Microenvironment and Immunology

Tumor Cell Invasion Is Promoted by Interstitial Flow-Induced Matrix Priming by Stromal Fibroblasts

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Abstract

Interstitial flow emanates from tumors into the microenvironment where it promotes tumor cell invasion. Fibroblasts are key constituents of the tumor stroma that modulate the mechanical environment by matrix remodeling and contraction. Here, we explore how interstitial fluid flow affects fibroblast–tumor cell interactions. Using a 3-dimensional invasion assay and MDA-MB-435S cells cocultured with dermal fibroblasts in a collagen matrix, we showed a synergistic enhancement of tumor cell invasion by fibroblasts in the presence of interstitial flow. Interstitial flow also drove transforming growth factor (TGF)-β1 and collagenase-dependent fibroblast migration, consistent with previously described mechanisms in which flow promotes invasion through autologous chemotaxis and increased motility. Concurrently, migrating fibroblasts enhanced tumor cell invasion by matrix priming via Rho-mediated contraction. We propose a model in which interstitial flow promotes fibroblast migration through increased TGF-β1 activation and collagen degradation, positioning fibroblasts to locally reorganize collagen fibers via Rho-dependent contractility, in turn enhancing tumor cell invasion via mechanotactic cues. This represents a novel mechanism in which interstitial flow causes fibroblast-mediated stromal remodeling that facilitates tumor invasion. Cancer Res; 71(3): 1–12. ©2011 AACR.

Introduction

The tumor microenvironment contains numerous cell types and a complex extracellular matrix (ECM), and plays a key role in cancer progression (1, 2). Stromal fibroblasts synthesize tumor-permissive ECM, and promote tumor invasion through secretion of cytokines and matrix metalloproteinases (MMP; ref. 3). Less appreciated are biomechanical changes that accompany tumor growth, such as increased matrix density and fluid flow, yet these have significant effects (4–6). Normally, fibroblasts support the epithelium by maintaining the ECM, secreting cytokines, and making cell–cell contacts (7). Altered tissue mechanics, such as that caused by collagen reorganization at the tumor-stromal interface and increased matrix stiffness, can disrupt epithelial structures and cause tumorigenesis and invasion (8–11). Because fibroblasts play a key role in sensing mechanical stress and modulating the biomechanical microenvironment (7, 12), we asked how the biomechanical force of interstitial flow affected fibroblast interactions with tumor cells.

Interstitial fluid flow through the ECM is omnipresent in tissues and caused by lymphatic drainage. Because of excessive interstitial fluid pressure in tumors, interstitial flow and lymphatic drainage are increased in the tumor margin (13, 14). Interstitial flow can redistribute bioactive molecules and alter gradients (15, 16). This in turn may drive cancer cell invasion by skewing autologously secreted chemokines, causing chemotaxis in the flow direction (17). Interstitial flow can also affect stromal cells, causing cell and matrix alignment (18, 19), increasing fibroblast motility via MMP-1 (20), and inducing myofibroblast differentiation via transforming growth factor (TGF)-β1 (18).

Because interstitial flow has significant effects on both fibroblast and tumor cell behavior, we hypothesized that interstitial flow would have synergistic effects on tumor cell–fibroblast interactions by altering cell behavior and the 3-dimensional (3D) matrix through which they communicate and migrate. For example, physical changes wrought by fibroblasts under interstitial flow (18) could increase matrix stiffness, density, and alignment, leading to increased tumor invasion (8, 10, 21). We used in vitro models that combine tumor cells and fibroblasts in a 3D environment with interstitial flow, and showed that flow drives fibroblasts to invade in a TGF-β1- and MMP-dependent manner, and simultaneously enhance tumor cell invasion by Rho-dependent matrix priming.
Methods

Cell isolation and culture
Human foreskin–derived dermal fibroblasts were isolated using dispase I and Liberase Blendzyme 1 (Roche), selected with antifibroblast-specific antigen microbeads (Miltenyi Biotec) and used at passages 3 to 10. GFP-MDA-MB-435S human melanoma cells were used as previously described (22). MDA-MB-435S cells, IMR-90 fetal lung fibroblasts, DU-145 prostate carcinoma, and MDA-MB-231 breast adenocarcinoma cells (ATCC) were maintained at 37°C, 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) with L-glutamine and sodium pyruvate (PAA Laboratories), 10% fetal bovine serum (FBS), and antibiotics (Invitrogen).

3D invasion assay
Modified 3D Boyden chambers, as previously described (17), were used to measure tumor cell and fibroblast invasion. Tumor cells (5 × 10⁵ cells/mL) and/or fibroblasts (2.5 × 10⁹ cells/mL) were suspended in 1.8 mg/mL rat tail tendon collagen and 0.5 mg/mL Matrigel (BD Biosciences). In some cases, Matrigel was omitted. For fibroblast density experiments, between 5 × 10⁴ and 50 × 10⁴ fibroblasts/mL were used. The cell–ECM mixture was added to culture inserts with 8-µm pores (Millipore). After gelation, DMEM with 5% FBS (serum-free for TGF-β1) was added. A pressure head of 5 mm H₂O drove tumor-relevant interstitial flow of approximately 0.1% bovine serum albumin for exogenous FBS (serum-free for TGF-β1) with 8–10⁵ cells/mL were suspended in 1.8 mg/mL rat tail tendon collagen and 0.5 mg/mL Matrigel (BD Biosciences). In some cases, Matrigel was omitted. For fibroblast density experiments, between 5 × 10⁴ and 50 × 10⁴ fibroblasts/mL were used. The cell–ECM mixture was added to culture inserts with 8-µm pores (Millipore). After gelation, DMEM with 5% FBS (serum-free for TGF-β1) was added. A pressure head of 5 mm H₂O drove tumor-relevant interstitial flow of approximately 0.1% bovine serum albumin for exogenous TGF-β1 experiments) was added. A pressure head of 5 mm H₂O drove tumor-relevant interstitial flow of approximately 0.5 µm/s (13). As appropriate, 0.45 mg/mL heparan sulfate, 0.45 mg/mL chondroitin sulfate A, 0.3 U/mL heparinase III, 100 µg/mL blebbistatin, 10 µmol/L ML-7 (Sigma-Aldrich), 1 µg/mL mouse anti-human TGF-β1 antibody or mouse IgG1 isotype control (R&D Systems), rhTGF-β1 (PeproTech), 10 µmol/L GM6001 (Millipore), and 2 µg/mL cell-permeable C3 transferase (Cytoskeleton Inc.) were added to the matrix and medium. Fibroblasts were labeled with 1 µmol/L CellTracker Green CMFDA or 2 µmol/L CellTracker Red CMPTX (Invitrogen).

TGF-β1 measurement
Total TGF-β1 was measured using the Human TGF-β1 DuoSet ELISA (R&D Systems). Active TGF-β1 was measured using transformed mink lung cells (TMLCs) that produce luciferase under the plasminogen activator inhibitor-1 promoter (23). TMLCs (2.5 × 10⁶ cells/mL) were cocultured with tumor cells and/or fibroblasts under static or flow conditions. Cell lysate was incubated with luciferin (Promega) and luminoscence was measured on a Centro LB luminometer (Bertold Technologies).

Dye-quenched collagen proteolysis assay
A total of 2.5 × 10⁶ fibroblasts/mL were suspended in the previously described collagen/Matrigel ECM with 100 µg/mL DQ collagen type I (Invitrogen) and seeded into a modified radial flow chamber (19). Cells were exposed to static or flow conditions for 18 hours and then imaged using an LSM 510 META confocal microscope (Carl Zeiss AG). The fluorescence intensity was quantified around each fibroblast using ImageJ after subtracting background fluorescence.

Gelatin zymography
Medium samples from the 3D invasion assay were resolved on a 10% gelatin/SDS-PAGE gel (Bio-Rad) under denaturing, nonreducing conditions. The MMPs were renatured, and the gels were incubated overnight at 37°C in developing buffer to induce proteolysis. Gels were stained with 0.5% Coomassie R-250 and imaged with a ChemiDoc XRS (Bio-Rad).

Confocal and live-cell microscopy
For live-cell microscopy experiments, cell-seeded radial flow chambers were placed on the confocal microscope in a 37°C, 5% CO₂ chamber for time-lapse fluorescence and reflectance imaging under static or flow conditions. Images were taken every 10 to 15 minutes for 18 hours. Afterwards, 2% paraformaldehyde was flowed through the chambers for later imaging.

Data and statistical analyses
All data are presented as mean ± SEM. For 3D invasion assay results, cell counts were normalized to the matching controls from each experiment, as noted in each figure legend. Statistical analyses were done using Matlab R2008 (MathWorks); t tests and multifactor analysis of variance (ANOVA) were used. For experiments that used multiple batches of cells and lots of matrix, an additional blocking variable was included to account for these variations. If the ANOVA showed significance at P < 0.05, a Tukey’s Honest Significant Difference test was used for post hoc multiple comparisons.

Results

Interstitial flow stimulates fibroblast and concomitant tumor cell invasion
We first verified that MDA-MB-435S tumor cells, cultured for 18 hours alone in a 3D collagen/Matrigel matrix, were more invasive under a physiologic level of approximately 0.5 µm/s interstitial flow (Fig. 1A) than in static conditions, a phenomenon we previously identified in 3D Matrigel culture and coined “autologous chemotaxis” (17). Without Matrigel, which is rich in heparan sulfate and binds many chemokines and growth factors (24), tumor cells did not respond to interstitial flow (Fig. 1A). Because in our earlier study, we found that autologous chemotaxis was dependent on the chemokine CCL21 (17), we hypothesized that matrix binding of CCL21 via heparan sulfate may play an important role in autocrine gradient formation and amplification (15). Consistent with this hypothesis, we found that soluble heparan sulfate, but not chondroitin sulfate, significantly decreased interstitial flow-mediated tumor cell invasion (Supplementary Fig. 1A), presumably by competitively inhibiting CCL21 binding to...
matrix-associated heparan sulfate for subsequent pericellular gradient formation. Treatment with heparinase III, which selectively cleaves heparan sulfate and thus prevents CCL21 binding to matrix heparan sulfate, also decreased interstitial flow-mediated tumor cell invasion (Supplementary Fig. 1B). Thus, interstitial flow stimulated tumor cell invasion through a 3D collagen/Matrigel matrix in a heparan sulfate-dependent manner, and we hypothesize that this was due to its chemokine binding activity that is required for local gradients to form.

Next, we examined the effects of cocultured fibroblasts on tumor cell invasion. We found that in static conditions, 2.5 × 10^5 fibroblasts/mL cocultured with MDA-MB-435S cells resulted in a small increase in tumor cell invasion (Fig. 1B); however, under 0.5 μm/s interstitial flow, they markedly increased tumor cell invasion compared with tumor cells cultured alone under interstitial flow (Fig. 1B). Note that the response elicited by combining fibroblasts and interstitial flow was significantly greater than the summative effects of either fibroblasts or flow. Similar effects were observed using IMR-90 fibroblasts (Supplementary Fig. 2) and MDA-MB-231 and DU-145 cell lines (Supplementary Fig. 3). This fibroblast-enhanced tumor cell invasion was not due to soluble factors, because the effect was abolished when fibroblasts were cultured in an adjacent gel upstream of tumor cells (Fig. 1B). Increased tumor cell invasion correlated to increased fibroblast migration, as interstitial flow also increased fibroblast migration, and even more so in the presence of tumor cells (Fig. 1B). Thus, we hypothesized that increased fibroblast migration and physical interactions between tumor cells and fibroblasts were necessary for fibroblasts to enhance tumor cell invasion under flow.

Unlike tumor cells, flow-enhanced fibroblast invasion was independent of Matrigel (Fig. 1C). As a result, tumor cells continued to invade in the presence of fibroblasts and flow.

Figure 1. Fibroblasts enhance tumor cell invasion in the presence of interstitial flow. Normalized cell invasion for MDA-MB-435S tumor cells (TC) and human dermal fibroblasts (Fb). In all graphs, invasion was normalized to the leftmost condition except in D, in which invasion was normalized to the lowest fibroblast density in static conditions. A, tumor cell invasion (without fibroblasts) in the presence or absence of 0.5 μm/s interstitial flow through a 1.8 mg/mL collagen matrix containing either 0.5 mg/mL Matrigel (MG) or no Matrigel. B, tumor cell (left) or fibroblast (right) invasion in coculture in the presence or absence of flow. Left, the cocultured fibroblasts were either mixed throughout the matrix with the tumor cells or present in a contiguous, but separate matrix upstream of the tumor cells. C, effects of Matrigel on flow-enhanced tumor cell (left) or fibroblast (right) invasion. D, effects of cocultured fibroblast density on flow-enhanced invasion of tumor cells. *, P < 0.05 versus matching static condition; #, P < 0.05 between indicated groups (A–C) or versus lowest fibroblast density (D).
even when Matrigel was absent (Fig. 1C); this is in contrast to tumor cells alone, which did not respond to flow without Matrigel (and its heparan sulfate proteoglycans; Fig. 1A). This suggests that fibroblasts enhanced tumor cell invasion via a mechanism independent of tumor cell autologous chemotaxis (17). Finally, fibroblast- and flow-enhanced tumor cell invasion was dependent on fibroblast density being greater than 50,000 cells/mL (Fig. 1D). Fibroblast density had no effect on tumor cell invasion present in static conditions, consistent with the hypothesis that increased tumor cell invasion due to fibroblasts and flow was dependent on fibroblast migration.

Flow- and fibroblast-enhanced tumor cell invasion depends on TGF-β1

Because previous work has shown that interstitial flow can stimulate fibroblast TGF-β1 expression (18) and mechanical stress can activate latent TGF-β1 (25), we hypothesized that flow-enhanced fibroblast invasion involved TGF-β1 activation. When we blocked TGF-β1 activity with a neutralizing antibody, the fibroblast-enhanced response of tumor cells to interstitial flow was abrogated (Fig. 2A). TGF-β1 blocking did not affect tumor cell invasion in the absence of flow or fibroblasts, but only when both were present (Fig. 2A). Similarly, flow-enhanced fibroblast migration was also inhibited by TGF-β1 blocking (Fig. 2A). These results suggest that TGF-β1 was indirectly enhancing tumor cell invasion by directly affecting fibroblast migration in the presence of interstitial flow.

To determine how TGF-β1 affected cell migration, we quantified the concentration and activity of TGF-β1. Total TGF-β1 did not change between static and flow conditions, and was resident in the matrix and produced by fibroblasts (Fig. 2B). Using reporter TMLCs that produced luciferase in response to active TGF-β1, we observed increased TGF-β1 activity in response to interstitial flow, regardless of the cell types present (Fig. 2C). The lower TGF-β1 activation seen in gels containing fibroblasts (Fig. 2C) compared with TMLCs alone was likely due to competition between the 2 cell types for active TGF-β1, because fibroblasts consume TGF-β1. Significantly, increased TGF-β1 activation occurred even when reporter TMLCs alone were present (Fig. 2C), suggesting either that interstitial flow can directly activate latent TGF-β1 (which is present in serum and Matrigel), independent of tumor cells and fibroblasts, or that interstitial flow increased TGF-β1 availability through improved mass transport.

To determine whether increased TGF-β1 was directly inducing tumor cell invasion, we exposed tumor cells to exogenous TGF-β1, either uniformly (Fig. 2D, left) or in a gradient (Fig. 2D, center). In both cases, tumor cell invasion was either insensitive to or inhibited by exogenous TGF-β1. Combined with the lack of an effect when neutralizing TGF-β1 in tumor cells alone (Fig. 2A), this suggested that TGF-β1 was acting primarily on the fibroblasts. Treating fibroblasts with TGF-β1, or tumor cells cocultured with fibroblasts, did not stimulate fibroblast or tumor cell invasion (Fig. 2D, right). Exogenous TGF-β1 combined with interstitial flow elicited a bimodal fibroblast invasion response, with peaks at 0 and 2 ng/mL TGF-β1, and a similar pattern of tumor cell invasion in the presence of fibroblasts (Fig. 2D, right). However, over the range of concentrations tested, fibroblast and tumor cell invasion never exceeded control values, and, in general, TGF-β1 inhibited fibroblast and tumor cell invasion. Biphasic migration responses have been previously reported in both fibroblasts and endothelial cells (26, 27), but these results showed peak migration at an intermediate, optimal concentration of TGF-β1. Given that fibroblasts invaded more under flow than in the presence of TGF-β1 (with or without flow), but neutralizing TGF-β1 completely abolished flow- and fibroblast-enhanced tumor cell invasion (Fig. 2A), we hypothesized that localized, active TGF-β1 gradients, rather than an increase in TGF-β1 concentration, may drive flow-enhanced fibroblast migration. This increase in fibroblast migration, in turn, is correlated with enhanced tumor cell invasion.

Fibroblast- and flow-enhanced tumor cell invasion depends on MMPs

MMPs (secreted by fibroblasts and other cells) are often implicated in tumor invasion (28). We found that the broad spectrum MMP inhibitor GM6001 significantly decreased tumor cell invasion when both interstitial flow and fibroblasts were present, but had no effects in the absence of either fibroblasts or interstitial flow (Fig. 3A). GM6001 also decreased flow-induced fibroblast invasion (Fig. 3A). Previous studies have shown that interstitial flow increases fibroblast motility through upregulation of MMP-1 (20), so we next used DQ collagen to determine whether collagenolysis was altered with flow. Indeed, fibroblasts exposed to interstitial flow exhibited a 70% increase in pericellular collagen degradation, and collagen degradation tended to coincide with increased matrix consolidation around fibroblasts (Figs. 3B and C). However, gelatin zymography revealed minimal levels of active MMP-2/9, which are often implicated in tumor-stromal interactions (29), and no differences in total MMP-2 or MMP-9 activity between static and flow conditions were found, or between tumor cells cultured alone versus with fibroblasts (Fig. 3D). This is consistent with previous reports that MMP-1, and not MMP-2, was responsible for interstitial flow-mediated enhancement of fibroblast motility (20). Thus, interstitial flow not only increased fibroblast invasion via TGF-β1, but also through increased collagen degradation.

Rho-dependent fibroblast contractility drives flow-enhanced tumor cell invasion

Cell contraction is an important mechanism by which fibroblasts alter their surroundings (30). ECM contracture is also important in tumor invasion, because stromal stiffness correlates with tumorigenesis and invasion (8–10), and the ability to deform the ECM is instrumental for tumor cell and fibroblast invasion (11, 21, 31). We inhibited fibroblast contraction pathways by targeting Rho activation with C3 transferase, myosin light chain kinase (MLCK) with ML-7, and nonmuscle myosin IIA with blebbistatin. Rho and MLCK are upstream effectors of fibroblast contraction (32, 33), whereas myosin IIA is the downstream cog in actomyosin interactions (34). We observed significant differences in fibroblast morphology on treatment with C3 transferase and blebbistatin compared with ML-7 (Fig. 4A). C3- and blebbistatin-treated
Figure 2. TGF-β1 is necessary for flow-enhanced tumor cell invasion only when fibroblasts are present. Invasion results are normalized to the leftmost condition in each graph unless otherwise noted. A, effects of TGF-β1 neutralization on MDA-MB-435S (TC) invasion (left) or fibroblast (Fb) migration (right) using a function-blocking antibody (α-TGF-β1 block). B, total TGF-β1 levels as measured by ELISA after 18 hours culture with 5 x 10^5 tumor cells/mL and 2.5 x 10^5 fibroblasts/mL. C, active TGF-β1 levels as measured by TMLC luciferase reporter assay, after 18 hours culture with 2.5 x 10^6 reporter cells/mL. The first condition contains only TMLC reporter cells; all other conditions contain the cells noted plus TMLCs. D, left, effects of exogenous TGF-β1 on the invasion of tumor cells alone. Middle, effects of an exogenous TGF-β1 gradient on the invasion of tumor cells alone. Right, effects of exogenous TGF-β1 on tumor cell invasion (in the presence of fibroblasts) and fibroblast invasion alone. Tumor cell and fibroblast invasion are normalized to their respective flow, no TGF-β1 condition. *, P < 0.05 versus matching static condition; #, P < 0.05 between indicated groups.
fibroblasts showed significantly altered cell morphology, reduced collagen matrix contraction, and few stress fibers (Fig. 4A). In contrast, ML-7–treated fibroblasts exhibited normal morphology and still contracted the matrix (Fig. 4A).

The morphologic differences induced by the different inhibitors matched the tumor cell invasion and fibroblast migration results. When we specifically inhibited Rho activation in fibroblasts with a cell-permeable C3 transferase, the flow response of tumor cells was impaired (Fig. 4B). This effect was independent of fibroblast migration, as C3 treatment actually increased fibroblast migration (Fig. 4B). This was consistent with previous findings showing that Rho/Rho-kinase inhibition can actually increase cell migration (33, 35). Similarly, blebbistatin treatment specifically inhibited only flow- and fibroblast-enhanced tumor cell invasion (Fig. 4C). Like C3 treatment, blebbistatin-treated fibroblasts exhibited increased migration (Fig. 4C). Conversely, ML-7 decreased tumor cell and fibroblast invasion under all conditions (Fig. 4D), showing that MLCK was not specifically necessary for flow- and fibroblast-enhanced tumor cell invasion.

Figure 3. MMP activity is necessary for flow- and fibroblast-enhanced tumor cell migration. A, effects of MMP inhibition on MDA-MB-435S tumor cell (TC) invasion (left) or fibroblast (Fb) migration (right). Invasion is normalized to the leftmost condition in each graph. B, confocal fluorescence images of fibroblasts (red, F-actin) and degraded collagen [green, fluorescein isothiocyanate (FITC)]. Scale bar, 25 μm. C, quantification of FITC intensity (normalized to the static condition) for DQ collagen experiments. D, gelatin zymogram on supernatants from 3D cultures. *, P < 0.05 versus matching static condition; #, P < 0.05 between indicated groups.
invasion. These findings show that Rho-mediated fibroblast contractility is essential to fibroblast- and flow-enhanced tumor cell invasion.

**Fibroblasts mediate ECM reorganization**

To determine whether fibroblast contractility was affected by flow, we used confocal reflectance microscopy to observe the collagen fiber matrix around fibroblasts and tumor cells. We observed fibroblasts contracting ECM fibers in their immediate vicinity in both static and flow conditions (Fig. 5). Fibers aligned with spindle-shaped fibroblasts or with cell processes when fibroblasts adopted a dendritic morphology. Flow itself did not affect ECM contraction by fibroblasts, as determined both microscopically (Fig. 5) and with a collagen gel contraction assay (Supplementary Fig. 4). This shows that two factors—increased fibroblast migration in response to interstitial flow and the fibroblast’s innate propensity for cell contraction—must interact to enhance tumor cell invasion.

In many instances, tumor cells extended processes toward fibroblasts, and in other cases tumor cells and fibroblasts made direct contact (Fig. 5). Tumor cell processes generally extended along the direction of fibroblast-aligned matrix fibers. In live-cell imaging experiments, tumor cells under static and flow conditions showed limited capacity to deform the matrix (Supplementary Movies 1 and 2). When fibroblasts were present, we observed significant ECM displacement, likely due to fibroblast contraction (Supplementary Movie 3).
3). When both fibroblasts and flow were present, the ECM showed significant displacement (Supplementary Movie 4). Notably, the matrix appears more degraded than in other conditions, suggesting that increased proteolysis, as we and others have previously shown (20), is involved in the invasion and remodeling process. We also observed tumor cells interacting with and aligning alongside fibroblasts (Supplementary Movie 4). The changes and interactions in Supplementary Movie 4 were representative. We conclude that fibroblasts locally remodeled the collagen matrix by applying traction forces to their surroundings, causing local changes in matrix density and fiber alignment, and in turn facilitating interactions with tumor cells.

Discussion

The importance of the tumor microenvironment on cancer growth and invasion are well appreciated (1–3), and the stiffness of the tumor stroma has been shown to drive tumorigenesis and invasion (8–10). However, in addition to matrix stiffness, interstitial flow is an important mechanical stress in the tumor stroma (5). By examining the interplay between tumor cells, fibroblasts, and interstitial flow, we showed that flow guides fibroblast invasion, leading to concurrent invasion of MDA-MB-43SS tumor cells through the ECM. Without interstitial flow, fibroblasts did not affect tumor cell invasion.

TGF-β1 regulates a variety of tumor suppressive and promoting effects, including epithelial homeostasis, epithelial-to-mesenchymal transition, myofibroblast differentiation, and metastasis (36). TGF-β1 was necessary for interstitial flow-enhanced fibroblast invasion (Fig. 2A) but only indirectly involved in tumor cell invasion (Fig. 2D). We hypothesize that TGF-β1 may increase fibroblast invasion through both TGF-β1–driven autologous chemotaxis (17, 37) and increased cell motility (38). Because exogenous TGF-β1 did not enhance (and in some cases inhibited) tumor cell and fibroblast invasion, this suggests that flow-generated gradients of TGF-β1, which would be washed out by exogenous TGF-β1, may be crucial. We also determined that interstitial flow leads to an apparent increase in TGF-β1 activation (Fig. 2C). This may be due to the direct effect of interstitial flow, consistent with other findings that fibroblast contraction and fluid flow can activate TGF-β1 (25, 39), which typically involves proteases and integrins (40, 41). Although the shear stress generated by the levels of interstitial flow examined here are likely extremely low (42), matrix fiber organization can strongly increase local shear stresses on cells and their pericellular matrix (43), potentially leading to mechanical activation of TGF-β1. Interstitial flow may also increase the availability of TGF-β1 by facilitating transport of active growth factor, which would likewise lead to an apparent increase in TGF-β1 activity. Thus, it is likely that the flow-enhanced fibroblast invasion was caused by increased TGF-β1 activation or improved transport, leading to increased fibroblast motility and/or chemotaxis.

Collagen degradation was also necessary for flow-induced fibroblast invasion and concurrent tumor cell invasion (Fig. 3A). Consistent with previous work with adventitial
fibroblasts, myofibroblasts, and smooth muscle cells (20), we found increased pericellular collagen degradation by fibroblasts in response to flow (Fig. 3B). Although collagen proteolysis may be important primarily for cell motility (20), it may also facilitate ECM remodeling, as has been previously shown with fibrosarcoma and breast carcinoma cells (44).

Our findings suggest that, similar to prior work with other tumor cell types (31, 45), fibroblasts may locally remodel the matrix via Rho-dependent mechanisms as they migrate through the ECM, priming the matrix such that invading tumor cells invade much more rapidly. Fibroblasts control ECM remodeling (7, 12) partly by exerting traction on the ECM via Rho-dependent contractility (30, 32). When fibroblasts contract, fibers align with their pseudopodia and stress fibers (30). Previous studies have shown that Rho and MLCK play nonredundant roles in regulating myosin activation: Rho and Rho kinase are involved in central stress fiber formation and cell contraction, whereas MLCK affects stress fibers at the cell periphery and cell motility (32, 33, 46, 47). Inhibition of Rho and myosin IIA actually increases cell motility (33–35); this is likely due to the fact that Rho promotes stress fiber formation for traction force generation, whereas migration may require more selective adhesion and less force exertion. These results corroborate our findings that Rho, but not MLCK, was necessary for fibroblast matrix contraction and subsequent matrix priming (Fig. 4B–D). Rho and nonmuscle myosin IIA inhibition increased fibroblast invasion even while simultaneously decreasing fibroblast- and flow-enhanced tumor cell invasion (Fig. 4B and C). These data also suggest that fibroblast migration is necessary, but not sufficient, to enhance tumor cell invasion. Fibroblasts must also remodel the matrix through Rho-dependent cell contraction; thus, if fibroblasts are migrating more but contracting less as they move (as shown in Fig. 4), they would be unable to enhance tumor cell migration.

On the basis of our findings, we propose a mechanism in which interstitial flow drives TGF-β1- and MMP-dependent fibroblast invasion, during which fibroblasts remodel the ECM through Rho-mediated cell contractility to prime the microenvironment for tumor cell invasion (Fig. 6). Fibroblasts secrete latent TGF-β1; fibroblast contraction and interstitial flow may enhance TGF-β1 activation, and interstitial flow can also increase the availability of TGF-β1. When TGF-β1 is activated pericellularly and nonuniformly redistributed due to interstitial flow, this drives fibroblast chemotaxis in the flow direction. Interstitial flow also stimulates fibroblast collagenolysis, leading to increased cell motility (20). Simultaneously, tumor cells invade the matrix by CCR7-dependent autologous chemotaxis (17); thus, both fibroblasts and tumor cells are invading concurrently but independently. As the fibroblasts invade, they also apply traction forces to the collagen matrix, creating local lines of tension resulting in alignment of collagen fibers and increased matrix density near the fibroblast (Fig. 5). Previous work has shown that active fibroblast contraction, combined with the nonlinear properties of ECM and local anisotropy, creates mechanical gradients, stiffens the matrix both locally and globally, and transmits forces between cells up to a distance of 500 μm (48). This is supported by time-lapse confocal images (Supplementary Movies 3 and 4), in which fibroblasts generate matrix deformation and prime the microenvironment for tumor cell invasion, which depends on collagen alignment, matrix density, and stiffness (11, 21, 31, 49, 50). Importantly, we did not observe increased fibroblast contraction by interstitial flow; however, fibroblasts are already sufficiently contractile under static conditions to meaningfully remodel the ECM (Fig. 5; Supplementary Movies 3 and 4). Instead, the key event in the proposed model is stimulation of increased fibroblast invasion by interstitial flow. When fibroblasts migrate more, they effectively increase their range of influence, encountering more tumor cells and coincidentally priming the local matrix to encourage tumor invasion.

In summary, we present a novel mechanism whereby the biomechanical force of interstitial flow enhances fibroblast invasion and leads to the priming of the matrix microenvironment, making it more permissive for tumor cell invasion. Interstitial flow acts as a biomechanical “switch,” inducing fibroblasts to prime the stroma for tumor invasion. Thus, interstitial flow can drive the invasive behavior of tumor cells, not only through autologous chemotaxis (17) but also by changing the nature of tumor–fibroblast interactions. These findings expand our current view of the tumor microenvironment, which not only should encompass cells, soluble factors, and the ECM, but also must include

![Diagram of tumor cell invasion](image_url)
biomechanical forces like interstitial flow and how these forces interact with the tumor and the other facets of the microenvironment.

**Disclosure of Potential Conflicts of Interest**

The authors have no conflicts of interest to disclose.

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