Mechanistic adaptability of cancer cells strongly affects anti-migratory drug efficacy

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Cancer metastasis involves the dissemination of cancer cells from the primary tumour site and is responsible for the majority of solid tumour-related mortality. Screening of anti-metastasis drugs often includes functional assays that examine cancer cell invasion inside a three-dimensional hydrogel that mimics the extracellular matrix (ECM). Here, we built a mechanically tuneable collagen hydrogel model to recapitulate cancer spreading into heterogeneous tumour stroma and monitored the three-dimensional invasion of highly malignant breast cancer cells, MDA-MB-231. Migration assays were carried out in the presence and the absence of drugs affecting four typical molecular mechanisms involved in cell migration, as well as under five ECMs with different biophysical properties. Strikingly, the effects of the drugs were observed to vary strongly with matrix mechanics and microarchitecture, despite the little dependence of the inherent cancer cell migration on the ECM condition. Specifically, cytoskeletal contractility-targeting drugs reduced migration speed in sparse gels, whereas migration in dense gels was retarded effectively by inhibiting proteolysis. The results corroborate the ability of cancer cells to switch their multiple invasion mechanisms depending on ECM condition, thus suggesting the importance of factoring in the biophysical properties of the ECM in anti-metastasis drug screenings.

1. Introduction

Metastasis is the leading cause of cancer mortality, accounting for 90% of cancer deaths in patients with solid tumours [1]. Therefore, understanding the mechanisms of cancer spread and developing anti-metastasis drugs have the potential to greatly improve cancer treatment. Metastatic cancer cells show upregulated motility [2], which enables cell migration and invasion through tumour stroma, followed by intravasation into and extravasation out of the blood vessels. Recently, tumour therapeutic targets have expanded into the non-cytotoxic domain with the objective of curbing cancer metastasis [3], in addition to the traditional chemotherapy and radiotherapy approaches. To screen for such non-cytotoxic drugs that can specifically inhibit pathological cell movement, it is necessary to develop a functional assay that can quantify the extent of cell migration and invasion through the tissue environment under the influence of these drugs [4,5].

Conventionally, cell migration assays that allow dynamic cell tracking and quantitative analysis were conducted on two-dimensional substrates. Although the flat and artificially stiff surfaces in two-dimensional monolayer cell culture are simple to set up, they are far from sufficient to emulate the physical and biochemical complexity and dynamics in vivo, where cells typically face a complex three-dimensional microenvironment, consisting of heterogeneous, fibrous networks of extracellular matrix (ECM) and other types of coexisting cells [6]. As such, three-dimensional models have been increasingly developed for modelling cancers [7] and testing therapeutics [8], as these models offer the potential to maximize the physiological relevance of in vitro cell culture, and better simulate the
spatio-temporal gradient of cues for cell migration found in the native tissue context. Indeed, three-dimensional cancer models have shown numerous advantages in terms of recapitulating cancer malignancy in vitro. For example, molecular approaches to revert abnormally regulated genes in cancer cells were found to be only effective in three-dimensional cultures but not on two-dimensional substrates [9]. Moreover, tracking of cancer malignancy progression showed that cell traction, adhesion and migration were correlated to cancer cell malignancy in three-dimensional culture but not on two-dimensional substrates [10].

To date, in vitro three-dimensional cell culture models have been developed for cell-based tests of drug safety, toxicity [11] and anti-viral effects [12]. But further assay characterizations and developments are still required for reliable testing of drugs targeting specific cell functions, such as migration. There is clear in vivo evidence that the ECM in different tissue locations exhibits diverse structural features [13]. Therefore, therapeutic measures against cancer cell migration must be examined in a variety of tissue-like contexts. Technically, it is possible to modulate many aspects of the three-dimensional culture models in order to simulate the complex tissue-like environments [14], through varying biophysical and biochemical properties that cells experience within scaffolds built with different methods and materials [15]. Such in vitro approaches have provided important insights for understanding cancer migratory behaviour. In particular, tissue mechanics has been reported to influence cancer cell growth, morphological development [16] and malignancy development [17,18]. Moreover, it has been found that cell motility depends on matrix mechanics [19], and, additionally, migrating cells significantly deform and remodel the surrounding hydrogel matrix [20], thus actively altering the physical [21] and mechanical [22] properties of their immediate microenvironment. But, at the same time, there is a surprising lack of knowledge on the interplay between metastasis-targeting drug effects and ECM mechanics. It has recently been acknowledged that proper modulation of the mechanical milieu is important in cell-based drug screening [23], and that the stiffness of the two-dimensional substrate significantly influences cancer and smooth muscle cells’ responses to drugs [24]. Because many anti-metastasis drugs antagonize specific factors influencing cell motility, it is important to assess the role of matrix mechanics in three-dimensional anti-metastasis drug assays that often involve cell motility measurement.

Here, we attempt to answer whether and to what extent the physical and mechanical factors of the three-dimensional substrate play a role in the effectiveness of anti-metastasis drugs. Among a number of biomaterials adopted as three-dimensional culture models, collagen is a widely used model for examining cancer cell invasive migration [25], partly because collagen is the major mass component of the stroma ECM and also largely determines the tensile strength of tissues [26]. In addition, collagen has important pathological implications, as altered production and regulation of collagen were found during cancer progression [27]. We built a mechanically tuneable collagen-based ECM equivalent and evaluated cancer cell invasion under the influence of a panel of compound drugs that interfere with known cell migration mechanisms. Strikingly, with varied ECM conditions, we find that highly metastatic breast cancer cells, MDA-MB-231, show differentiated levels of sensitivity to the pharmaceutical intervention of cell migration. Our results corroborate that biophysical factors of the ECM need to be carefully considered in developing anti-metastasis drug assays and in interpreting results from these assays. Moreover, our study also provides guidance for the design of three-dimensional cell culture models for effective drug screening.

2. Material and methods

2.1. Cell culture and three-dimensional invasion assay

Malignant human breast cancer cell line MDA-MB-231 was maintained in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech), supplemented with 10% FBS and 1% penicillin/streptomycin, under 37 °C humidified atmosphere with 5% CO₂. Nutagen collagen solution (6.4 mg ml⁻¹, pH approx. 2) was obtained from Inamed Biomaterials (Fremont, CA, USA). The main components were bovine collagen-I (97%) and collagen-III (3%). All other chemical was obtained from Sigma-Aldrich (St Louis, MO, USA), unless specified otherwise.

To set up the three-dimensional invasion assay, the collagen solutions were prepared according to the protocol provided by the manufacturer (Inamed Biomaterials, 2006). The collagen solution was adjusted to the desired pH with NaOH and mixed with 10 × PBS. Cells were harvested through trypsin/EDTA treatment and homogeneously resuspended in the prepared collagen solution on ice. The final mixture contained 1.5 million cells per millilitre and 2.5 mg ml⁻¹ collagen, from which 20 µl were transferred to the centre of the well in a glass-bottom dish (MatTek, Ashland, MA, USA). Incubation for 45 min in 37 °C humidified chamber led to the self-assembly of a semi-spherical cell-seeded collagen gel (‘inner gel’). A total of 180 µl of cell-free collagen solution was next added to cover the inner gel and polymerized under the same conditions to form a dome-shaped acellular ‘outer gel’. The microstructure and mechanical properties of the outer gel were varied by varying the collagen concentration (1.5, 2.5 or 4.0 mg ml⁻¹) and the polymerization pH (6.0, 7.4 or 9.0). The geometrical configuration of the concentric two-gel model is illustrated in figure 1a. One hour after polymerization, 2 ml phenol-red-free DMEM (GIBCO, Life Technologies Corporation, Carlsbad, CA, USA) containing 10% FBS and 1% penicillin/streptomycin was added to the dish, and the medium was refreshed every 3 days.

2.2. Characterization of gel mechanics

The mechanical properties of the collagen gels were measured by performing oscillatory shear rheology using an AR-G2 rheometer (TA Instruments, New Castle, DE, USA). The gels were polymerized in situ by loading collagen solution onto the bottom plate and bringing down the 60 mm steel top plate to a final gap size of 250 µm. Gel loading was performed immediately before the series of measurement procedures, which included a 40 min time-sweep at 1% strain, a frequency-sweep at 10% strain with the angular frequency ω ranging from 2π × 10⁻³ to 100 rad s⁻¹ and a strain-sweep at ω = 1 rad s⁻¹, where the amplitude of shear strain was logarithmically increased from 1% to the level causing sample failure. The plates were maintained at 37 °C throughout the tests.

2.3. Live cell imaging

Cells were allowed to invade the acellular outer gel in the first 7 days of culture, during which the cells spread radially outward. Afterwards, we carried out time-lapse confocal microscopy on eight volumes-of-views (VoVs) in the outer gel, each measuring X × Y × Z = 647 × 647 × 100 µm³, which were distributed around the circular periphery of the spreading cell population with uniform angular intervals in between (figure 1b). All
imaging of cells in hydrogel was conducted with a 20× dry Apochromatic lens (NA = 0.75) on an inverted microscope, Nikon TE-2000 C1 system (Nikon, Tokyo, Japan) equipped with confocal laser scanning microscopy. For confocal fluorescence imaging, cells were labelled in situ with 5 μM fluorescence CellTracker orange CMTMR (Molecular Probes, Life Technologies, Carlsbad, CA, USA). Samples were kept at 37°C and 5% CO2 atmosphere throughout the 8 h of imaging using a custom-built chamber mounted on the microscope.

2.4. Cell morphology and track analysis
To analyse cell morphology, Z-stack images were linearly processed using NIS-Elements AR (Nikon, Tokyo, Japan) through background adjustment and maximum intensity projection. Contrast enhancement and image segmentation necessary for three-dimensional cell tracking were performed in Imaris (Bitplane, Zurich, Switzerland). Using the built-in tracking algorithm in Imaris, three-dimensional cell tracks (figure 1c) were generated based on the Z-stacks of time-lapse confocal fluorescent images with close human supervision. To eliminate random and system noise, cell tracks shorter than 3500 s were discarded, and all tracks were corrected for overall drift whenever necessary.

The cell migration tracks were quantitatively parametrized in terms of several geometrical metrics (figure 1d). Euclidean distance (ED) that measures the net displacement of a cell track was defined as the length of the vector directly connecting the start and end of the cell paths, whereas accumulative distance (AD) was calculated by summing up the lengths of cell movement vectors in each time step. These are mathematically represented as

$$ED = |\Delta r| = |d_i - d_{i-1}|$$

and

$$AD = \sum_{n=0}^{n-1}|d_{n+1} - d_n|$$

respectively, where i is an arbitrary time point in the n time points in the tracks, and $d_i$ is the cell position vector. The cell migratory behaviour was further characterized using two indices representing migration speed and directionality. Cell speed $S$ was calculated from AD using $S = AD / T = (\sum_{n=1}^{n-1}|d_{n+1} - d_n|)/(n \cdot \Delta t)$, where $T$ is the total duration of the track and $\Delta t$ is the time interval, which was fixed at 10 min in this study. To characterize cell migration directionality, cell track straightness $k$ was calculated as $k = ED / AD$. With this definition, an upper-limit $k$ value of 1 represents a straight track, whereas $k = 0$ corresponds to a cell movement without net displacement.

2.5. Pharmaceutical interventions of cell migration
On the seventh day, after cells have spread significantly into the outer gel, four drugs targeting four different cell migration mechanisms were added to the complete culture media. For inhibition of actomyosin contractility, cells were treated with 20 μM Y27632 (Santa Cruz Biotechnology, Inc., San Francisco, CA, USA), a Rho-associated protein kinase (ROCK) inhibitor. To interrupt filamentous actin (F-actin) polymerization, we used 2 μM cytochalasin D (Sigma-Aldrich). A broad-spectrum matrix metalloprotease (MMP) inhibitor, 25 μM GM6001 (or galardin) (Enzo Life Sciences International, Inc., PA, USA), a Rho-associated protein kinase (ROCK) inhibitor. To interrupt filamentous actin (F-actin) polymerization, we used 2 μM cytochalasin D (Sigma-Aldrich). A broad-spectrum matrix metalloprotease (MMP) inhibitor, 25 μM GM6001 (or galardin) (Enzo Life Sciences International, Inc., PA, USA) was added to interfere with proteolysis of collagen. Lastly, 2 μM nocodazole (Sigma-Aldrich) was applied to disrupt microtubules stability. The latter three drugs were dissolved in DMSO to prepare stock solutions and were further diluted 1000 times in the medium. We also analysed cell migration in the presence of an
empty vehicle as a control, where cells were supplied with normal medium containing 0.1% DMSO. An hour after each drug was administered, cell morphology was examined and cell migration in the gel was monitored by confocal microscopy in the following 8 h in the presence of the drug.

2.6. Statistical analysis

The drug-treated cell movement was compared to the respective control cases in the same collagen condition to detect drug-induced changes in $S$ and $k$. As the data were not normally distributed, we employed Mann–Whitney $U$ tests for the paired drug-control data. For each condition, data were obtained from multiple cell tracks ($n > 450$) obtained from more than three independent experiments.

3. Results

3.1. Mechanical characterization of collagen matrices

In this study, by varying two factors during collagen gel polymerization, namely, collagen concentration and pH condition, we modulated ECM microstructure and stiffness to create five conditions of three-dimensional collagen gel. Hereinafter, we denoted these five gel conditions by their polymerization pH (for brevity, gels formed at pH 7.4 will hereafter referred to as ‘pH 7’) and collagen concentration. The self-assembled collagen gels were predominantly elastic, as evident from the low phase angle ($\delta = \tan^{-1}(G''/G') < 20^\circ$), and exhibited weak frequency dependence (figure 2a), consistent with previous report [28]. Furthermore, rheological characterization showed that, just by modifying the two polymerization conditions, we were able to tune the elastic modulus $G'(\omega)$ of the gels over almost two orders of magnitude, ranging from a few Pa to approximately 100 Pa (table 1). This magnitude range of the measured elastic modulus was consistent with previous reports on collagen-I gels polymerized in similar conditions [31,32]. In general, the gel becomes stiffer as the concentration and the polymerization pH were increased.

The dramatic variations in the gel stiffness resulted from alterations in the density and microstructure of the collagen networks. It must be noted, however, that varying collagen concentration changes both matrix porosity and stiffness at the same time. While it has been shown that cell spreading, translocation and migration are differentially regulated by matrix stiffness and porosity [33], how cancer cell invasive behaviour is differentially affected by these factors has not been thoroughly studied. To fill this gap, we looked for combinations of collagen concentration and polymerization pH that result in comparable rheological behaviour. By varying polymerization pH and fixing collagen concentration at 2.5 mg ml$^{-1}$, we essentially altered the network porosity (table 1) without changing protein content. We found that collagen polymerized at pH 9 formed a dense network with small (1.7 ± 0.1 $\mu$m) pore size and showed comparable mechanical behaviour to a high-concentration (4 mg ml$^{-1}$) gel formed at pH 7, whereas collagen polymerized at pH 6 exhibited large (4.9 ± 0.2 $\mu$m) pore size and similar mechanical behaviour to a low-density (1.5 mg ml$^{-1}$) gel (figure 2d). By making use of these five collagen conditions, we can thus delineate the effect of matrix stiffness and porosity on the cancer cell migration behaviour.

We also performed strain-sweep measurement of the collagen gels up to levels of strain causing sample failure, and found that the response is highly nonlinear (figure 2b). More specifically, at small strains (less than 10%) the gels soften slightly with an increase in strain, followed by a rapid increase in the elastic modulus with strain. Both strain-softening [31] and strain-stiffening [31,34] responses have been previously reported in collagen networks, although the microstructural origins are still under intense investigations. Importantly, these observations imply that collagen gels are nonlinear elastic materials [35], implying that its mechanical property can be altered by the amount of deformations in the gels. This is particularly relevant in three-dimensional cell cultures, as cells are known to deform their surrounding matrices through

![Figure 2](http://rsif.royalsocietypublishing.org/)

**Figure 2.** Mechanical characterization of self-assembled collagen gels. (a) The frequency dependence of the elastic modulus $G'(\omega)$ of the collagen gels with different polymerization conditions. (b) The nonlinear softening and stiffening of collagen gels, measured for gels polymerized at pH 7 at $\omega = 1$ rad s$^{-1}$. (Online version in colour.)

**Table 1.** Collagen gel properties. All values are mean ± s.d.

<table>
<thead>
<tr>
<th>condition</th>
<th>$G'$ (Pa)</th>
<th>$\delta$ (°)</th>
<th>pore size ($\mu$m) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH7—4 mg ml$^{-1}$</td>
<td>74.4 ± 1.2</td>
<td>6.5 ± 0.3</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>pH9—2.5 mg ml$^{-1}$</td>
<td>61.0 ± 2.7</td>
<td>10.9 ± 0.3</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>pH7—2.5 mg ml$^{-1}$</td>
<td>20.1 ± 1.0</td>
<td>11.4 ± 0.5</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>pH6—2.5 mg ml$^{-1}$</td>
<td>5.7 ± 0.3</td>
<td>13.1 ± 0.7</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>pH7—1.5 mg ml$^{-1}$</td>
<td>6.1 ± 0.3</td>
<td>11.3 ± 0.9</td>
<td>4.5 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$Obtained using bubble analysis [29,30] from confocal reflectance images of unlabelled, hydrated collagen samples.
contractile forces [20], often in the stress and strain levels corresponding to the nonlinear regimes of the gels.

3.2. Cell migration in different collagen matrices

We used the above five gel conditions to test the role of biophysical factors in the ECM on cell migration by seeding highly invasive cancer cells and examining the migratory behaviour of the cells in these three-dimensional collagen matrices. After 7 days of three-dimensional culture, the cells had spread to the outer gel. We then tracked the migration of these cells in the outer gel and quantitatively analysed the trajectories in terms of the migration speed $S$ and directionality $k$. The cell speed distribution was slightly right-tailed, with an average speed of approximately 13 $\mu$m h$^{-1}$ and a maximum of 72 $\mu$m h$^{-1}$. Comparing the results obtained with different outer gels, we found that the migration pattern did not vary dramatically with collagen conditions, and generally cell speed was only slightly reduced as the collagen concentration and polymerization pH increased (figure 3$a$–$b$). It is worth noting that the peak of $S$ in the highest collagen density (4.0 mg ml$^{-1}$) was lower than in other ECM conditions, indicating the larger fraction of cells that moved more slowly in 4.0 mg ml$^{-1}$ collagen. In addition, the percentage of fast-moving cells (defined as $S > 15$ $\mu$m h$^{-1}$) was the lowest in the pH7—4.0 mg ml$^{-1}$ gel, whereas the pH7—1.5 mg ml$^{-1}$ and pH6—2.5 mg ml$^{-1}$ gels had the highest percentage of such tracks. To check whether this was a result of the difference in outer gel stiffness, we plotted $S$ as a function of the linear elastic modulus $G$ as measured using rheology. The plot (figure 3$c$) reveals a slight negative correlation between $S$ and $G$, indicating that cell speed was weakly influenced by matrix stiffness.

In addition to the cell speed, we also quantified the migrational directionality of the individual cells using the order parameter $k$. In denser matrices, cells were likely to encounter physical barriers more frequently, and this could lead to more frequent changes of direction and therefore lower directional persistence. We plotted the probability distribution function (PDF) of $k$ for the different outer gel conditions in figure 3$d$–$e$. The distributions were very similar for all outer gel conditions except for the pH7—4.0 mg ml$^{-1}$ gel, where most of the cell tracks exhibited low straightness ($k < 0.25$). Indeed, when plotted against gel stiffness (figure 3$f$), there was no consistent trend in $k$, in contrast to our earlier result on cell speed. These results confirm our supposition that high collagen density is correlated with low migration directionality. Indeed, from the trajectories of each migrating cell, we observed that cell directional persistence was lowest in the pH7—4.0 mg ml$^{-1}$ gel compared with other conditions.

3.3. Matrix-dependent drug effects

Several molecular mechanisms are known to be involved in cell migration machinery, including ROCK-mediated cytoskeletal (actin and microtubule) reorganization and protease-dependent remodelling of the matrix. Aiming at antagonizing these cell motility mechanisms, we selected the following four small molecule compounds (MW < 500 g mol$^{-1}$) for optimal diffusion-based delivery in three-dimensional collagen gel.

3.3.1. Y27632

Rho family members are involved in the formation of focal adhesion complexes, actin stress fibres, lamellipodia and filopodia—essential instruments in promoting cell migration [36–38]. We found that inhibition of ROCK by Y27632 lead to a significant decrease in cancer cell migration speed in all collagen matrices tested (figure 4$a$). This is consistent with recent observations for epithelial cells [39] and fibroblasts [40] in three-dimensional...
3.3.2. Cytochalasin D

Actin protrusion, in conjunction with the subsequent integrin-mediated adhesion and myosin-dependent contraction, forms the basis of cell migration mechanism on two-dimensional surfaces [41] and has been suggested to be critical in three-dimensional matrices [42]. Our assays showed that inhibition of F-actin polymerization by cytochalasin D resulted in a significant decrease in three-dimensional cancer cell migration speed for all collagen matrices tested (figure 4b), confirming the crucial role of F-actin in three-dimensional cell migration and cancer invasion. Similar to the response to Y27632 treatment, some cells exhibited dendritic morphology, where collagen gels. The treated cells exhibited reduced cytoplasm spreading due to the more relaxed cytoskeletal tension, but with exaggerated cellular processes (arrowheads in figure 5), which resulted in dendritic morphology. Interestingly, cell track straightness was only significantly affected in three of the five gel conditions. More specifically, the most pronounced difference was found between the track straightness of control cells and the treated cells in the pH6—2.5 mg ml⁻¹ and pH7—1.5 mg ml⁻¹ gels. These gels had the sparest matrices compared to the other gel conditions, suggesting that contact guidance by the fibrillar matrix may play a role in ROCK-independent migration strategy of the cancer cells.
curvy processes at the ends of the cell body were no longer aligned with the main axis of cell body (two-way arrows with dashed lines in figure 5). However, the cell track straightness was only affected in two out of the five gel conditions. In contrast to the result for Y27632 treatment, cytochalasin D affected cell track straightness in the densest matrices, namely, the pH9—2.5 mg ml\(^{-1}\) and pH7—4.0 mg ml\(^{-1}\) gels.

While the molecular basis for how inhibition of actin polymerization differentially affects the migration phenotype of the cancer cells is unclear, the discrepancy with the response to ROCK inhibition indicates that direct perturbation to the actin polymerization machinery affects different migration mechanisms of the cells.

### 3.3.3. Nocodazole

In addition to actin, microtubules are also part of the cell cytoskeleton, which are believed to be important for establishing and maintaining cell polarity, and thus cell migration efficiency and directionality [43]. Previous studies have found that nocodazole-induced depolymerization of microtubules disrupts tubulin polarity and cell-generated forces [44], and results in decreased migration velocity in cell-derived matrix [40]. In our assays, destabilization of microtubules resulted in significant cell rounding (figure 5), but we did not find any general trend on how nocodazole treatment affected the migration speed of the cancer cells (figure 4c). By contrast, the cell track straightness in the three sparsest matrices (i.e. the pH7—2.5 mg ml\(^{-1}\), pH7—1.5 mg ml\(^{-1}\) and pH6—2.5 mg ml\(^{-1}\) gels) was clearly decreased, consistent with the hypothesized loss of cell polarity in the absence of stable microtubule network. It is interesting to note that both the cell speed and directionality in the pH7—4.0 mg ml\(^{-1}\) matrices were intriguingly increased, although we did not observe a marked difference in the qualitative migration behaviour compared to the cells in other matrices. This result could be an indication that under certain mechanochanical conditions cancer cells can modify their migration strategy and turn it to their advantage.

### 3.3.4. GM6001

One important cell migration strategy is proteolytic migration, where cells secrete active proteases, such as MMPs [45], to degrade the surrounding ECM and create macroscopic cavities that allow for their movement. Collective migration of cancer cells into dense matrices were reported to require localized MMP activity [46], although its necessity has been debated [39,46–48]. In our assays, we found that inhibition of MMP by the broad-spectrum MMP inhibitor GM6001 led to cell rounding (figure 5). A consistent decrease in both cell speed and track straightness was only found in the densest and stiffest gels (i.e. pH7—4.0 mg ml\(^{-1}\)), while the responses in other gels were less consistent (figure 4d). This result can be understood by considering that MMP-dependent migration is most needed when the cells are trapped in the network mesh and face a physical barrier from the surrounding fibres. In the pH9—2.5 mg ml\(^{-1}\) gels, although the matrix might also be dense, the self-assembled fibres were thin and may be physically remodelled by the cells without requiring proteolytic mechanisms. These results further emphasize the need to consider the matrix microenvironment when analysing cell migration data.

### 3.4. The efficiency of anti-migratory drugs in different gel conditions

We now compare the efficiencies of the different drugs in limiting cell migration within the same gel conditions. The drug-induced changes in cell migration speed \(\Delta S\) and in cell track straightness \(\Delta k\) with respect to the control are shown in figure 6 for the five gel conditions. In gels of intermediate density (pH7—2.5 mg ml\(^{-1}\)), cytochalasin D and Y27632 significantly reduced \(\Delta S\) by 60% and 33%, respectively, whereas...
nocodazole and GM6001 did not lead to more than approximately 10% reduction. Interestingly, treatments of nocodazole and GM6001 caused reductions of $k$ by 49% and 29%, respectively, considerably larger than the effects of cytochalasin D and Y27632. This result suggests that the two groups of drugs affect different pathways of cell migration strategies. Disruption of Rho-mediated actin structure formation and turnover impacts the cell’s contractility-dependent migration, and disruption to the cell’s microtubule dynamics or proteolytic activity affects the cell’s migration only indirectly by reducing directional persistence.

A similar effect on the cell migration speed was found in the pH9—2.5 mg ml$^{-1}$, pH7—1.5 mg ml$^{-1}$, and pH6—2.5 mg ml$^{-1}$ gels: cytochalasin D and Y27632 were able to reduce cell speed by 40–70%, whereas nocodazole and GM6001 did not cause more than 20% reduction. In addition, the effects of Y27632, nocodazole, and GM6001 on cell directionality were very similar in the two softest and sparsest gels (i.e. the pH7—1.5 mg ml$^{-1}$ and pH6—2.5 mg ml$^{-1}$ gels). In these gels, Y27632 and nocodazole decreased $k$ drastically, whereas GM6001 did not cause significant reduction of $k$. Interestingly, cytochalasin D treatment led to an increase of cell track straightness in the sparsest gel (i.e. the pH7—1.5 mg ml$^{-1}$ gels) by almost 60%. This suggests that disrupting F-actin polymerization can in fact help cells to migrate in a directionally more persistent manner in certain microenvironments (e.g. sparse matrix), possibly by forcing them to assume another, matrix-dependent advantageous molecular mechanism.

In the gels of highest collagen density (4.0 mg ml$^{-1}$), the results were markedly different from those in other gels. Inhibition of MMPs activity by GM6001 reduced $S$ and $k$ by 36% and 29%, respectively, making GM6001 the most effective anti-migratory drug in this gel condition. This result indicates that protease-dependent degradation of the collagen matrix, which may not play as important a role in sparser gels, is crucial for effective cell migration in these dense collagen gels, since the network mesh size decreases with increasing collagen concentration. Surprisingly, introduction of nocodazole resulted in the complete opposite effect, i.e. it increased $S$ and $k$ instead of inhibiting cell migration. Most notably, the cell track straightness was increased by a factor of two, as indicated by the increase of almost 100% in $\Delta k$. One possible explanation for this result is that the disruption of microtubules leads to an increase in cell polarity and contractile force [49], and such increases in force can overcome the increased matrix stiffness in these dense gels. Alternatively, the cells may use a different migration strategy (e.g. more amoeboid-type migration) when the cytoskeletal rigidity is compromised to be able to squeeze through the smaller pores in these gels.

Importantly, our results provide clear evidence that the effects of anti-migratory drugs targeting different pathways in cell migration can vary considerably in different matrices. It is noteworthy that, in the four matrices with moderate-to-low gel density (concentration of 2.5 mg ml$^{-1}$ or lower), cytochalasin D and Y27632 performed consistently...
in reducing $S$ by at least 30%. Overall, in the range of conditions tested, cells in softer gels reacted more sensitively to drugs that perturb cytoskeleton systems for the regulation of cell motility, including actomyosin-controlled contractility, actin polymerization and microtubule stability. In gels of higher collagen density, however, MMPs inhibitor effects were more significant.

4. Discussion

While it is generally believed that matrix mechanics plays a significant role in cell functions, it has been increasingly appreciated that other spatio-temporally varying biophysical and biochemical factors of the ECM can also influence cell migration as well as cancer cell invasiveness [50,51]. In the range of ECM conditions tested, we observed weak dependence of cancer cell motility on matrix mechanics without drug treatment. This could be due to a number of reasons. A previous study of normal and transformed fibroblasts has reported that the growth and survival of transformed cells are less sensitive to substrate flexibility [52], suggesting that cancer cells possess better mechanical adaptability to survive and proliferate in a wider range of conditions in vivo. Similarly, our observation that the three-dimensional migration of highly metastatic cells are not much affected by gel mechanics in the tested range may also indicate that these cells have developed a robust migratory mechanism that is less environment-dependent, thus giving them 'migration advantage'. This is especially relevant in vivo, as metastatic cells must migrate through a wide range of microenvironment with highly heterogeneous collagen microarchitecture, with fibre spacing varying from 10 to 1000 $\mu$m$^2$ [13].

We further speculate that the robust migration ability of MDA-MB-231 cells lies in the flexibility of the cells to employ multiple strategies to adapt to different ECMs. For instance, in a very dense ECM a cell can rely on enzymatic digestion of the surrounding matrix to provide space for movement, whereas in a less dense ECM environment it may primarily use contractile forces to deform the matrix to compensate for the low matrix stiffness. Indeed, there have been reports that the cell-exerted traction forces may change the local matrix mechanical properties, making it significantly different from the bulk property of the gel [22,53,54]. In addition, as little as 30% strain can already stiffen 1.5 mg ml$^{-1}$ collagen matrix by twofold (figure 2b), owing to the nonlinear properties of collagen. Therefore, strain-stiffening mechanisms may also provide a means by which cancer cells can actively modulate their immediate microenvironment and optimize it for their survival and invasion. Another possible explanation is the dual role that collagen density plays in influencing cell migration. The stiffness of collagen gels is generally larger in denser gels, i.e. gels with smaller pores, and studies have reported close correlation between matrix mechanics and cell motility [55,56]. However, the reduction of ECM pore size simultaneously increases steric barriers for moving cells [19], thus impeding three-dimensional cell migration [56,57]. We also note that cell contractile forces are known to result in a denser matrix immediately surrounding the cells, as observed in our study. Therefore, to a certain extent, the interplay between these biophysical factors and cellular remodelling can potentially compensate one another, resulting in an ECM microenvironment that is amenable for effective invasion by cancer cells.

The variety of cell migration mechanisms and the apparent flexibility with which cells can switch from one mechanism to another led us to hypothesize that the effectiveness of anti-migratory drugs on cancer cell invasive behaviour may be affected by ECM conditions. In particular, depending on the ECM contexts, different cell migratory strategies and cellular factors may be activated to different extents, and thus different cellular mechanisms (e.g. matrix degradation, cell contractility, actin polymerization or microtubules stability) targeted by the drug may function as the leading pro-migratory factor in different gel conditions. Indeed, we discovered that the effectiveness of anti-migratory drugs is sensitive to the biophysical conditions of the three-dimensional cell culture environment, as modulated through the various collagen gel preparation methods used in this study. For example, inhibiting MMPs activity is more effective in high-density collagen, suggesting that cells rely more on MMPs-mediated matrix degradation to migrate in denser networks; whereas disrupting actomyosin-mediated force generation mechanisms limited cell migration speed more significantly in gels of moderate-to-low density. It is imperative that such mechanistic adaptability be systematically and biochemically verified, for us to gain better understanding of three-dimensional cell migration in general, and cancer metastasis and its potential pharmaceutical interventions in particular.

Our results relate well with previous reports, and in fact may provide an explanation for the seemingly contradictory findings in the literature. First, the effectiveness of MMP inhibition is low in gels of low collagen density or with large pore size, in support of the hypothesized ‘protease-independent motility’ [13,56]. In this case, cell movement is not sterically hindered and cells primarily employ actomyosin contractility to deform the cell body and the surrounding ECM fibres to make paths for migration [35,45]. Indeed, we found that, in the collagen gels with large (approx. 5 $\mu$m) pores, cell migration was more significantly retarded with the inhibitors of actin polymerization or myosin motor activity, compared with the effects of other drugs. On the other hand, MMP inhibition was found to be highly effective in dense gels. Another interesting finding is that, in the collagen gels of highest density (4 mg ml$^{-1}$), but not necessarily only with small (1–2 $\mu$m) pore size (i.e. pH9—2.5 mg ml$^{-1}$ gels), the disruption of microtubule stability with nocodazole even promoted cell migration slightly. Since microtubules contribute to maintaining cell polarity, which facilitates mesenchymal cell migration, the finding lends further support to the claim that cells in matrices with small pore size did not require significant polarity in order to migrate [35]. The drugs targeting actin polymerization and myosin motor activity induced similar influences in the cell speed in gels of moderate-to-low collagen density. A possible reason is that perturbing one type of cytoskeleton element often simultaneously weakens other elements that are required for traction force generation [44].

Quantitative measurement of in vitro cell migratory behaviour in three-dimensional biomimetic matrices is a direct approach for determining the efficacy of anti-metastatic drugs on cancer cell migration. Designing such in vitro assays requires building a physiologically relevant tissue-like microenvironment, where cancer cells manifest their intrinsic migratory capabilities. Reconstituted biological hydrogels have proved to be excellent for such purposes. Our findings suggest
that the assessment of anti-migratory cancer drugs in three-dimensional hydrogels should be interpreted with careful consideration of the ECM biophysical factors. Furthermore, our results suggest that, for each drug, there may be an optimal range of collagen gel conditions to test drug effects with high sensitivity. For example, collagen gels with moderate-to-low density and stiffness seem to be the best for testing drugs that act on cytoskeleton, while MMP inhibitors may be better tested in dense collagen matrices. On such basis, our work may provide guidance for in vitro drug-screening models, as well as for testing the efficacy of other drugs intervening specific molecular mechanisms, using similar experimental set-ups.

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References

23. Velegol D, Lanni F. 2001 Cell traction forces on soft biomaterials. I. Microchemistry of type I collagen


45. Salve F et al. 2004 Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. J Cell Biol 167, 769 – 781. (doi:10.1083/jcb.200408028)


