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Solid stress facilitates fibroblasts activation to promote pancreatic cancer cell migration

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Abstract and Key Terms

Pancreatic fibroblasts are continuously gaining ground as an important component of tumor microenvironment that dynamically interact with cancer cells to promote tumor progression. In addition, these tumor-infiltrated fibroblasts can acquire an activated phenotype and produce excessive amounts of extracellular matrix creating a highly dense stroma, a situation known as desmoplasia. Desmoplasia, along with the uncontrolled proliferation of cancer cells, leads to the development of compressive forces within the tumor, generating the so-called solid stress. Solid stress is previously shown to affect cancer cell proliferation and migration, however there is no pertinent study taking into account the effects of solid stress on fibroblasts and whether these effects contribute to tumor progression. In this work, we applied a defined compressive stress on pancreatic fibroblasts, similar in magnitude to that experienced by cells in native pancreatic tumors. Our results suggest that solid stress stimulates fibroblasts activation and strongly upregulates Growth Differentiation Factor-15 (*GDF15*) expression. Moreover, co-culture of compression-induced activated fibroblasts with pancreatic cancer cells significantly promotes cancer cell migration, which is inhibited by shRNA-mediated silencing of *GDF15* in fibroblasts. Conclusively, our findings highlight the involvement of biophysical factors, such as solid stress, in tumor progression and malignancy revealing a novel role for GDF15.

Keywords

mechanical compression; metastasis; co-culture system; tumor microenvironment; TGFβ; GDF15

Introduction

Pancreatic cancer is among the deadliest forms of cancer worldwide 15. Most pancreatic tumors contain an extremely dense extracellular matrix (ECM) consisting mainly of collagen and hyaluronan. ECM along with a number of non-cancerous, stromal cells, such as immune

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cells, endothelial cells and fibroblasts comprise the tumor microenvironment, which is thought to be responsible for the highly malignant phenotype of this cancer type 18. In fact, fibroblasts are of great importance for pancreatic cancer biology, being responsible for ECM synthesis (mainly collagen type I and fibronectin), as well as for other processes such as wound healing and tissue fibrosis 18. However, tumor-infiltrated fibroblasts, in particular, tend to acquire a constantly activated phenotype producing large amounts of ECM proteins 18 which are accumulated within the tumor leading to a stiffer matrix, a condition known as **desmoplasia**.

Fibroblasts, once activated, produce contractile forces which when combined with the dense matrix in desmoplastic pancreatic tumors and the uncontrolled proliferation of cancer cells in the restricted environment of the host tissue, lead to the development of mechanical compressive stress (i.e. force per unit area) within the tumor interior 16, 41, 46. This stress, derived from the structural components of a tumor, is known as solid stress and can be divided in two parts. A part of it, known as growth-induced stress, is generated owing to microscopic interactions among viscoelastic components of the tumor microenvironment and remains in the tumor even after its export from the organ 16, 41. Moreover, as a tumor grows and exerts forces on the adjacent host tissue, a reciprocal compressive stress is applied from the host tissue to the tumor, which is known as externally-applied stress 16, 45.

Several studies have dealt with the effect of compressive stress on cancer cells 6, 8, 9, 12, 43 using different experimental setups including (i) tumor spheroids growing in a matrix 6, 8, 12 (ii) controlled mechanical compression applied on single cells or spheroids embedded in a matrix 6, 9 and (iii) controlled mechanical compression applied on a cell monolayer 6, 29, 43. To date, results indicated that solid stress impairs cancer cell proliferation and promotes cancer cell migration 6, 8, 9, 12, 22, 43, however there are no studies taking into account the effect of solid stress on other cellular components of the tumor microenvironment, such as fibroblasts. In fact, tumor fibroblasts are increasingly gaining ground as an important component of tumor microenvironment 18. This is based on the fact that among others, fibroblasts continuously produce fibrillar proteins such as fibronectin and collagen type I providing cell-matrix interactions which in turn promote cancer cell invasion through the extracellular matrix 3, 18, 34. At the same time, they remodel the ECM by altering fibers orientation and by producing matrix degrading proteins, such as matrix metalloproteinases (MMPs). These events enable cancer cells to migrate and invade into the matrix in order to escape the highly desmoplastic primary tumor site 10, 16, 18, 19, 34. Moreover, fibroblasts secrete cytokines and growth factors, such as TGF β , that directly promote the proliferation and migration of cancer cells 3, 18, 21. Indeed, in an orthotopic model of pancreatic cancer, the size of the primary tumor as well as the number of distant metastasis were greater when cancer cells were co-injected with fibroblasts 14, 44. Thus, it is well established that tumor-infiltrated activated fibroblasts dynamically interact with cancer cells to promote tumor progression and malignancy 14, 27, 31, nevertheless it is still unclear whether solid stress affects tumor-stromal interactions.

A plausible hypothesis is that solid stress regulates the expression of specific factors in fibroblasts, which in turn mediates cancer cell behaviour. One such candidate could be the Growth Differentiation Factor 15 (*GDF15*), which is known to regulate responses to cellular

stress as well as responses to morphological and cytoskeletal changes, but it has never been linked to solid stress 2, 28. *GDF15*, also known as macrophage inhibitory cytokine 1 (*MIC-1*), belongs to the Transforming Growth Factor β (*TGF β*) superfamily of cytokines and has attracted much attention because of its role in several physiological or pathological processes 28. More specifically, *GDF15*, like other *TGF β* members, has been reported to play a dual role in cancer, either by inducing apoptosis and inhibiting tumor growth 25, 28, 33 or by stimulating cancer cell proliferation, invasion and metastasis 28, 33, 38. In particular, it has been suggested that *GDF15* can regulate cellular responses via the ligand binding to *TGF β* receptors type I and II 28. These receptors can subsequently recruit and phosphorylate downstream signaling molecules such as Smad2/3 or Smad1/5/8. In turn, the phosphorylated Smads can create heteromeric complexes with Smad4, that are subsequently translocated into the nucleus acting as transcription factors regulating the expression of genes implicated in the proliferation, migration and invasion of cancer cells 23, 28, 33. *GDF15*, like *TGF β* , can also regulate cancer cell metastasis by Smad-independent signaling pathways 33 such as PI3K-Akt 33, 48, MEK-Erk1/2 33, 37 and FAK-RhoA signaling pathways 33, 38. Indeed, inhibitors against PI3K and ERK1/2 signaling pathways impaired *GDF15*-induced proliferation and invasion of liver and breast cancer stem-like cells 37, 48. *GDF15* was also found to drive the invasiveness of breast cancer cells by binding to the Insulin Growth Factor Receptor (IGF-1R), nevertheless the exact activated intracellular pathways in this case are yet to be determined 35. In pancreatic cancer, Twist was found to stimulate the expression of *GDF15* through the p38 MAPK signaling pathway to promote the invasiveness of pancreatic cancer cells and drug resistance, while treatment with an appropriate p38 MAPK inhibitor significantly reversed these effects 17.

Interestingly, *GDF15* has been found to be upregulated in several aggressive tumor types including glioblastoma, pancreatic, prostate, breast and colorectal cancers, while high levels of *GDF15* in serum samples from cancer patients have been associated with poor prognosis and patient survival 4, 7, 24, 39, 47. Recent studies have indicated that fibroblast-derived *GDF15* stimulates prostate cancer cell growth, migration and invasion *in vitro* and *in vivo* 5, however the underlying molecular mechanism that promotes *GDF15* upregulation in the tumor microenvironment remains elusive.

Hence, in order to investigate the effect of solid stress on fibroblasts and its implication in tumor-stromal interactions, we employed a previously described transmembrane pressure device 6, 29, 43 to simulate the compressive solid stress encountered in the tumor microenvironment 16, 30, 41. Using this experimental setup, we were able to identify the molecular effect of a defined compressive stress on normal pancreatic fibroblasts, as well as on two distinct pancreatic cancer cell lines, CFPAC-1 and MIA PaCa-2, using a co-culture system. Our results suggest that fibroblast-derived *GDF15* is upregulated in response to solid stress, and it is necessary to induce migration of pancreatic cancer cells *in vitro*.

Materials and Methods

Cell culture

Human normal pancreatic fibroblasts were obtained from Neuromics (Edina, MN) and were maintained in Vitro Plus III medium (Neuromics) supplemented with 1 % antibiotics. MIA

PaCa-2 and CFPAC-1 pancreatic cancer cell lines were obtained by American Type Culture Collection (ATCC) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % antibiotics. For co-culture experiments, both cell lines were cultured in 2 % FBS-containing DMEM. All cells were incubated at 37°C and 5 % CO₂ in a humidified incubator.

***In vitro* transmembrane pressure device**

For the application of a defined and controlled compressive solid stress on fibroblasts, we employed a previously described transmembrane pressure device used in pertinent studies 6, 29, 43. Briefly, 2-4x10⁵ cells were cultured overnight in the inner chamber of a 24 mm diameter transwell insert (Greiner Bio-one,) with 0.4 µm pores, which permits nutrient and oxygen diffusion and prevents cell migration. A 2 % low melting agarose cushion was placed on top of the cells preventing any direct contact between piston and cells, and providing a uniform distribution of the applied force. A 24 mm diameter piston of adjustable weight was placed on the top of the agarose gel (Figure 1A) and the cells were subjected to 1.0, 2.0, 4.0 or 6.0 mmHg stress for 6 hours, or to 4.0 mmHg stress for 48 hours. These solid stress levels were similar in magnitude to those estimated in pancreatic tumors 30, 46. Control cells were covered with an agarose cushion only (i.e., 0.0 mmHg).

Co-culture experiments

For the co-culture experiments, approximately 2x10⁵ fibroblasts or *GDF15*-knockdown fibroblasts and MIA PaCa-2 or CFPAC-1 cells, were separately seeded in transwell inserts and 6-well plates, respectively. After overnight incubation, cells were set in a co-culture system and fibroblasts were subjected to a compression of 4.0 mmHg (Figure 1B). Cells were allowed to grow for 48 hours in 2 % FBS-containing DMEM at 37 °C and 5 % CO₂ in a humidified incubator.

Alamar Blue Assay

Fibroblasts were subjected to a cell viability test following compression using Alamar Blue reagent (Thermo), according to the manufacturer's instructions.

Quantitative Real Time PCR

Total RNA was extracted from both fibroblasts and cancer cells using Trizol (Invitrogen) and reverse transcribed to cDNA using Superscript Reverse Transcriptase (Invitrogen). Quantification of gene expression was performed by real-time PCR using SYBR Green Supermix (KAPA Biosystems) in a real-time PCR detection system (BioRad). The primers for the *β-actin* housekeeping gene were used as an internal control. Each sample was measured in triplicate for each gene. The relative quantification of gene expression was analyzed by the Ct quantification method, using a relevant calibrator as specified in each figure legend. Real-time PCR primers for target genes are listed in Supplementary Table 1.

Western Blotting

For protein expression analysis, total cell lysates from fibroblasts compressed by 4.0 mmHg for 48 hours were obtained using radio immunoprecipitation assay (RIPA) buffer containing

a protease inhibitor cocktail tablet (Sigma). Protein concentration was determined by the BCA protein assay kit (Pierce) and cell lysates were run on a 12 % acrylamide gel and transferred to a PVDF membrane using the BioRad Semi-dry transfer system. Membrane was blocked in 5 % non-fat milk or Bovine Serum Albumin (BSA) in TBS-T buffer and then incubated with anti- α -smooth muscle actin (α -SMA) (Abcam), anti- Collagen I (Abcam) or anti-GDF15 (Cell signalling) antibodies overnight. Following standard western blot procedure steps, the detection of antibodies was performed with enhanced chemiluminescent system (Pierce) using Kodak Biomax light films. Relative protein expression of GDF15, Collagen I and α -SMA was quantified with β -tubulin or β -actin as loading control using ImageJ software. The mean relative protein expression from different immunoblots from at least 2 independent experiments was used.

Immunofluorescence staining

To determine the effect of compression on α -SMA protein expression in fibroblasts, cells were fixed in 4% paraformaldehyde, permeabilized in PBS containing 0.25 % Triton X-100 and blocked with PBS containing 0.1 % Tween-20 and 1 % BSA. Cells were then stained with anti- α -SMA (Abcam) or anti-Collagen I (Abcam) antibody diluted in blocking buffer for 1 hours at room temperature. Alexa 647-donkey anti-rabbit antibody was used as a secondary antibody and nuclei were stained using DAPI. Images were obtained using an Olympus BX53 fluorescent microscope.

Cloning of shRNA-expressing vector and transient transfection of fibroblasts

To generate vectors expressing shRNA against *GDF15*, AgeI/EcoRI-digested pLKO.1-puro vector was ligated with 58-base pair annealed oligos. The sequence of the forward oligo was CCGGGCAAGAACTCAGGACGGTGAAGTTCACCGTCCTGAGTTCTTGCTTT TTG and sequence for the reverse oligo was AATTCAAAAAGCAAGAACTCAGGACGGTGAAGTTCACCGTCCTGAGTTCT TGC. Ligated plasmids were transformed into XL10 gold competent bacteria and selected on ampicillin-containing LB agar plates (100 μ g/ml). Single colonies were grown for 16 hours in LB broth and plasmids were isolated using a NucleoSpin[®] Plasmid QuickPure kit (Macherey-Nagel). The presence of each insert was tested by PCR (KAPA Biosystems) using pLKO.1 Forward primer: GGAATAGAAGAAGAAGGTGGA and GDF15 Reverse primer: GCAAGAACTCAGGACGGTGAA. Following verification by DNA sequencing (Macrogen, Netherlands), transient transfection of fibroblasts was performed with pLKO-shScrambled vector (or shSCR, used as a control) or pLKO-shGDF15 vector using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's guidelines. Cells were allowed to grow overnight in antibiotics-free medium before the co-culture with cancer cells.

Wound Healing assay

A wound healing assay was performed on cancer cells co-cultured with compressed or uncompressed fibroblasts based on published protocols [11]. By the end of the co-culture period, fibroblasts were removed from the co-culture system, conditioned medium was collected, centrifuged to remove cell debris and stored at 4 °C until use. Cancer cells were then washed twice with PBS followed by treatment with 10 μ g/mL Mitomycin-C in 2 %

FBS-containing medium 2 hours prior to the generation of the wound in order to avoid any effect of cell proliferation in wound closure. Cell-free space was then created by generating a wound on the monolayer with a 200 μ L pipette tip and cells were washed twice with PBS to remove debris. Cells were subsequently stimulated with the corresponding conditioned medium for 24 hours, which is thought to include all secreted factors. Images from 4 different fields per condition were taken at 0 hours and 24 hours. The cell-free area from at least 2 independent experiments was quantified using the ImageJ software. Quantification was performed for each condition using the following formula:

$$(\text{Width of the wound at 0 hours} - \text{Width of the wound at 24 hours}) / (\text{Width of the wound at 0 hours}).$$

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was performed in conditioned medium from the co-culture system upon completion of the co-culture period, using the Quantikine ELISA human GDF15 (R & D systems,) following the company's guidelines.

Statistical Analysis

Results are represented as mean $\pm$ standard error (SE). Significant changes were determined by Student's *t*-test using two-tail distribution. Differences with *p*-values <0.05 were considered as significant (indicated by an asterisk *).

Results

Solid stress regulates gene expression of normal pancreatic fibroblasts

In order to study the effect of solid stress on normal pancreatic fibroblasts, cells were subjected to a constant mechanical compression similar in magnitude to that experienced by cells in the tumor interior 30, using an established transmembrane pressure device 6, 29, 43 (Figure 1A). Cells were exposed to a stress ranging from 1.0 to 6.0 mmHg 30 and the expression of several genes known to be upregulated in tumor-infiltrated activated fibroblasts was evaluated by qPCR. First, we tested the expression of *TGF β* which has been shown to be implicated in ECM synthesis and mediates cancer cell proliferation and migration 32. Moreover, we measured the mRNA expression of genes encoding proteins of the ECM, such as *Collagen I* and *Fibronectin I*, as well as *Periostin*, which has been shown to regulate matrix elasticity, stimulates the expression of ECM proteins and promotes cancer progression 13, 20, 26, 40. Our results revealed a trend for increased mRNA expression in all these genes in response to increasing levels of mechanical compression (Figure 2, **dashed red line**). More specifically, a modest but significant increase in the expression of *TGF β* , *Collagen I*, *Fibronectin I* and *Periostin* (Figure 2A-D) was more pronounced when cells were subjected to stresses ranging from 2.0 to 6.0 mmHg. However, within the same range of compression, *GDF15* which regulates cellular responses to stress 2 exhibited the highest mRNA expression of all genes tested (Figure 2E). Interestingly, *α -SMA*, one of the most established markers for fibroblast activation 18, was upregulated when cells were compressed from 1.0 to 6.0 mmHg (Figure 2F), suggesting that fibroblasts can immediately

get activated, as early as 6 hours post application without any effects in cell viability (Supplementary Figure 1A).

Solid stress maintains fibroblasts activation, induces desmoplasia and upregulates the expression of *GDF15*

To examine whether fibroblasts remain activated after application of mechanical compression for longer time periods, we repeated the experiment for 48 hours using the 4.0 mmHg stress condition, as this was the minimum stress condition in which *α-SMA* exhibited the highest mRNA expression level (Figure 2F). Under these experimental conditions, *α-SMA*, *GDF15* and *Collagen I* were upregulated, not only at the RNA (Figure 3A-C) but also at the protein level (Figure 3D-F). Quantification of protein levels is shown in Figure 3G-I. The expression of *α-SMA* and *Collagen I* was also evaluated by immunofluorescence staining, which confirmed that these proteins become upregulated in compressed compared to uncompressed cells (Figure 3J). Consistent with the data from the short-term application of stress (Figure 2), the expression of *TGFβ*, *Fibronectin I* and *Periostin* exhibited a modest increase at the mRNA level (Supplementary Figure 2), while cell viability showed no significant difference between compressed and uncompressed fibroblasts (Supplementary Figure 1B). Collectively, our findings suggest that solid stress not only stimulates but also maintains the activation of fibroblasts, which subsequently can produce larger amounts of fibrillar proteins such as Collagen I. In addition, solid stress strongly upregulates the expression of *GDF15*, and to a lesser extent it induces the expression of genes implicated in ECM synthesis.

Compression-induced activated fibroblasts stimulate the migration of CFPAC-1 and MIA PaCa-2 pancreatic cancer cells

It has been proposed that tumor-infiltrated activated fibroblasts interact with cancer cells to promote tumor growth and invasion [5], hence we wanted to test whether fibroblasts activation by mechanical compression affects adjacent cancer cells through fibroblasts-derived factors, such as *GDF15*. To this end, we developed a novel co-culture system (Figure 1B) which consists of compressed fibroblasts and two distinct pancreatic cancer cell lines, CFPAC-1 or MIA PaCa-2 (see Figure 4A for the experimental design). In this setup, cells were allowed to interact for 48 hours and then a wound healing assay was performed on cancer cells. Both cell lines were pretreated with mitomycin-C, in order to avoid any effect on wound closure due to cancer cell proliferation. As presented in Figure 5A, both pancreatic cell lines exhibited higher migratory ability when co-cultured with compressed compared to uncompressed fibroblasts (Figure 5A-C). Interestingly, this was associated with the higher *GDF15* mRNA expression in compressed fibroblasts co-cultured with CFPAC-1 and MIA PaCa-2 (Figure 5D). This result indicated that secreted *GDF15* may act in a paracrine fashion to stimulate the migration of adjacent cancer cells. However, to eliminate the possibility that *GDF15* could be secreted by cancer cells and act in an autocrine manner, we tested its expression in cancer cells. Real-time PCR analysis showed that *GDF15* mRNA levels were unchanged when both CFPAC-1 or MIA PaCa-2 were co-cultured with uncompressed or compressed fibroblasts (Figure 5E). Moreover, to verify whether *GDF15* is produced and secreted by fibroblasts, we performed ELISA to quantify *GDF15* protein levels in the conditioned medium. We found that *GDF15* was present in higher concentration

when MIA PaCa-2 or CFPAC-1 cells were co-cultured with compressed fibroblasts compared to the system where cells were co-cultured with uncompressed fibroblasts (Figure 5F, Supplementary Figure 3). Our results suggest that solid stress not only activates normal fibroblasts but it can also induce the secretion of GDF15 from them.

GDF15 secreted by compressed fibroblasts is required for the migration of CFPAC-1 and MIA PaCa-2 pancreatic cancer cells

Finally, we investigated whether GDF15 expression and secretion from fibroblasts is necessary for pancreatic cancer cells to migrate. For this purpose, we constructed a plasmid vector expressing shRNA against *GDF15*, and transiently transfected fibroblasts to inhibit its upregulation by solid stress. We then co-cultured compressed fibroblasts, transfected either with shRNA against *GDF15* or scrambled shRNA (control), with CFPAC-1 or MIA PaCa-2 for 48 hours (see Figure 4B for the experimental design). A wound healing assay showed that neither CFPAC-1 nor MIA PaCa-2 cells could migrate when co-cultured with sh*GDF15*-transfected compressed fibroblasts compared to pancreatic cancer cells co-cultured with sh*SCR*-transfected compressed fibroblasts (Figure 6A-6C). Finally, real time PCR analysis and Western Blotting verified that *GDF15* was successfully silenced by sh*GDF15*-expressing construct (Figure 6D-G). Our results suggest that the upregulation and the subsequent secretion of GDF15 from fibroblasts as a response to solid stress are necessary for the migration of pancreatic cancer cells *in vitro*.

Discussion

Solid stress developed within tumors, as a result of increased ECM density and uncontrolled cancer cell proliferation, has been shown to promote cancer cell migration *in vitro* 43. However, solid stress is also exerted on other cellular components of the tumor microenvironment, including fibroblasts, which acquire an activated phenotype fueling tumor progression¹⁸.

In this study, we employed a previously described transmembrane pressure device^{6, 29, 43} as a tool to apply compressive solid stress in a measurable and predefined manner, similar in magnitude to the stress applied on cells in the tumor interior³⁰. The level of compressive stress used in this study ranged from 1.0 to 6.0 mmHg, which was in agreement with experimental measurements in *in vivo* tumor models^{30, 46}.

Our results reveal that solid stress facilitates the activation of normal pancreatic fibroblasts as indicated by the upregulation of *α-SMA*, the most established marker of fibroblasts activation¹⁸, and promotes desmoplasia as revealed by the upregulation of Collagen I expression (Figures 2B,F, and 3A,C,D,F,J). Furthermore, we show that solid stress strongly upregulates the expression of *GDF15* in fibroblasts (Figures 2E, and 3B, E), which has been previously shown to be regulated by cellular stress, morphological or cytoskeletal changes^{1, 2}, but it has never been linked to solid stress. Finally, we demonstrate that both CFPAC-1 and MIA PaCa-2 pancreatic cancer cell lines exhibited higher migration rates when co-cultured with compressed fibroblasts (Figure 5A-C), while this effect was not observed when cancer cells were independently treated with the conditioned medium of control and compressed fibroblasts (Supplementary Figure 4A-B). This phenomenon indicates that

compressed fibroblasts continuously interact with cancer cells and induce cancer cell migration possibly through secretion of fibroblasts-derived factors. Notably, GDF15 levels in fibroblasts of both co-culture systems (CFPAC-1 or MIA PaCa-2) were elevated (Figure 5D). In fact, it is of note that while compressed fibroblasts co-cultured with CFPAC-1 showed increased mRNA expression of GDF15, fibroblasts co-cultured with MIA PaCa-2 showed an even more dramatic increase in GDF15 expression (4 times that of fibroblasts co-cultured with CFPAC-1) (Figure 5D). We further verified the levels of secreted GDF15 from fibroblasts co-cultured with MIA PaCa-2 cells by ELISA and found an also dramatic elevation compared to the co-culture system of CFPAC-1 (Figure 5, Supplementary Figure 3). Next, to assess the direct effect of GDF15 on cancer cells, we treated both cancer cell lines with recombinant GDF15 (rGDF15) and we found that rGDF15 can induce pancreatic cancer cell proliferation and migration in the absence of compressed fibroblasts. Nevertheless, the co-culture of cancer cells with fibroblasts, which is more physiologically relevant to the native tumor microenvironment, is necessary to enhance their GDF15-induced migratory ability (Supplementary Figure 5A-D and Supplementary Figure 6). Strikingly, this effect was significantly decreased when cancer cells were co-cultured with compressed fibroblasts in which *GDF15* had been silenced (Figure 6A-B). Our findings suggest, for the first time, that compression-induced secretion of GDF15 by activated fibroblasts could be responsible for the migration of cancer cells. Interestingly, multiple myeloma cells co-cultured with bone marrow stromal cells treated with a neutralizing antibody against GDF15 were associated with decreased colony formation and GDF15 null mice exhibited prolonged survival compared to wild-type mice 42. Similarly, in a more recent study, when mice were injected with *GDF15*-knockdown pancreatic tumor cells or treated with a neutralizing antibody against GDF15 exhibited delayed tumor formation, reduced tumor growth and increased survival³⁶. Therefore, *GDF15* can be a promising target for prevention of compression-induced pancreatic tumor progression.

Collectively, we propose that solid stress developed within tumors is able by itself to activate normal fibroblasts, which in turn produce excessive amounts of ECM proteins leading to desmoplasia (Figure 3A, C, J). Desmoplasia along with the uncontrolled proliferation of cancer cells leads to the development of solid stress within the tumor, to create a feedback loop (Figure 7). To date, there is no pertinent study taking into account the effect of solid stress on fibroblasts and thus, this is the first study showing that solid stress directly activates fibroblasts to promote pancreatic cancer cell migration. More importantly, we provide evidence that this effect is mediated, at least in part, by the secretion of high levels of GDF15 in response to mechanical stimuli (Figure 7). So far, it was only known that GDF15 stimulates cancer cell migration and invasion *in vitro* and *in vivo*⁵, but the exact mechanisms by which GDF15 is upregulated in pancreatic tumor microenvironment were unclear.

Therefore, our findings highlight the involvement of biophysical factors, such as solid stress, in tumor progression and malignancy, revealing a novel regulatory mechanism of *GDF15* expression. Further studies are needed in order to shed light upon the mechanisms by which fibroblast-derived GDF15 stimulates cancer cell migration. Thus, identification of the molecular pathways involved in GDF15-induced cancer cell migration will certainly provide information that could be exploited therapeutically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. A schematic of the *in vitro* transmembrane pressure device.

(A) Fibroblasts were grown as a monolayer on the transmembrane of a 0.4 μm transwell insert and a piston of adjustable weight was applying a compressive stress. Control cells were covered with an agarose cushion only. (B) The experimental set-up of the co-culture system consisted of fibroblasts and pancreatic cancer cells (MIA PaCa-2 or CFPAC-1) in the upper and lower chamber of a transwell insert, respectively. A piston with adjustable weight, applying 4.0 mmHg of compressive stress on fibroblasts for 48 hours is shown. A co-culture

system consisting of fibroblasts and cancer cells without a compressive load was used as a control.

Figure 2. Solid stress regulates gene expression of normal pancreatic fibroblasts.

Normal fibroblasts were subjected to 1.0, 2.0, 4.0 and 6.0 mmHg of compressive stress for 6 hours. qPCR was used to measure the mRNA expression of *TGFβ* (A), *Collagen I* (B), *Fibronectin I* (C), *Periostin* (D), *GDF15* (E) and *α-SMA* (F). The expression in each sample was analyzed with the $\Delta\Delta C_t$ method relative to the expression of control sample (cells compressed by the agarose cushion only). The mean fold change was calculated and plotted for each gene. Each bar indicates the mean fold change $\pm$ SE of two independent experiments (n=6). Asterisk (*) indicates a statistically significant difference (p<0.05).

Figure 3. Solid stress maintains fibroblasts activation, induces desmoplasia and upregulates the expression of *GDF15*.

(A-C) Fibroblasts were compressed at 4.0 mmHg for 48 hours and total RNA and protein were extracted. qPCR was used to measure *a-SMA* (A), *GDF15* (B) and *Collagen I* (C) mRNA expression. The expression in each sample was calculated with the Ct method using the expression of uncompressed cells as a reference. Each bar indicates the mean fold change $\pm$ SE of three independent experiments (n=9); (D-F) Representative western blot showing *a-SMA* (D), *GDF15* (E) and *Collagen I* (F) protein expression. B-tubulin or β -actin

was used as a loading control. **(G-I)** Quantification of α -SMA (G), GDF15 (H) and Collagen I (I) protein expression was normalized to the β -tubulin or β -actin loading control using the ImageJ software. The mean intensity was quantified from 3 immunoblots (n=3); **(J)** Representative immunofluorescent staining of fibroblasts with anti- α -SMA antibody (green), anti-Collagen I (red) and DAPI (blue) for visualization of the nuclei. Pictures for α -SMA and Collagen I were taken under 40x and 20x objective respectively. Scale bar: 0.1 mm. Asterisk (*) indicates a statistically significant difference ($p<0.05$).

Figure 4. Diagrammatic representation showing the experimental design of co-culture systems. (A) CFPAC-1 or MIA PaCa-2 were co-cultured for 48 hours with compressed fibroblasts (4.0 mmHg) on the lower and upper chamber of a transwell insert, respectively. Co-culture of CFPAC-1 or MIA PaCa-2 with uncompressed fibroblasts (agarose cushion only) was used as a control condition. (B) CFPAC-1 or MIA PaCa-2 were co-cultured for 48 hours with compressed fibroblasts (4.0 mmHg) transfected with shGDF15 vector on the lower and upper chamber of a transwell insert, respectively. Co-culture of CFPAC-1 or MIA PaCa-2 with compressed fibroblasts transfected with shSCR vector (control) was used as a control.

Figure 5. Compression-induced activated fibroblasts stimulate the migration of CFPAC-1 and MIA PaCa-2 pancreatic cancer cells.

(A) Control and compressed fibroblasts (FBs) were co-cultured with CFPAC-1 (A) and MIA PaCa-2 (B) in 2 % FBS containing DMEM for 48 hours. FBs were then removed and cancer cells were subjected to wound healing assay for 24 hours. Pictures were taken with Nikon Eclipse TS100 optical microscope. Scale bar: 0.1 mm. (B-C) Graphs show the percentage of wound closure of CFPAC-1 (B) and MIA PaCa-2 (C) as quantified with the ImageJ software. At least 3 different images from three independent experiments were analyzed. Asterisk (*) indicates a statistically significant difference in wound closure of CFPAC-1 or

MIA PaCa-2 co-cultured with compressed FBs compared with CFPAC-1 or MIA PaCa-2 co-cultured with control FBs ($p < 0.05$). **(D-E)** RNA was extracted from both FBs (D) and cancer cells (E), reversed transcribed into cDNA and qPCR was used to measure the *GDF15* mRNA expression in all cell lines. The expression in each sample was calculated with the C_t method using the expression of cells from the co-culture system of CFPAC-1 or MIA PaCa-2 with control FBs as a reference. Each bar indicates the mean fold change $\pm$ SE of at least three independent experiments ($n=9$); **(F)** Human GDF15 protein (pg/ml) secreted in the conditioned medium was quantified using ELISA. Two independent experiments were performed. Asterisk (*) represents a statistically significant difference ($p < 0.05$).

Figure 6. GDF15 secreted by compressed fibroblasts is required for the migration of CFPAC-1 and MIA PaCa-2 pancreatic cancer cells.

(A) Transfected fibroblasts (FBs) with shSCR (control) and shGDF15 vectors were compressed and co-cultured with CFPAC-1 (A) and MIA PaCa-2 (B) in 2 % FBS containing DMEM for 48 hours. FBs were then removed and cancer cells were subjected to wound healing assay for 24 hours. Pictures were taken with Nikon Eclipse TS100 optical microscope. Scale bar: 0.1 mm. (B-C) Graphs show the percentage of wound closure of CFPAC-1 (B) and MIA PaCa-2 (C) as quantified with the ImageJ software. At least 3

different images from three independent experiments were analyzed. Asterisk (*) indicates a statistically significant difference in wound closure of CFPAC-1 or MIA PaCa-2 co-cultured with compressed FBs knockdown for GDF15 (shGDF15) compared with CFPAC-1 or MIA PaCa-2 co-cultured with compressed shSCR FBs ($p < 0.05$). **(D-E)** RNA was extracted from shGDF15-transfected FBs co-cultured with CFPAC-1 (D) and MIA PaCa-2 (E), reversed transcribed into cDNA and qPCR was used to measure the GDF15 mRNA expression. The expression in each sample was calculated with the 2^{-Ct} method using the expression of FBs transfected with shSCR vector as a reference. Each bar indicates the mean fold change $\pm$ SE of a representative experiment ($n=3$); Asterisk (*) represents a statistically significant difference ($p < 0.05$). **(F-G)** Representative western blot verifying successful knockdown of GDF15 at the protein level, between shGDF15 compressed fibroblasts and shSCR compressed fibroblasts. B-actin has been used as a loading control.

Figure 7. Diagram showing the working hypothesis of the present study.

Mechanical forces in the tumor microenvironment activate normal fibroblasts, which in turn produce excessive amounts of ECM proteins (such as Collagen I and fibronectin), leading to desmoplasia. Desmoplasia along with the uncontrolled proliferation of cancer cells in the confined space of the host tissue, leads to the development of compressive stress within the tumor, thus creating a feedback loop. At the same time, mechanical compression upregulates GDF15 expression in activated fibroblasts, which is secreted to the ECM and stimulates the migration of adjacent cancer cells.