collagen I gels confirmed that collagen gels were consisted of fibers with natural characteristics in their entire volume (Figure 7B). Also collagen fibers presented the characteristic D band of ~67 nm (Figure 7B), which is a fundamental element of the natural forming collagen fibers and it has been proposed to be recognized by cells (50).

Cell spheroids were then generated in the presence or absence of TGF- β using the “hanging drop” method (31, 33–35, 51) and were embedded in the 3D collagen gels. Six hours (6h) later, images of spheroids invading through surrounding collagen were obtained (Figure 7C) showing that TGF- β had negligible effects on the invasive capacity of FBs (Figure 7C) but significantly increased CAFs' invasion (Figure 7C&D), which is consistent with the changes observed in lamellipodia formation and cell spreading.

To examine the molecular mechanism by which TGF- β promotes CAF spheroid invasion in 3D collagen gels, we performed gene expression analysis in cells cultured in 3D, being embedded in the gels (52), and allowing them to grow for 2 days with and without TGF- β treatment. As shown in Figure 8, FBs (Figure 8A) and CAFs (Figure 8B) were indeed embedded in collagen gels growing at different levels in all three dimensions within the collagen matrix.

Thus, we set out to investigate how proteins implicated in migration and invasion are affected by TGF- β treatment. To that regard, we tested the mRNA expression of members of the Rho GTPase molecular pathway (namely, RhoA, Rho associated protein kinase-ROCK, and cell division control protein 42-Cdc42) were selected as this pathway has been previously proposed to mediate ECM remodeling by CAFs (53) and their regulation is likely different in activated FBs compared to normal FBs (5). Furthermore, our previously-described results (alterations in stress fibers, cell shape and lamellipodia) make these genes interesting candidates for investigation. Our findings indicate that although a small increase in the ROCK mRNA expression was recorded, none of the selected genes was statistically affected by TGF- β treatment in FBs cultured in 3D collagen gels (Figure 8C). However, significant increase was observed in RhoA and ROCK mRNA expression in CAFs after TGF- β treatment (Figure 8D), suggesting that the effect seen in lamellipodia, cell spreading and cell invasion in CAFs following TGF- β treatment is likely mediated by RhoA and ROCK, which are both associated with regulation of stress fibers formation and motility-related processes (46, 54, 55).

Taken together, all TGF β -induced morphological changes, such as cell elongation, lamellipodia formation, stiffness and stress fiber orientation, are essential for cell migration

and invasion indicating the significantly positive effects of TGF- β on CAFs at the designated *in vitro* experimental conditions.

Discussion

Desmoplasia is known to promote tumor growth and metastasis and inhibit drug delivery to the tumor (56), while CAFs and TGF- β are thought to be key players in this process (14–17). Thus, identifying proper molecular targets to prevent tumor desmoplasia can improve the systemic delivery of drugs and hinder metastasis (57–59). However, targeting CAFs and/or TGF- β in the clinic remains a difficult task since the exact tumor-promoting mechanisms of the desmoplastic microenvironment are only partially understood. In the present study, we investigated the effect of TGF- β on pancreatic FBs and CAFs with regard to morphological characteristics that contribute to a more desmoplastic and aggressive phenotype.

In terms of *FBs and CAFs characterization and comparison* our results demonstrated that pancreatic CAFs present higher levels of α -SMA expression and are more elongated than FBs. They also exhibit oriented stress fibers and increased number of lamellipodia, while at the same time being less stiff. Furthermore, as it has been demonstrated that cancer cells are significantly “softer” than normal cells (37, 39, 40, 60) and AFM is increasingly emerging as an important tool in cell mechanics and imaging, a significant part of this work was performed using AFM (61). To the best of our knowledge, this is the first time that AFM was applied for characterizing CAFs in comparison to normal FBs. Although the reported range of modulus ratio (normal/cancer) for different cells studied by AFM is very broad from 0.5 to 32 (40), in our results the modulus ratio (FBs/CAFs) was found to be ~ 1.3 , a value that is similar the modulus ratio (normal/cancer) for breast cells (62, 63), prostate cells (38) and chondrocytes (64). Even though both FBs and CAFs present a dense network of stress fibers, we found a significant difference in the Young’s modulus between the two cell types. This observation must be owing to the fact that the Young’s modulus of cells depends not only on the density of stress fibers but also on their orientation (40, 43–45, 65, 66). Since, the morphological characteristics that we studied (elongation, stress fibers, lamellipodia and stiffness) play a crucial role in cell migration and invasion (67), our findings suggest that CAFs bear morphological characteristics that justify their migratory and invasive phenotype.

In terms of the *effect of TGF- β* on FBs and CAFs, our findings indicate that the levels of α -SMA was increased in FBs following TGF- β treatment verifying the fact that TGF- β activates FBs into a myofibroblast-like phenotype (Fig. 3B). Our results also demonstrated that the TGF- β treated FBs present similar α -SMA expression levels to those of control CAFs (Supplementary Figure 1). However, TGF- β treatment down-regulated α -SMA in CAFs, perhaps due to the fact that CAFs are already in an activated state and they thus cannot be activated further in terms of α -SMA expression. Concerning *morphology and stiffness*, our results demonstrated that although both FBs and CAFs become stiffer in the presence of TGF- β , TGF- β initiates a differential response with regard to cell morphology in the two cell types. More specifically, we found that TGF- β treatment promotes cell elongation and lamellipodia formation only in CAFs. Our results are in agreement with

previous studies investigating the effect of TGF- β on different cell types using AFM (18, 20, 68). More specifically, Thoenig *et al.*, studied Epithelial to Mesenchymal Transition (EMT) in proximal kidney tubule epithelium cells after TGF- β stimulation (68). They demonstrated an increase in stress fiber formation and cell elongation. In another study, Buckley *et al.* measured a significant increase in stiffness of alveolar epithelial cells following TGF- β treatment, while cells presented a rougher surface profile with notable protrusions (18). Also, Schneider *et al.* found that the apical tension increases and the excess surface area of the apical membrane decreases visibly with increasing treatment duration, during TGF- β induced EMT in an epithelium cell line (20). As a consequence, stiffer cells are generated, compared to the ordinary polarized epithelial cells. Additionally, Wu *et al.* (2014) used a microplate measurement system and demonstrated that NMuMG cells became stiffer after TGF- β due to thicker and more abundant F-actin (69). Concerning, cell stiffness, although in the case of cancer cells the majority of tumor cells are softer than normal ones (39, 70), this is not always true (40). In fact, things are more complicated as there are studies correlating cell stiffness with metastatic potential (70, 71), while others do not indicate such a correlation (64, 72, 73). Consequently, the TGF- β -induced increase in stiffness is not necessarily associated with inhibition of metastatic potential. According to Wu *et al.* cells with increased stiffness upon TGF- β treatment exhibited accelerated EMT and significantly enhanced migration and invasion (69). Interestingly, it has been postulated that cells reorganize their cytoskeleton prior to invasion thus gaining properties that enable them to invade through surrounding tissues where they encounter higher levels of stress (18, 69). It is therefore hypothesized that higher cell stiffness prepares the cells for invasion through stiffer environments and enables them to withstand the increased deformation occurring during transition (69).

Concerning *the functional effect of TGF- β on cell shape modulation* our results showed that TGF- β promotes cell spreading in CAFs through upregulation of Rac, while it has no effect on FBs, indicating that CAFs as activated fibroblasts respond better to TGF- β , modulating their cell shape and spreading capacity. Furthermore, *regarding the effect of TGF- β on the metastatic characteristics of cells such as cell invasion*, our results demonstrated that TGF- β treatment promotes CAFs but not FBs spheroid invasion through collagen gels. Notably, our spheroid invasion data are supported by gene expression analysis showing a significant increase in RhoA and ROCK mRNA expression following TGF- β -treatment in CAFs but not in FBs.

Finally, our findings highlight the fact that although TGF- β treatment activates FBs into myofibroblasts (α -SMA expression) (5), this alone is not sufficient to make them acquire all the characteristics associated with CAFs. This suggests that other factors and molecular constituents, such as matrix stiffness (5, 14) and Thy-1 may also be required for the transition from FBs to CAFs (10, 74).

Conclusions

Our results show that CAFs present specific myofibroblast-like characteristics such as α -SMA expression, while also being more elongated with more lamellipodia and softer than normal FBs. Moreover,, we show for the first time that TGF- β induced morphological

changes in CAFs, which include cell elongation and lamellipodia formation, both of which are critical for cell migration and invasion, which in fact is significantly increased in CAFs following TGF- β treatment, as shown by the spheroid invasion assay results. Furthermore, it was demonstrated that TGF- β promotes CAFs' but not FBs' spreading through Rac. Finally, these changes in spreading and spheroid invasion were verified by gene expression analysis showing an upregulation of Rac, RhoA and ROCK following TGF- β treatment. The results of this study demonstrate the effect of TGF- β on CAF's morphology, behavior and stiffness providing new insights into the mechanisms involved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

α-SMA	Alpha-Smooth-Muscle Actin
AFM	Atomic Force Microscopy
DAPI	4',6-Diamidino-2-Phenylindole
DMSO	dimethylsulfoxide
EMT	Epithelial to Mesenchymal Transition
ECM	Extracellular Matrix
FBs	Fibroblasts
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
ROCK	Rho associated protein kinase
TGF-β	Transforming Growth Factor beta

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Highlights

- CAFs are more elongated and express higher levels of α -SMA than FBs
- CAFs have oriented stress fibers and are softer than FBs with more lamellipodia
- TGF- β promotes cell spreading in CAFs through Rac
- TGF- β makes CAFs more elongated with more lamellipodia and promotes their invasion
- RhoA and ROCK expression is altered in CAFs treated with TGF- β

Figure 1. Characterization of Cell Morphology.

(A) Optical Microscope imaging of FBs and CAFs, (B-C) cell elongation and percentage of elongated cells, (D) topography AFM imaging of FBs and CAFs (fixed cells in air in contact mode with PicoPlus AFM). (E) α-SMA stained cells by immunofluorescence, (F) analysis of the corrected total α-SMA cell fluorescence from fluorescence images and (G) relative mRNA α-SMA expression. Asterisks indicate a statistically significant difference between compared groups ($p < 0.05$).

Figure 2. CAFs have oriented stress fibers and lower Young's modulus than FBs

(A) phalloidin-stained cells (B) computationally processed images from panel A, presenting stress fiber orientation (each color corresponds to a different fiber orientation), (C) percentage of cells forming lamellipodia, (D) amplitude AFM image of CAFs (fixed cells in air in contact mode with PicoPlus AFM) (E) relative cell Young's modulus of live cells as it was measured by AFM. Asterisks indicate a statistically significant difference between compared groups ($p < 0.05$).

Figure 3. Effect of TGF- β on α -SMA expression.

(A, B) α -SMA immuno-fluorescence staining in control and TGF- β treated FBs and CAFs, respectively (C) relative α -SMA mRNA expression. Asterisks indicate a statistically significant difference between compared groups ($p < 0.05$).

Figure 4. Effect of TGF-β on cell morphology.

(A and B) cell elongation and percentage of elongated cells, (C) relative cell stiffness of live cells as it was measured by AFM, (D) phalloidin-stained cells and (E) ratio of cells forming lamellipodia compared to total number of cells counted. Asterisks indicate a statistically significant difference between compared groups ($p < 0.05$).

Figure 5. Fluorescence and AFM imaging of spread cells.

(**A and B**) phalloidin-stained cells during spreading and computationally processed images, presenting the stress fiber orientation (each color corresponds to a different fiber orientation), for FBs and CAFs, respectively, (**C and D**) AFM contact mode images under PBS and force maps of spread FBs and CAFs.

Figure 6. TGF- β treatment increases cell spreading of CAFs but not that of FBs. (A and B) representative optical microscope images of control and TGF- β spread FBs and CAFs, (C) quantitative analysis cell spreading from optical microscope images and (D) relative Rac mRNA expression. Asterisks indicate a statistically significant difference between compared groups ($p < 0.05$).

Figure 7. Effect of TGF- β on tumor spheroid invasion.

(A) AFM topography image of collagen type I gel (1.0 mg/ml) that were used as ECM models and (B) AFM deflection image presenting collagen fibers with the D-band (~67 nm) from histological section from collagen type I gel (contact mode in air with PicoPlus AFM), (C) FBs and CAFs cell spheroids embedded in collagen gels of 1.0 mg/ml at time zero and at 6h post embedding, respectively (D) Percentage (%) change in FBs and CAFs cell spheroids' size within 6h post embedding, respectively. Asterisks indicate statistically significant changes (p value < 0.05).

Figure 8. Gene expression analysis in CAFs and FBs cultured in 3D collagen gels following TGF- β treatment.

(**A and B**) Optical microscope images of FBs and CAFs cultured in 3D collagen type I gels (1mg/ml), respectively. (**C-D**) relative RhoA, ROCK and CDC42 mRNA expression in FBs and CAFs cultured in the 3D collagen gels after TGF- β treatment. Asterisks indicate a statistically significant difference between compared groups ($p < 0.05$).