Inhibition of tumor cell proliferation and motility by fibroblasts is both contact and soluble factor dependent

Twana Alkasaliasa,b, Emilie Flaberga, Vladimir Kashubaa,c, Andrey Alexeyenkoa,d, Tatiana Pavlova, Andrii Savchenkoa, Laszlo Szekelya, George Kleinb,1,2, and Hayrettin Guvena,1

aDepartment of Microbiology, Tumor and Cell Biology, Karolinska Institutet, 17177 Stockholm, Sweden; bDepartment of Biology, College of Science, Salahaddin University, 44002, Irbil, Kurdistan-Iraq; cInstitute of Molecular Biology and Genetics, Ukrainian National Academy of Sciences, 03680, Kiev, Ukraine; and dBioinformatics Infrastructure for Life Sciences, Science for Life Laboratory, 17165 Stockholm, Sweden

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Normal human and murine fibroblasts can inhibit proliferation of tumor cells when cocultured in vitro. The inhibitory capacity varies depending on the donor and the site of origin of the fibroblast. We showed previously that effective inhibition requires formation of a morphologically intact fibroblast monolayer before seeding of the tumor cells. Here we show that inhibition is extended to motility of tumor cells and we dissect the factors responsible for these inhibitory functions. We find that inhibition is due to two different sets of molecules: (i) the extracellular matrix (ECM) and other surface proteins of the fibroblasts, which are responsible for contact-dependent inhibition of tumor cell proliferation; and (ii) soluble factors secreted by fibroblasts when confronted with tumor cells (contacted conditioned media, CCM) that contribute to inhibition of tumor cell proliferation and motility. However, conditioned media (CM) obtained from fibroblasts alone (noncontacted conditioned media, NCM) did not inhibit tumor cell proliferation and motility. In addition, quantitative PCR (Q-PCR) data show up-regulation of proinflammatory genes. Moreover, comparison of CCM and NCM with an antibody array for 507 different soluble human proteins revealed differential expression of growth differentiation factor 15, dickkopf-related protein 1, endothelial-monocyte-activating polypeptide II, ectodysplasin A2, Galectin-3, chemokine (C-X-C motif) ligand 2, Nidogen1, urokinase, and matrix metalloproteinase 3.

Here we report the surprising finding that the inhibitory effect of normal fibroblasts is retained partially after fixation. We also show that live fibroblasts release soluble factors upon confrontation with tumor cells that increase their inhibitory effect and identify a number of the proteins and cytokines that could be involved in this intriguing process.

Materials and Methods

Cell Lines. The prostate carcinoma cell line PC3 (from bone metastasis) used as the tumor target in our functional assays was obtained from the American Type Culture Collection. To be able to distinguish tumor cells from unlabeled fibroblasts in coculture, PC3 cells were transfected with recombinant histone H2A-red fluorescein protein (H2AmRFP) carrying plasmid. PC3 cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% (vol/vol) heat-inactivated FBS and penicillin, streptomycin, and gentamicin at 100, 100, and 50 μg/mL concentrations, respectively (complete medium).

Isolation of Subclones of BJHTERT Fibroblasts. Isolation of fibroblast subclones was described before (7). Briefly, subclones were obtained by plating 1,000 recombinant telomerase-transfected immortalized human fibroblast line (BJHTERT) cells that has been generated by introducing a human telomerase encoding vector [myeloproliferative sarcoma virus (MPSV)-HTRT] into human foreskin fibroblasts (9) in a 10-cm tissue culture dish in 10 mL of complete medium. A few colonies started to appear after 2–3 wk. After 4 wk, colonies were checked under light microscope and 10 colonies were complete.

Significance

Normal microenvironments can restrict cancer development and progression. Inhibition of tumor cell growth and motility by normal fibroblasts is one measurable manifestation of this multicomponential control. Here we show that inhibition withstands formalin fixation and can be augmented by the addition of conditioned medium derived from live cultures of tumor cells confronting the stromal fibroblasts. We describe a number of molecules involved in this process. This study lays the foundation for further mechanistic studies of this important phenomenon and its contribution to possible dormancy and the tumor’s resistance to therapy.


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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE56832).

1G.K. and H.G. contributed equally to this work.

2To whom correspondence should be addressed. Email: georg.klein@ki.se.

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removed from the dish by the tip of a pipette and cultured further. Of the 10 BJhTERT thyri trabecles, we separated three that showed the highest inhibitory capacity.

Production of Conditioned Medium. Conditioned medium (CM) was prepared from BJhTERT thyri trabecles. Briefly, confronted CM (CCM) was prepared by plating 0.5 × 10^5 of BJhTERT thyri trabecles in collagen-coated 10-cm² dishes. After 5–6 d when full and aged monolayer of fibroblasts was obtained, 0.5 × 10^3 PC3 mRFP cells were added to the full monolayer of fibroblasts. These cocultures were kept for 2 d in complete medium and after 2 d, the cocultures were washed twice with PBS and a fresh serum-free medium was added. After 24 h, conditioned medium was collected, spun down for 10 min at 750 × g and then filtered (0.45 μM) and stored in −80 °C until future use. Nonconfronted CM (NCCM) from fibroblasts alone was prepared in an identical setup without tumor cell confrontation. A total of 2.5% (vol/vol) PBS was added to CM just before starting the experiments.

Coculture Assay. Tumor cell proliferation on fibroblast monolayers was analyzed in 384-well plates. Fibroblasts were plated in 80 μL complete medium and cultured for 5–6 d to form confluent and aged monolayers. After formation of full confluent and aged monolayer, the monolayer was used either after fixation with 4% (vol/vol) formaldehyde for 20 min followed by washing with PBS three times and then overnight incubation with serum-free medium or without fixation. H2AmRFP-labeled PC3 tumor cells were plated in fresh 80 μL complete medium on top of the fibroblast monolayers. The control wells contained 200 labeled tumor cells without fibroblasts.

Automated Microscopy. Every well of the 384-well plate was imaged using a modified version of the automated microscope system previously developed by us (7, 8). Briefly, images at 2.5× magnification (NA 0.08), covering the entire bottom area of a well, were captured after seeding of tumor cells (day 0) and after 5 d of coculture with fibroblasts. At each time point, both transmitted light and fluorescence images were acquired (excitation at 560 nm and emission at 600–620 nm for mRFP-labeled cancer cells). The microscope platform was built using a Nikon microscope, a programmable XY table (Märzhauser), and a Retiga-4000RV camera (QImaging).

Image Analysis and Quantification. Quantification of tumor cell numbers was done at the single cell level, using the find maxima algorithm in ImageJ (National Institutes of Health). For optimal quantitation of the red-labeled nuclei of the tumor cells, all images were identically processed for quality enhancement using rolling ball background subtraction and 5 × 5 median filtering (ImageJ). The proliferation ratio was calculated by dividing the number of tumor cells on day 5 with the number of tumor cells on day 0 and presented as the mean of measurements in at least 10 individual wells from each experiment of three separate experiments. All results are presented together with the SEM.

Extended Field Live Cell Movie. Fibroblasts were seeded on round coverslips (30 × 0.17 mm in a six-well plate; 18–20 × 10^4 BJhTERT thyri trabecles were grown for 5–6 d. After formation of full confluent and aged monolayer, the monolayer was fixed with 4% formaldehyde for 20 min followed by washing with PBS three times and then overnight incubation with serum-free medium. The next day, 45,000 PC3 mRFP cells were seeded on top of the monolayer (for control experiment, 45,000 PC3 mRFP cells were seeded on round coverslip without any fibroblasts underneath). After 1–2 h, when tumor cells attached to the fibroblast monolayer, the coverslip was removed and inserted into a closed “perfusion open and closed” (POC)-mini chamber system. The motility of the tumor cells was followed for 60 h, with images captured every 52 min. For each time point in the movie, a field of 49 images, covering a total area of 4.5 × 5.9 mm² (26 mm²), was captured using 10× magnification. The movie was captured using a program for multifield extended field capture (multifield 10x), developed by us using Openlab Automator (Perkin-Elmer).

Real-Time PCR. Total RNA was purified from flow cytometry sorted BJhTERT whirly and with and without PC3 mRFP confrontation using the RNA Purification kit (Ambion) according to the manufacturer’s instructions. One microgram of total RNA was used for the CDNA synthesis using a First Strand CDNA Synthesis kit (Thermo Scientific). For Q-PCR, the total reaction volume was 25 μL and the primer concentration was adjusted to a final concentration of 0.3 μM. Quantitative real-time PCR (Q-PCR) was performed using the SYBR Green Master mix and the 7500 Real-Time Thermocycler (Applied Biosystems) under the following conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. The PCR primers for genes were obtained from the quantitative real-time PCR primer database (primerdesigner.nci.nih.gov).

CT values were determined for the internal control (glyceraldehyde-3-phosphate dehydrogenase or TATA-binding protein) and for the test genes at the same threshold level in the exponential phase of the PCR curves. Relative quantification [comparative Ct (ΔΔCt) method] was used to compare the expression level of the test genes with the internal control. Dissociation curve analysis was performed after every run to check the specificity of the reaction.

Human Antibody Array. The kit (L-Series Human Antibody Array L-507 Membrane Kit) was purchased from RayBiotech. The kit detects 507 soluble proteins simultaneously in cell culture supernatants (the proteins include cytokines, chemokines, adipokine, growth factors, angiogenic factors, proteases, and other soluble factors and receptors).

The staining was done according to the manufacturer’s instructions. Briefly, the first step in using human L-507 is to biotinylate the primary amine of proteins in the sample. Then the membrane (already preprinted with capture antibodies) was blocked with blocking buffer. The biotin-labeled sample was loaded onto the membrane and incubated, allowing the target proteins to interact properly. After that, the membrane was treated and incubated with HRP-conjugated streptavidin and followed by adding the substrate or detection solution. A Luminescent Image Analyzer Las-1000 instrument (Fuji Film) and Las-1000 Image Reader software have been used to detect the dots on the membrane.

Affymetrix Microarrays. The Affymetrix Gene Chip WT Sense Target Labeling and Control Reagents kit (P/N 900652) was used for preparation of CDNA from 150 ng of total RNA. Array hybridization, washing, staining, and scanning were performed on the Gene Titan system using the Gene Chip Human Gene 1.1 ST Array Plate. Summarization, normalization, and background correction were performed in Affymetrix Expression Console (v. 1.3.1) using the robust multiarray average (RMA) method. The GEO accession no. for the Affymetrix results is GSE56832.

Statistical Analysis. Data for tumor cell proliferation were transformed to arcsine of the square root of the original value. Statistical analysis was performed using general linear models. The significance effect between group means and for each treatment.
Confronted CM, respectively. PC3 mRFP tumor cells during 0 all five color-coded images shows the total motility (full trajectories) of 36 h), blue (36 h), and red (48–60 h). A maximum projection of all five color-coded images shows the total motility (full trajectories) of the PC3 mRFP tumor cells during 60 h for BJhTERT whirly nonconfronted and confronted CM, respectively.

Fig. 2. Conditioned medium from confronted fibroblast cultures significantly inhibited the motility of tumor cells. Tumor cell motility was studied by extended field live cell microscopy. Trajectories of tumor cells growing on top of fixed BJhTERT whirly fibroblast monolayer. Trajectories of PC3 mRFP tumor cells during 12-h intervals (A) treated with confronted CM or (C) with nonconfronted CM. Color-coded images show a 60 time point projection of the red-labeled tumor cells: yellow (0–12 h), green (12–24 h), magenta (24–36 h), blue (36–48 h), and red (48–60 h). (D) A maximum projection of all five color-coded images shows the total motility (full trajectories) of the PC3 mRFP tumor cells during 0–60 h for BJhTERT whirly nonconfronted and confronted CM, respectively.

We observed that both live and formaldehyde fixed full confluent monolayers of whirly fibroblasts inhibit the proliferation of PC3 mRFP tumor cells. As shown in Fig. 1 (blue line), live monolayer of whirly fibroblasts inhibited the growth of PC3 mRFP tumor cells significantly, compared with PC3 mRFP control cells \( P = 0.00002 \). The ratio of tumor cell proliferation on day 5 of coculture was 0.22 compared with the PC3 mRFP control cells (0.45). After fixation, whirly fibroblasts still inhibited the tumor cell proliferation significantly (0.31) compared with the PC3 mRFP control cells \( P = 0.00002 \). However, fixed whirly fibroblasts were significantly less inhibitory compared with live whirly monolayer \( P = 0.00002 \). Moreover, tumor cell proliferation was inhibited significantly by fixed fibroblasts regardless of whether CM was present (Fig. 1, blue, green, and red lines). Interestingly CM from confronted cultures (CCM) increased the inhibitory capacity of fixed whirly fibroblasts significantly, compared with fixed fibroblasts treated with either NCM or medium alone \( (P = 0.00002) \). The ratio of tumor cell proliferation on day 5 of coculture was 0.24 for CCM compared with NCM (0.31) and medium alone (0.32). In contrast, NCM did not show any effect on the inhibitory capacity of fixed fibroblasts compared with medium alone \( (P = 0.97) \) (Fig. 1, fixed BJhTERT, green, and blue lines). More interestingly, fixed fibroblasts fully regained their maximum inhibitory capacity after CCM treatment (Fig. 1, fixed BJhTERT, whirly red line with live BJhTERT, whirly blue line). In addition, when CCM and NCM were tested on PC3 mRFP cells alone (no monolayer of fibroblasts underneath), both CM did not show any inhibitory effect \( (P = 0.99) \) on tumor cell proliferation compared with the medium-alone treatment (Fig. 1, PC3 alone/ control). Results so far clearly show that CM from confronted cultures (CCM) is able to inhibit tumor cell proliferation only in the presence of fibroblast monolayer underneath. This could mean that optimum inhibition of tumor cell proliferation requires not only soluble factors produced by confronted cultures but also some surface and extracellular matrix (ECM) factors produced by fibroblasts.

CCM but Not NCM Restricted Motility of Tumor Cells on Fixed Fibroblasts. To investigate whether inhibition of tumor cell motility is in concordance with the inhibition of tumor cell proliferation, we studied differences in the motility of PC3 mRFP cells by time lapse imaging combined with our extended field live cell microscopy technique. The PC3 mRFP cell movement was recorded for 60 h continuously. For visualization of the motility, these 60 time points were subdivided into five color-coded trajectories each illustrating 12-h intervals. A marked difference in the motility of tumor cells was seen when cocultured cells (fixed monolayer of fibroblast plus live tumor cells) were treated with CCM but not NCM (Fig. 2). CCM inhibited the motility of tumor cells on top of fixed fibroblast monolayer (Fig. 2A and B). In contrast, NCM did not affect the motility of PC3 mRFP cells on top of fixed monolayer of fibroblasts (Fig. 2C and D). As shown in Fig. 3 and Movie S1, starting 24 h after CCM treatment, the motility of tumor cells was significantly inhibited (Fig. 3, red line) \( (P = 0.04) \). On the contrary, NCM treatment did not show any effect on the motility of tumor cells for 60 h (Fig. 3, blue line).

Fig. 3. Kinetics of tumor cell motility and proliferation. (A) Motility of PC3 mRFP cells when treated with NCM (blue line) and CCM (red line). Motility of tumor cells has been quantified by calculating the area of cell trajectories normalized for average number of cells in 12-h intervals. \( \text{P-value} = 0.04 \). Factorial ANOVA followed by Tukey’s HSD test were performed to determine the significance effect between group means for all intervals. (B) Number of PC3 mRFP cells proliferated during 60 h (time points). Factorial ANOVA followed by Tukey’s HSD test were performed to determine the significance effect between group means for all intervals.
Fig. 4. Conditioned medium (CM) has no effect on tumor cell motility when cultured alone without fibroblasts underneath. Tumor cell motility was studied by extended field live-cell microscopy. Trajectories of tumor cells cultured alone. Trajectories of PC3 mRFP tumor cells during 12-h intervals (C) treated with confronted CM and (A) with medium-only color-coded images show 50 time points projection of the red labeled PC3 tumor cells: yellow (0–12 h), green (12–24 h), magenta (24–36 h), blue (36–48 h) and red (48–60 h). (D and B) A maximum projection of all five color-coded images shows the total motility (full trajectories) of the PC3m RFP tumor cells during 0–60 h for confronted CM and medium only, respectively.

Identification and Verification of Fibroblast Genes Involved in Inhibition of Tumor Cell Proliferations. To identify important genes that might be involved in the control of tumor cell inhibition by BJhTERT whirly fibroblasts, the expression levels of the whole genome of BJhTERT whirly fibroblasts were compared before and after tumor cell confrontation using microarray analysis with Affymetrix Gene Chips. We showed that 617 genes were up-regulated, whereas 402 genes were down-regulated upon tumor cell confrontation (Dataset S1). To further verify the results from microarray data, Q-PCR was run for some of the genes (Table 1). We have tested 11 genes mainly belonging to the proinflammatory gene signature as well as some ECM and matrix metalloproteinase (MMP) genes. Eight genes (IL-8, IL-1b, ICAM1, CXCL1, CXCL2, COL15A, MYO10, and IL-6) showed up-regulation; however, three genes (TNFRSF11B, ADAMTS1, and SPP1) showed down-regulation as depicted in Table 1.

Identification of Differentially Produced Soluble Proteins in CM. To identify proteins or factors secreted by fibroblasts that inhibit both tumor cell proliferation and motility, we used the L-Series Human Antibody Array L-Membrane Kit for 507 soluble proteins in both confronted and nonconfronted CM (Fig. 5). The analysis revealed that two CM differ in their protein expression level. Nine proteins showed differential expression. As shown in Fig. 5, MMP3 was the only protein that showed down-regulation upon confrontation. The remaining eight proteins growth differentiation factor 15 (GDF-15), dickkopf-related protein 1 (DKK1), endothelial-monoocyte-activating polypeptide II (EMAP-II), ectodysplasin A2 (EDA-A2), Galectin-3, chemokine (C-X-C motif) ligand 2 (CXCL2), Nidogen1, and urokinase (uPA) showed up-regulation upon confrontation with tumor cells.

Discussion

Previously we reported that confluent monolayer of fibroblasts can efficiently inhibit proliferation of tumor cells in vitro (7, 8). Here we significantly expand our previous findings and show that inhibition of both proliferation and motility of tumor cells by fibroblasts is controlled by different mechanisms that require both soluble and cell-to-cell contact-dependent factors by fibroblasts. Cell surface-dependent interactions have existed throughout evolution. It has been shown that nectins and nectin-like molecules play important roles in contact inhibition of cell proliferation (6). It has also been found that sponge cells can reject juxtaposed xenogeneic and allogeneic cells. Thus, contact inhibition of cell movement and proliferation is a recurrent theme during evolution (10). Confirming our previous findings, we show that full monolayers of fibroblasts inhibit tumor cell growth in vitro (7, 8). To characterize the nature of this inhibitory effect and the relative significance of contact-dependent factors as well as fibroblast-derived secreted factors, we exposed PC3 mRFP tumor cells to fixed fibroblast monolayers of BJhTERT. The fixed fibroblasts inhibited the growth of PC3 mRFP cells in coculture at a reduced level, compared with live fibroblasts. This may open up ways for molecular analysis of the fixation resistant structures that can contribute significantly to the inhibitory effect.

Table 1. Differentially expressed genes in BJhTERT whirly fibroblasts after confrontation with PC3 mRFP cells

<table>
<thead>
<tr>
<th>Genes</th>
<th>BJhTERT whirly alone</th>
<th>BJhTERT whirly + PC3 mRFP</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL8</td>
<td>14.3</td>
<td>554.3</td>
<td>38.9</td>
</tr>
<tr>
<td>IL-1b</td>
<td>2.3</td>
<td>114.6</td>
<td>49.9</td>
</tr>
<tr>
<td>ICAM1</td>
<td>1.8</td>
<td>24.9</td>
<td>14.0</td>
</tr>
<tr>
<td>CXCL1</td>
<td>0.9</td>
<td>6.8</td>
<td>7.1</td>
</tr>
<tr>
<td>CXCL2</td>
<td>14.0</td>
<td>10.1</td>
<td>7.1</td>
</tr>
<tr>
<td>COL15A</td>
<td>0.2</td>
<td>0.8</td>
<td>5.1</td>
</tr>
<tr>
<td>MYO10</td>
<td>21.2</td>
<td>57.7</td>
<td>2.7</td>
</tr>
<tr>
<td>IL-6</td>
<td>13.0</td>
<td>15.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Down-regulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFRSF11B</td>
<td>161.4</td>
<td>4.0</td>
<td>40.2</td>
</tr>
<tr>
<td>ADAMTS1</td>
<td>65.1</td>
<td>18.0</td>
<td>3.6</td>
</tr>
<tr>
<td>SPP1</td>
<td>0.7</td>
<td>0.2</td>
<td>2.9</td>
</tr>
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(P = 0.81). Comparing the effect of CCM to NCM, CCM treatment reduced the motility of tumor cells significantly during 60 h (P = 0.004). In the control setup, CCM did not inhibit the motility of tumor cells when cultured alone (no monolayer of fibroblast underneath (Fig. 4). Interestingly, when the movement of tumor cells was analyzed closely, it was apparent that tumor cells moved in a more restricted straight path as long as they were cocultured with fixed fibroblasts regardless of CM treatment (Movies S1 and S2), but the movement of tumor cells was much more diverse when cultured without fibroblasts underneath. Instead of a straight movement, the tumor cells move in a circular pattern (Movies S3 and S4). These results also show that motility of tumor cells are inhibited relatively early in CCM compared with NCM, where it took place relatively late.
In our previous study on 107 fibroblast isolates from different individuals (8) we found differences in inhibitory activity, depending on the site of origin (skin, hernia), and also the derivation from normal skin versus cancerous prostate. The latter difference is particularly interesting because it may stem from the "corruption" of fibroblasts by adjacent tumor cells in vivo. A switch from inhibitory to noninhibitory or stimulatory fibroblast phenotype appears to be crucially involved in tumor development.

We show here that when fibroblasts are fixed with formaldehyde, they still show some degree of inhibition, although less than live fibroblasts. This suggests that the inhibition is partially due to the interaction of insoluble ECM and the surface proteins of the fibroblasts with tumor cells. Our suggestion is based on the fact that formalin fixation keeps the ECM and the surface proteins structurally intact. This is in line with some of the previous findings by other groups. Among them, Allard et al. showed that growth suppression of transformed cells was dependent on direct contact with confluent cultures of 3T3 cells. They showed that this was caused by a progressive elongation of the cell cycle leading to arrest in G2/M in the transformed cells (11). In addition, Alexander et al. (2004) showed that direct physical contact between transformed and nontransformed cells was required for growth suppression of transformed cells (12). Moreover, they showed that gap junctional communication was not required (11, 12). Instead, nontransformed cells increased the expression of serum deprivation response protein (SDPR) and transcription factor 4 (TCF4) and a half LIM domain 1 (FHL1) in tumor cells. Their results suggest that normal cells may block Wnt signaling, inhibit insulin-like growth factor activity, and promote host recognition of neighboring tumor cells (12). In our current study, we show that live monolayers of BJhTERT whirlly fibroblasts were more inhibitory when confronted with PC3 mRFP cells compared with fixed monolayers of the same fibroblast cells. This implies the presence of additional inhibitory factors along with the surface molecule and/or ECM. Thus, to explain the differences in the inhibitory capacity of fixed and live fibroblast cultures, CM from nonconfronted and confronted fibroblast cultures were tested, respectively. CM collected from BJhTERT whirlly confronted with PC3 mRFP, enhanced the inhibition capacity of fixed BJhTERT whirlly cells. It is particularly interesting to highlight that fixed fibroblasts regained their full inhibitory capacity after treatment with CCM, which was similar to the inhibitory capacity of live fibroblasts (Fig. 1). This finding in turn shows that inhibitory capacity of fibroblasts is determined by multiple factors (surface, ECM, and soluble) and soluble factors are inducible after confrontation of live fibroblasts with tumor cells. In contrast, nonconfronted CM from BJhTERT whirlly culture did not enhance the inhibition. We therefore suggest that soluble factors secreted by BJhTERT whirlly cells upon tumor cell confrontation enhance the inhibitory capacity of fibroblasts. Apart from our results, Pessina et al. showed that CM from human skin-derived fibroblasts treated with Paclitaxel (a chemotherapeutic drug) is a potent inhibitor of tumor cells in vitro (13). In addition, other groups showed that CM from CAFs has a growth-promoting effect on tumor cells due to factors secreted in the culture medium (14, 15). Among these factors, IL-6, TGFbeta, COX-2, and EMAP-II (4) have already been mentioned (16–20). Interestingly, we show that our results are in accordance with Erez et al. in that the proinflammatory gene signature was up-regulated in our fibroblasts in vitro as well, except for SPP1, which showed down-regulation upon tumor cell confrontation (21).

In addition to the proliferation, we studied the effect of CM on tumor cell motility in a POC-mini chamber using extending field live cell microscopy. We previously showed that tumor cell motility decreased when cocultured with inhibitory fibroblasts compared with noninhibitory fibroblasts (8). In the present study we show that confronted CM inhibits tumor cell motility as well as tumor cell proliferation in the presence of fibroblast monolayer underneath. However, this effect was not seen when the
tumor cells were cultured alone without a fibroblast monolayer underneath. Based on these results, we suggest the following hypothesis (Fig. 6): The inhibition of tumor cells by fibroblasts requires two signals; the first signal originates either from the surface molecules on the fibroblasts or from the structural molecules of the ECM, whereas the second signal is inducible and originates from soluble factors secreted as a result of the cross-talk between tumor cells and fibroblasts. On the other hand, others showed that soluble factors secreted from CAFs may promote tumor growth and motility. One of the soluble factors secreted by CAFs is exosomes, which promote breast cancer cell motility via Wnt-planar cell polarity signaling (22). Furthermore, activated fibroblasts addicted to hypoxia secrete several cytokines, which increase the invasiveness of melanoma cells and are active players in attracting melanoma cells to different locations (23). In agreement with our study, Cheng et al. has already shown that p53 attenuates the motility of PC3 tumor cells by inducing the expression of GDF-15 (24).

Interestingly, PC3 mRFP cells exhibit different patterns of movement when cultured alone or in coculture with fibroblast monolayer. In coculture with fibroblast monolayer, the PC3 mRFP cells move straight either forward or backward (Movies S1 and S2). In contrast, PC3 mRFP cells show random and colonial multidirectional movement when cultured alone (Movies S3 and S4). This may be due to the presence of fibroblasts that may have some polarity-specific molecules, which could determine the path for tumor cells. It has been found that CAFs generate some tracks through which the squamous cell carcinoma moves and such tracks enable the invasion of the tumor (25).

It also looks striking that proliferation of tumor cells was reduced in parallel with reduction in the motility of tumor cells when these tumor cells were treated with CCM with fibroblasts underneath. Moreover, reduction in motility as well as increase in inhibition of tumor cell proliferation started already after 24 h and stopped moving totally at 48 h in CCM cultures (Fig. 3B, red line and Movie S1), whereas in NCM cultures, motility was slightly reduced after 40 h (Fig. 3B, blue line and Movie S2).

The finding that CM derived from the BJhTERT whirly cultures confronted with the PC3 mRFP tumor cells enhanced the inhibition may open up means of identification of soluble factors involved in the inhibitory effect. It is particularly interesting that confronted CM shows different expression levels of nine proteins (eight up-regulated and one down-regulated) compared with CM derived from BJhTERT whirly cells cultured alone. Taken together, these findings indicate that the ability of fibroblasts to inhibit tumor cell growth may be subjected to epigenetic, disease-related variation. Although it can only reflect one aspect of the complex microenvironmental control, the simple in vitro test of fibroblast-mediated inhibition of tumor growth and migration opens the way to a disease-related analysis of the phenomenon.

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Supporting Information

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Movie S1. The motility of PC3 mRFP cells on fixed BJhTERT whirly fibroblast monolayer was significantly inhibited in confronted CM.

Movie S2. The motility of PC3 mRFP cells on fixed BJhTERT whirly fibroblast monolayer was not inhibited in nonconfronted CM.

Movie S1

Movie S2
The motility of PC3 mRFP cells was not affected in medium alone.

Movie S3

The motility of PC3 mRFP cells was not affected in confronted CM.

Movie S4

Dataset S1. Top up- and down-regulated genes in BJhTERT whirlly fibroblasts after confrontation with PC3 mRFP cells

Dataset S1