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Quantitative Imaging of Epithelial Cell Scattering Identifies Specific Inhibitors of Cell Motility and Cell-Cell Dissociation

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Abstract

The scattering of cultured epithelial cells in response to hepatocyte growth factor (HGF) is a model system that recapitulates key features of metastatic cell behavior *in vitro*, including disruption of cell-cell adhesions and induction of cell migration. We have developed image analysis tools to automatically track and characterize this process in live cells without the need for fluorescent tagging, in three different physical aspects: increase in cell motility, loss of cell-cell adhesion, and spatial dispersion of cells. These tools were used to screen a library of drugs. We identified several efficient inhibitors of scattering and classified them as selective inhibitors of either motility or loss of cell-cell adhesion, or as non-selective inhibitors. We validated the inhibitors and putative targets from this screen in two unrelated model cell lines using pharmacological treatments and RNA interference (RNAi). We found that non-steroidal anti-inflammatory drugs inhibited cell-cell dissociation, that indirubins inhibited cell motility, and that cyclin-dependent kinase 1 and ribosomal S6 kinase were signaling intermediates in HGF-induced cell scattering. The assay will be suitable for larger-scale screenings of chemical compounds or RNAi libraries.

Introduction

During malignant transformation of locally restricted, benign epithelial tumors, cells lose cell-cell adhesions and develop a motile phenotype that facilitates invasion(1). Drugs intended to prevent metastasis in cancer patients are generally designed to inhibit cell

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migration, for example by targeting matrix metalloproteinases (MMP)(2). Inhibitors that specifically target the initial loss of cell-cell adhesion have not been identified so far. Such drugs may have clinical potential, but both they and their targets are hard to pinpoint, in part because of a lack of an assay to accurately measure in a high-throughput fashion the state of cell-cell adhesion, which would allow the probing of related signaling pathways by small molecule inhibitors and RNAi.

A well-established *in vitro* model system that captures key aspects of malignant transformation in 2-dimensional cell culture is the scattering of Madin Darby Canine Kidney (MDCK) cells in response to hepatocyte growth factor (HGF)(3). HGF is a metastasis-promoting hormone that binds to the receptor c-MET, which has been implicated in various malignant human cancers (4). This model has been used to investigate pathways involved in tumor invasion, leading to the identification of oncogenes like Ras, PI3K and Src(5–7). HGF-induced scattering of MDCK cells occurs within 24 hours(3) and in three phases (Fig. 1a,b): (i) cell spreading; (ii) weakening and disruption of cell-cell adhesions; and (iii) increased cell motility(7). The combined effect of these processes was reflected in the average cell speed, which started to rise ~2 hours after HGF stimulation, reaching a plateau within 8–10 hours, with a half-time of 5–6 hours (Fig. 1c). When the formation of cell-cell junctions was prevented in low calcium conditions, the speed of cells increased more quickly after stimulation (half-time ~3 hours) (Fig. 1c, and Supplementary Movie S1). This suggests that the migration machinery is rapidly activated upon HGF stimulation, but that the migration speed is not increased efficiently until cell-cell adhesion is lost. The dissociation of adhesions is itself promoted by increased cytoskeletal contraction, which is part of the migratory response to HGF(8). Therefore, increased cell speed upon HGF stimulation is the combined response of adhesion loss and activation of the migration machinery. If a drug inhibits this increase of cell speed, it is not clear whether the drug prevents cell migration, cell-cell adhesion loss, or both.

To pinpoint the differential contributions of signaling pathways to cell motility and loss of cell-cell adhesion, which would enable the search for adhesion-specific inhibitors, we established a quantitative imaging assay of cell scattering. The assay is based on the analysis of cell trajectories acquired by low-magnification phase-contrast microscopy and automated cell tracking. The acquisition of individual cell tracks from phase-contrast images distinguishes our method from other non-fluorescent image analysis methods that have used population and clustering analyses to quantify cell scattering(9, 10). Our approach avoids the complications of attempting to directly assay cell-cell contacts in low-magnification images of multi-cellular aggregates. Sampling at 6 frames/hour is sufficient to identify changes in cell-cell adhesion. Hence the assay is suitable for image acquisition in a multi-well format on any robotic microscope. As a proof-of-concept and validation of the assay, we present a screen of small molecule inhibitors in MDCK cell scattering, which identified 29 efficient inhibitors, including 8 drugs that specifically affected cell motility and 9 drugs that specifically inhibited the loss of cell-cell adhesion. We confirmed inhibitor efficiency in a second HGF-responsive model cell line (DU145 prostate carcinoma cells) and directly assessed for specificity on cell-cell adhesion in MDCK cells with cell-cell junctions disrupted by knock-down of the junction protein α -catenin. Finally, several putative targets of efficient drugs were assessed by RNAi-mediated knock-down. These results support the

notion that COX-2 functions in formation as well as dissociation of cell-cell junctions, and identified the kinases CDK-1 and RSK as signaling intermediates in HGF-induced epithelial cell scattering.

Results

Three parameters derived from cell trajectories define the time course of cell scattering

The movement of individual cells was tracked in phase contrast images using the dark area generated by the nucleus (note S1). Cell detection and tracking performance were validated with respect to a hand-tracked data set (note S1). Before HGF stimulation, the agreement between computer and manual tracking was 90 – 95%. This discrepancy can partially be explained by rounding of cells during mitosis, which leads to shielding of the dark nuclear area (note S1). After HGF stimulation, the morphological transformation of the cells decreased the detection efficiency to an overall accuracy of 80–85% (Supplementary Fig. 1). The field of view of the images typically contained 6–10 epithelial cell-clusters composed of 4–12 MDCK cells, and some single cells and smaller clusters (Movie S1). Thus, at each time-point ~80 cell trajectories suitable for further processing could be harvested from one single time-lapse sequence. In each experiment, 3 separate time-lapse sequences were collected for each drug condition. This large pool of trajectories then allowed us to extract three parameters of cell motion that specifically reflected the three physical steps of cell scattering: motility increase, spatial dispersion, and adhesion loss. We confirmed that the increase of tracking error from before to after HGF stimulation had no systematic effect on any of these three parameters (note S1).

The motility parameter measures the change in the average speed of cell migration (Fig. 2a and note S2) during cell scattering. Before HGF stimulation, the histogram of individual cell velocities shows a single slow population; after stimulation, a second fast population emerges, the size of which increases over time (Fig. 2b; Movie S2). Because rounded cells were not detected, non-migrating mitotic cells were generally not included in the motility parameter. Thus, unless a drug application strongly affects the rate of cell division rates and duration of mitosis the motility parameter is robust against variation in cell cycle.

The spatial dispersal parameter measures the redistribution of cells during cell scattering. This parameter is based on the L-function(11), which is derived from Ripley's K-function(12) (note S2). In brief, the analysis tests in each time point of a movie whether the number of cells within a radially expanding distance from each cell in the field of view increases, on average, faster or slower than would be expected for a spatially random distribution of cells (Fig. 2c). Before HGF stimulation, adherent cells were clustered in groups, resulting in a distinct positive peak of the L-function at distance d_2 , which related to the average size of groups (Fig. 2d). For distances between 0 and d_1 the L-function value was negative, indicating the exclusion of neighboring nuclei at distances less than the average cell diameter. After HGF stimulation, the function flattened in 2 phases. First, cell spreading causes radial movement of cells away from the cluster center. Second, cells completely disperse in random directions when cell-cell junctions are disrupted. The rate of flattening in this second phase is the combined result of the velocity and persistence of cell migration. Rapid dispersal rates thus indicate high values for either or both of these cellular

behaviors. The flattening was quantified by the time course of the measured area under the L-function curve between the characteristic distances d_1 and d_2 (Fig. 2d; Supplementary Fig. 2; and Supplementary Movie S3). Measurement of the L-function required the correction of boundary effects caused by the systematic under-estimation of the number of neighboring cells near the border of the field of view. Furthermore, the effects of increasing cluster size due to cell proliferation were corrected by simulation and subtraction of L-function changes associated with cell division (note S3).

The adhesion loss parameter measures the degree of cell-cell adhesion in the cell population based on the motion correlation of neighboring cells (Fig. 2e). For each cell, a correlation value between -1 and $+1$ was determined by averaging the cosine of the angle between the cell's displacement and the displacements of its nearest neighbors (note S3). Strongly adherent cells in a cluster, which have a high probability of moving locally in parallel, systematically generate correlation values close to 1. In contrast, cells that are detached from one another move independently, and thus generate random correlation values with 0 mean. Accordingly, before HGF stimulation, the histogram of correlation values was skewed to positive values (Fig. 2f). 12 hours after stimulation, the histogram was nearly symmetrical about zero. Hence, at any given time point the histogram can be decomposed into a positively-skewed mode produced by cells that adhere to their neighbors; and a symmetrical mode produced by cells that do not adhere to their neighbors (Supplementary Fig. 3 and Supplementary Movie S4). The adhesion loss parameter is then defined as the ratio between the contributions of these two modes to the histogram.

To determine the potency of different drugs to inhibit scattering, the time courses of the three parameters were normalized to the 12 hour-effect of a reference sample in which cells were stimulated by HGF only (without any additional drug treatment). Accordingly, in a sample not stimulated by HGF ('no HGF'), the values of the normalized parameters remained close to 0 for the entire experiment (Fig. 2g-i). In the reference sample ('HGF'), the normalized parameter values increased from 0 to 1 over the course of 12 hours. The fastest increase was in the spatial dispersal parameter (half-time <3 hours), followed by the adhesion loss parameter (half-time ~ 4 hours) and the motility parameter (half-time <5 hours). If a tested drug was a potent scattering inhibitor, the parameter time-courses resembled the 'no HGF' condition; for a weak scattering inhibitor, the parameter time-courses resembled the 'HGF' condition.

The parameter of adhesion loss specifically reports the state of cell-cell attachment during cell scattering and is independent of motility and spatial dispersal

To establish that the adhesion loss parameter reports the adhesive state of the cell population, and that this parameter is independent of cell motility and spatial dispersal, we determined the parameters in MDCK cells in which the E-cadherin complex, and consequently all cell-cell junctions, were disrupted by shRNA-mediated knock-down of α -catenin. After addition of HGF, the motility parameter increased substantially faster in the absence of cell-cell junctions, suggesting that these cells no longer needed to overcome initial resistance to motility (Fig. 3a; Movie S5). Moreover, α -catenin depleted cells also reached a higher speed than wild-type MDCK cells, which likely reflects that loss of cell-cell adhesion is never

complete in a population of wild-type MDCK cells, and that scattering cells that encounter one another form functional, albeit transient cell-cell junctions even in the prolonged presence of HGF(8). Although the junction-compromised cells did cluster in the absence of HGF (Movie S5), the adhesion-loss parameter under these conditions was already close to 1, consistent with the absence of motion correlation between adjacent cells (Fig. 3b). The motility parameter before HGF stimulation or under unstimulated conditions in α -catenin depleted cells is similar to that in unstimulated and thus uniformly adherent wild-type cells (Fig. 3a). This confirms that the adhesion loss parameter specifically detects the status of cell-cell adhesion, independently of the motility or spatial distribution of cells.

To validate that the observed two modes, correlated and uncorrelated (Fig. 2f, Supplementary Fig. 3), represented sub-populations of attached and detached cells, and to further probe the relationship between cell velocities and cell-cell adhesion, we examined the difference in correlation value between sub-populations of different velocities. We first pooled the trajectories from 3–6 movies before and after HGF stimulation, and without and with α -catenin knockdown, over several tens of frames and partitioned cells into ‘slow’ and ‘fast’ populations. The partition threshold was set to $v_{thresh} = \bar{v} + 3 \cdot \sigma$, where $\bar{v}$ and σ are the average velocity and standard deviation measured before HGF stimulation (Fig. 3c). Because pre-stimulation velocities approximate a normal distribution, less than 1% of the unstimulated cells have velocities above the partition threshold. Then we tested whether the average motion correlation underlying the adhesion loss parameter was different between these two groups (Fig. 3d). We found that in unstimulated wild-type MDCK cells (which showed no loss of initial adhesion), the average correlation value (note S3) was high for both slow and fast populations (0.277 compared to 0.242); in unstimulated or HGF-stimulated α -catenin negative cells (which did not show adhesion from the onset of the experiment), the average correlation value was low for both slow and fast populations (0.034 compared to 0.018). Thus, under these two conditions in which adhesion was uniform and cell velocity was independent of cell-cell adhesion, the adhesion loss parameter correctly reported equally strong adhesion in unstimulated cells and equally weak adhesion in α -catenin depleted cells for the different velocity categories. In contrast, in HGF-stimulated samples (in which initial adhesion was lost over time), fast cells had a significantly lower average correlation score (0.074) than slow cells (0.194). This reflects the separation of HGF-treated MDCK cells in 2 subpopulations: (i) cells that have disrupted their adhesions to neighbors and are free to migrate at high speed, and (ii) cells that maintain a certain degree of cell-cell adhesion which inhibits their full migratory potential. Thus, also under the condition in which velocity increase and loss of cell-cell adhesion were related (Fig. 1) the adhesion loss parameter specifically reported the state of cell-cell adhesion of subpopulations of MDCK cells. Thus, combined with the motility parameter and the spatial dispersal parameter describing the overall status of the scattering process, these measurements can be used to investigate the effect of interventions of cell scattering as a whole and to identify targets that differentially affect the motility or cell-cell dissociation response.

A screen of small molecule inhibitors reveals motility- and adhesion-specific regulators of scattering

To identify signaling intermediates that are involved in the regulation of either cell motility or cell-cell dissociation, we used compounds from a commercially available library of small molecule inhibitors with known targets (Lopac 1280). We chose inhibitors of kinases, phosphatases, and mediators of calcium and lipid signaling that are putative intermediates of HGF-induced transformation. We complemented this selection with various commercially available inhibitors of kinases suspected to be activated downstream of the c-Met receptor (Supplementary Table S1) and included the cMET inhibitor PHA-665752 as a positive control. The efficiency of an inhibitor was defined as the ratio of the normalized parameter values of the drug-treated condition ('HGF+drug') compared the reference sample ('HGF') (Supplementary Fig. 4). Time-resolved efficiencies are shown as heatmaps for all drug conditions at various concentrations (Fig. 4). These heatmaps indicated that some drugs preferentially inhibited one parameter. For instance, the COX inhibitors nimesulide and diclofenac inhibited adhesion loss more efficiently than the increase in cell motility, whereas the PKC inhibitor GFX and FAK inhibitor PF573228 inhibited cell motility more efficiently. Some drugs (for example, the PKC activator oleic acid, the phosphatase inhibitor tetramisole hydrochloride, and the serine/threonine kinase inhibitor H-7 dihydrochloride) inhibited the spatial dispersal parameter at early timepoints, indicating a specific effect on the cell spreading. We speculate that this reflects the effect of isoquinoline sulphonamides on Rho-activated kinase (ROCK) and its downstream target actomyosin(13, 14), but other explanations are possible. The mechanism for the effects by other drugs remains unclear. Here, we focused our analysis on adhesion-loss and motility specific drugs.

Drugs were ranked based on their maximum efficiency, which we defined as the efficiency 12 hours after stimulation (Supplementary Fig. 5). We referred to those 39 drug conditions that exceeded 66% efficiency for at least one of the three parameters as 'effective inhibitors'. Hierarchical clustering of all effective inhibitors (using the three efficiencies as the features to measure distances between inhibitors) distinguished three groups of drugs (Fig. 5a). The first group (green) contained inhibitors of targets implicated in the regulation of cell-cell adhesion because their strongest effect was on the adhesion-loss parameters (also see Fig. 4 and Fig. S6). The second group (red) contained inhibitors of targets implicated in the regulation of cell motility. The third group (brown) contained inhibitors that had similar effects on all three parameters. In the clustering tree, the branches of the unspecific third group merged first with the motility group, which underscores that inhibition of cell motility may affect scattering both directly (because the speed of migration affects the speed of spatial dispersal of the cells) and indirectly (because certain aspects of the motility response, especially the induction of acto-myosin contraction, are needed for efficient breakdown of cell-cell junctions(8)).

Some drugs were already effective at non-maximal concentration and therefore yielded entries for multiple concentrations. In the case of the PKC inhibitor GFX, all efficient concentrations of the drug had a motility-specific effect. In other cases (the MEK inhibitor U0126, the PKC family inhibitor rottlerin, the Src family inhibitors PP1 and piceatannol), specificity towards one parameter at lower concentration was lost at higher concentration.

This could reflect a dual function with differential sensitivity to drug inhibition of the targeted protein in both aspects of scattering, or non-specific targeting of the inhibitors at higher concentrations. Overall, these analyses demonstrate the sensitivity of the three implemented parameters of cell scattering to classify signaling intermediates at the interface between loss of cell-cell adhesion and increased motility.

Comparison of motility of clustered and single cells confirms the specificity of adhesion loss parameter

To further validate drugs classified by hierarchical clustering as adhesion-specific, we implemented an alternative readout of adhesion specificity, which relied only on the difference in cell velocity between clustered cells and single cells (those detected as distinctly isolated by the initial image segmentation). If a drug inhibited the loss of cell-cell adhesion but not the motility increase, isolated cells would be expected to migrate faster than clustered cells. On the other hand, if a drug blocked both loss of adhesion and the increase in motility, the velocities of single cells and clustered cells would be expected to be similar (see Supplementary Fig. 6 for examples of velocity time-courses). Thus, the difference in velocities between isolated and clustered cells was also indicative of the adhesion specificity (quantified by the p-value of a two-sided t-test of the means of the two velocity distributions). Using this analysis, we found that adhesion-selective drugs (green) had p-values < 0.05 (Fig. 5b), showing that single cells had significantly higher velocities than clustered cells in samples treated with these drugs. Non-selective drugs (brown) generally scored lower on the significance scale.

Myricetin, PP1 and U0126, which were classified by hierarchical clustering as non-specific drugs, had high p-values in this measurement indicating a differential inhibitory effect on adhesion loss and motility. The different data interpretations by hierarchical clustering (Fig. 5a) and velocity-based analysis (Fig. 5b) were likely caused by the limitations of the purely velocity-based readout, which depends on an accurate segmentation of single cells compared to clustered cells. Due to stringent selection criteria, only a few fully non-contacting cells were found, which makes this measurement less robust. Also, a velocity-based readout is susceptible to motion artifacts caused by changes of cell morphology. Especially with isolated cells, changes in the cell shape induced by a sub-group of drugs can cause oscillations in the detected cell position. These two factors combined lead to an overestimation of single cell velocity, and thus a high p-value for the velocity difference between isolated and clustered cells. Thus, although this alternative approach of classifying inhibitors overall corroborates the distinction of adhesion-specific inhibitors, we suggest that the combination of all three efficiencies in a hierarchical clustering offers more robust results.

RNAi mediated disruption of cell-cell adhesion confirms drugs that specifically inhibit adhesion

To directly test the predictive value of our classification, we examined the effects on velocity increase of a subset of efficient inhibitors in α -catenin-depleted MDCK cells (Supplementary movie S5). Motility-selective drugs would be expected to have equally strong effects in these cell-cell adhesion-defective and wild-type cells, whereas adhesion-

loss selective drugs would be expected to be less effective in adhesion-defective cells. Plotting the ratio between percentage inhibition in wild-type and adhesion-defective cells for each drug at a concentration that was in the dynamic range of inhibition showed that adhesion-specific drugs (PP1 and diclofenac) were less effective on adhesion compromised cells than on adhesive cells (Figure 5c and Supplementary Fig. 8). Most other drugs, including two drugs previously classified as motility-specific, clustered around a ratio of 0.5, indicating that they retained about 50% of their inhibitory potential in junction-compromised cells. This may reflect that engagement of the motility machinery to increase actomyosin contraction also contributes to the efficient breakdown of cell-cell junctions in MDCK cells after HGF(8). Adhesion-defective cells may therefore be less efficiently inhibited by motility-targeting drugs as well. In sum, these analyses validate our classification based only on cell migration dynamics as a powerful tool for the identification of drugs that specifically target the loss of cell-cell adhesion downstream of HGF.

E-cadherin staining confirms the adhesion-loss specificity of inhibitors

To investigate whether the specificity of adhesion-loss inhibitors was reflected in molecular markers of cell-cell adhesion, we performed immunofluorescence staining of E-cadherin. We quantified the relative intensity of the E-cadherin signal in remaining junctions (a ~400 nm wide linescan along junctions) over the background signal near the junctions (a 2.6 μ m wide linescan along the junctions) (Fig. 5d). The relative abundance of E-cadherin in cell-cell junctions was variable among individual junctions and on average, decreased only slightly in response to HGF before junction breakage occurred (Fig. 5E, upper panel). In the presence of the motility specific inhibitor of FAK (PF573228), the abundance of E-cadherin decreased faster than in control cells. Conversely, in the presence of the adhesion-loss specific COX-2 inhibitor diclofenac, the abundance of E-cadherin decreased less rapidly and to a lesser extent. After 6 hours of HGF treatment, the remaining junctions in cells treated with the adhesion loss specific inhibitors such as diclofenac and nimesulide or the cMET inhibitor PHA-665752 had higher amounts of E-cadherin in cell-cell junctions than uninhibited or FAK inhibited cells (figure 5e, lower panel). These data indicate that differential effects on molecular parameters of cell-cell adhesion were exerted by adhesion-loss and migration specific inhibitors. However, because of the minor loss of E-cadherin it is questionable that this molecular measure is a reliable estimate of adhesion strength. This corroborates our earlier work on MDCK cell scattering, in which we found that the build-up of physical forces, rather than a gradual loss of E-cadherin by internalization, precedes cell-cell junction disruption(8). For a second independent validation of the adhesion-loss parameter, we quantified by immunofluorescence the number of cell-cell junctions at increasing times after HGF. The adhesion-loss specific inhibitors diclofenac and nimesulide and the cMET inhibitor PHA-665752 blocked the loss of cell-cell junctions after stimulation, whereas FAK inhibition did not (Fig. 5f). Together these analyses further establish the validity of the migration-based readout of cell-cell adhesion strength used in our screen, but they also demonstrate that an assessment of adhesion strength by immunofluorescence of adhesion components is more ambiguous than the proposed live cell assay.

Efficient inhibitors were verified in unrelated cells

DU145 cells are human prostate cancer cells that scatter in response to HGF. Because of the importance of HGF in prostate cancer, and the possible use of this cell line as a model for drug- or siRNA-based target discovery, we compared the scattering process between MDCK and DU145 cells. We tested 17 efficient drugs covering all predicted intracellular signaling intermediates from the screen on the human DU145 cell-line. Most drugs (except the COX inhibitor SC-560 and the adenylyl cyclase activator forskolin) reproduced their inhibitory effect on the HGF-induced scatter response as measured by velocity increase (Fig. 6), which suggests that cells from different species and from different organs respond to HGF through conserved molecular mechanisms that can be targeted by the same chemical inhibitors.

Drug targets were validated by RNAi

Several of the efficient inhibitors from our chemical screen target intermediates of scattering or cell migration such as Src, FAK and PI3K and PKC. The screen also identified various less characterized drug targets, which we explored in more detail. Indirubin-3-oxime and kenpaullone, two efficient non-selective inhibitors of scattering, are CDK inhibitors with side effects on other kinases, including glycogen synthase kinase (GSK) 3 β , LCK, and serum- and glucocorticoid-induced protein kinase (SGK). We eliminated some of these possible targets by showing that the CDK-2 inhibitor roscovitine and the GSK-3 β specific inhibitor SB216763 (both included in the screen) did not inhibit scattering. Because CDK-5 has been previously implicated in prostate cancer cell migration(15), we used siRNA mediated knockdown in DU145 cells to directly test the role in HGF-induced scattering of CDK-5 and a close homolog, CDK-1, which is also targeted by these drugs. siRNA-mediated knockdown of CDK-1, but not CDK-5, significantly inhibited scattering (Fig. 7a,b and movie S6). Because CDK-1 is a regulator of cell cycle progression, we were concerned that the effects of RNAi on the motility parameter could be related to an increased population of non-migrating cells arrested in mitosis (note S2). The rate of cell division was affected by CDK-1 RNAi (Fig. 7c). However, the percentage of tracked cells that undergo mitosis was too low to affect the average velocity and was not altered by CDK1 depletion (as judged from the individual velocity profiles of all cells that were fully tracked between 3 and 6 hours after HGF) (Fig. 7d). Also, the duration of mitosis was not altered by CDK1 RNAi as judged from the individual cell velocity profiles (Fig. 7e). Thus, these results identify CDK-1, but not CDK-5, as an intermediate in HGF-induced scattering of DU145 cells.

We also examined COX-2 inhibitors, several of which emerged as selective inhibitors of the loss of cell-cell adhesion in the screen. Indeed, COX-2 abundance was increased after 2 hours of HGF stimulation of MDCK cells (Fig. 7f). Using shRNA constructs against canine COX-2, we reduced its abundance in HGF-stimulated cells to below its abundance in unstimulated cells (Fig. 7f). The effect of COX-2 inhibition by RNAi, however, was opposite that of drug-induced inhibition of COX-2: COX-2 RNAi resulted in a more rapid and higher increase in cell velocity than in control cells, similar to the effect of E-cadherin depletion in MDCK cells (Fig. 7f and movie S7). Because two unrelated shRNAs targeting COX-2 showed the same effect, these results are very unlikely due to an off-target effect of the shRNA constructs. We propose that COX-2 protects cell-cell junctions from HGF-induced

disruption in MDCK cells, whereas chemical inhibition of COX-2 prevents the efficient breakdown of cell-cell junctions by HGF. Whether the latter effect is through inhibition of COX-2 enzymatic activity or due to off-target effects of these drugs remains to be investigated.

An effective motility-selective drug was BI-D1870, which targets p90RSK family members. HGF induced rapid and prolonged phosphorylation of several residues in RSKs (Fig. 7g right panel), which is consistent with the upstream induction of mitogen-activated protein kinase (MAPK) signaling. Targeting the RSK1 and RSK2 by siRNA reduced the overall abundance and phosphorylation of RSKs, and inhibited the HGF response in DU145 cells (Fig. 7g and movie S8). Thus we conclude that the RSK family kinases are signaling intermediates in HGF-induced scattering that specifically mediate the cell motility response.

Discussion

We have developed a quantitative assay for epithelial cell-scattering based on phase-contrast imaging and tracking of cell nuclei, and demonstrated its use in a screen of chemical inhibitors to identify specific regulators of cell motility or loss of cell-cell adhesion downstream of HGF in MDCK cells. We identified 29 efficient inhibitors of scattering, including 8 inhibitors of motility response and 9 inhibitors of cell-cell-dissociation. Using the human prostate carcinoma cell line (DU145), we verified that drugs targeting the HGF response are efficient in an unrelated cell line from a different species and organ. Furthermore we have validated the predictive value of the cell-cell adhesion-specificity classifier with cell-cell junction defective cells and by direct observation of E-cadherin aggregation in junctions. Finally we have used RNAi to validate the drug targets CDK1 and RSK as signaling intermediates in HGF-induced cell scattering.

A key feature of our assay is the use of an indirect measure of cell-cell adhesion through the correlated movement of neighboring cells following the notion that cell-cell junctions restrict and coordinate migration of cells in a cluster. If correlated cell movement is also promoted by mechanisms beyond cell-cell junctions, like polarized paracrine signaling, effects of drugs on such a mechanism would also affect this parameter. To our knowledge, such mechanisms have been described neither for the MDCK nor the DU145 systems, but this possibility should be taken into account when using this assay. We have validated our assay by several alternative approaches (Fig. 5) including the use of junction compromised (α -catenin-depleted) cells. Subsequently, we applied the assay to identifying several drugs that specifically interfere with adhesion-loss during HGF-induced cell scattering.

For all tested drugs, we considered the possibility that effects on scattering were due to inhibition of other yet unidentified targets(13, 14). Controlling compound specificity in separate biochemical experiments was beyond the scope of this study, which aimed to provide a low-cost, high-yield screening method for the identification of drugs selective for inhibition of motility and loss of cell-cell adhesion. Nevertheless, in cases in which we identified several drugs targeting one protein family (such as Src, PKC, or COX), it is more likely that the observed effect was through inhibition of these specific proteins. It is interesting to note that inhibitors with different selectivity for the individual Src family

members are classified as motility specific (piceatannol), adhesion specific (emodin, PP1), or non-specific (su6656, 7-cyclopentyl-5-(4-phenoxy)phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine). This result underscores the differential roles these individual Src family members play, and highlights the potential for developing member-specific drugs.

A prominent group of efficient inhibitors of cell-cell dissociation we identified belong to the class of non-steroidal anti-inflammatory drugs (NSAIDs) which target cyclooxygenase (COX) enzymes: nimesulide, SC-560, and diclofenac sodium (movie S7). Of the 2 mammalian family members, COX-2 is regulated at the level of protein expression and is increased in several types of invasive cancer including colon cancer(16). Consistent with our result that COX-2 inhibitors slow the loss of cell-cell adhesion upon HGF stimulation, COX-2 reduces E-cadherin-dependent cell-cell adhesion by activating RhoA(17) or by negatively regulating E-cadherin expression through the transcription factors Snail and ZEB1(18). COX-2 has also been implicated in HGF-induced angiogenesis, which involves disruption of endothelial cell-cell junctions(19). Surprisingly, we found that knockdown of COX-2 resulted in increased scattering, comparable to the effect of E-cadherin knockdown. This suggests that COX-2 depletion is not compatible with the formation of stable cell-cell adhesion in MDCK cells. The difference in effects between chemical inhibition and RNAi-mediated interference could be caused by different effects of long term reduction of protein abundance compared to acute short term inhibition, and suggests that COX-2 functions both in formation as well as dissociation of cell-cell junctions. This also highlights the notion that target discovery by RNAi may yield different results than drug discovery by chemical screening. The alternative explanation for the difference in this case is that these NSAIDs target other enzymes to inhibit cell-cell dissociation. Nevertheless, NSAIDs are drugs that have a selective effect on the HGF-induced loss of cell-cell adhesion which makes them interesting lead compounds for anti-metastasis drug development.

Drugs based on the natural compound indirubin have been extensively studied and have been used as anti-cancer treatment predominantly in chronic myeloid leukemia. Because their main targets are the CDKs(20), their effect is thought to be inhibition of cell division. However, indirubin-based drugs target several other kinase families including GSK3 and the SGK family kinases. Our current results show that indirubin-3-oxime is a potent inhibitor of HGF-induced cell scattering, which adds a new spectrum to the clinical potential of indirubin-derived compounds. Whether indirubin-3-oxime's effect on scattering is mediated through inhibition of CDKs is not yet fully established. Based on our inhibitor screen we can exclude most of the alternative kinase targets as well as the cell cycle member CDK-2 as the indirubin-3-oxime target involved in inhibited scattering. Efficient knock-down of CDK-5 did not affect scattering, and thus, we can also exclude a role of CDK-5 in this process. On the other hand knock-down of CDK-1 inhibited DU145 cell scattering. CDK1 was previously shown to stimulate cell migration in a different prostate cancer cell line LNCaP(21).

RSK family kinases have multiple downstream substrates and multiple intracellular functions (22). They are crucial for the Ras-mitogen-activated protein kinase (MAPK) induced motility of MDCK cells(23) and pharmacological inhibition of RSKs inhibits the effect of 31 migration-inducing shRNA treatments (24). Our results show that RSK is

phosphorylated downstream of HGF and that its pharmacological inhibition as well as knock-down of two of its isoforms inhibits HGF-induced cell scattering by decreasing cell motility. This places RSK at a crucial position in the signaling cascade leading from cMET activation to cell scattering and underscores the potential of RSK family kinases as drug targets in cMET positive metastatic cancer(23).

In summary, we have developed a robust image analysis tool that can be used in any standard laboratory setting to track cells and distinguish inhibitory effects by drugs and RNAi on cell motility and cell-cell adhesion during epithelial transformation. The data from our screen confirms the involvement of a number of signaling molecules (such as MEK, PI3K, PKC and Src) in HGF-induced metastatic cell-behavior. They identify NSAIDs as drugs that specifically target the disruption of cell-cell adhesion downstream of HGF, identify Indirubins as drugs that target cell motility downstream of HGF and identify CDK1 and RSK as cMET signaling intermediates and putative drug targets.

Material and Methods

Phase-contrast microscopy

We used different microscopes systems: Zeiss Axiovert 200M, Leica AF7000, Nikon Ti all similarly equipped with robotic stages and controlled by company software (Axiovision, LAS-AF and NIS elements). Cameras on these systems were: a 10-bit CCD Axioacam camera (Zeiss) a 12-bit CCD DFC365 FX camera (Leica) and a 12 bit EMCCD Luka Camera (Andor). Images were acquired using comparable 10× 0.5 NA Plan objective lenses and compatible LWD condensers.

Cell Culture

MDCK cells were plated in DMEM (Gibco), supplemented with 0.5% FCS (Sigma) and 10mM Hepes pH 7, 24 hours prior to imaging at a density of 5500 cells/cm² in 3 µg/ml collagen type 1 (Sigma) -coated, non-tissue-culture-treated polystyrene 48 or 96-well plates (Nunc). DU145 cells were plated in RPMI medium (Gibco), supplemented with 10% FCS (Sigma), 48 hrs prior to imaging at a density of 5000 cells/cm² in 10 µg/ml collagen type 1 (Purecol) –coated, non-tissue-culture-treated polystyrene 48 well plates (Nunc). 24 hrs prior to imaging the medium was replaced by medium containing 0.5% FCS. Immediately prior to imaging, drugs were added and wells were completely filled with medium and sealed using silicon grease and a glass plate. Cells were imaged for 2 hrs before stimulation, then wells were opened briefly for HGF addition, after which imaging continued for at least 9 hrs. To establish low Ca²⁺ conditions, we used calcium-free DMEM (Gibco), supplemented with 0.06 mM CaCl₂, 0.5% FCS and 10mM Hepes pH 7.

RNAi

For RNAi in MDCK cells, 19 nucleotide target sequences were cloned into pSuper and transfected by 2 rounds of nucleofection (Lonza, DE) using buffer L and programme T-020. 4 days and 2 days prior to imaging. Sequences were: E-cadherin: 5′ – AGCAGTATGTCTTGACGT – 3′, COX-2 sh1: 5′ – ATGGGATGATGAGCGGTTA – 3′, COX-2 sh2: 5′ – GGGAGTCTGGAACATTGTC – 3′. MDCK cells stably expressing an α-

catenin KD sequence were a gift from James Nelson and was described previously(25). For RNAi in DU145 cells, RNA duplexes were transfected using HiPerfect (Qiagen) according to the manufacturers protocols 3 days prior to imaging. We used the following sequences for RSK-1: 5' - CCCAACATCATCACTCTGAAA - 3' and RSK-2: 5' - AGCGCTGAGAATGGACAGCAA - 3' that were previously tested in(26). For CDK1 and CDK5 we used an ON-TARGET plus SMART pools from Thermo Scientific Dharmacon.

Western blotting

For analysis of protein abundance we performed SDS-PAGE using NuPage gradient gels (invitrogen) and standard Western blotting on PVDF membranes. The antibody directed against CDK5 was from Cell Signaling (#2506), those directed against COX-2, CDK1 and E-cadherin (clone 36) were from BD Biosciences, and those directed against total RSK, pS380-RSK, and pT573-RSK were from Cell Signaling and actin (clone C4) was from Millipore.

Immunofluorescence analysis of E-cadherin and junction disruption

The IF staining and quantification of E-cadherin levels in junctions in Fig. 5d–f was as follows: Cells were grown on collagen (3 µg/ml) coated coverslips for 24 hrs and then stimulated for the indicated times with HGF. The appropriate drugs were added 10 min before addition of HGF (or 10 min before fixation if no HGF was added). Cells were washed once in ice cold PBS, then once in ice cold cytoskeletal buffer(27), once more in ice cold PBS and fixed in 4% paraformaldehyde. The E-cadherin antibody used was clone 36 (BD Biosciences). Imaging was performed on a Nikon Ti epi-fluorescence microscope equipped with a 12 bit EMCCD Luka Camera (Andor) using a 40× 1.3 NA plan fluor objective lens. The relative abundance of E-cadherin in cell-cell junctions was measured by drawing a line on top of a junction fragment (from one tricellular junction to the next, or along the full junction between 2 cells) and dividing the average pixel intensity in a 3 pixel linescan (corresponding to 400 nm) along this line over the average pixel intensity in a 20 pixel linescan (2.6 µm) along this line. Values were normalized to the unstimulated, drug-treated conditions. To quantify the average number of cell-cell junctions per cell in Fig. 5d, we determined for each cell in these images the number of contacts with neighboring cells that showed discernible E-cadherin signal (irrespective of the length of contact).

Image analysis and parameter identification

Details of the methods for image analysis and cell tracking are in note S1. The identification of parameters that specify the diverse physical aspects of cell scattering is explained in notes S2, S3 and S4. The metric used for hierarchical clustering of drug effects on cell scattering is defined in note S5, along with a discussion of cluster validation procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Physical events during cell scattering

a Snapshots from a phase-contrast time-lapse image sequence of MDCK cells scattering in response to HGF stimulation. Scalebar 50μm. **b** Sequence of physical events during cell scattering. **c** Time course of average migration velocity (5 frame running average to increase smoothness of curves) in response to HGF-stimulation, under normal (n=1,915 cells) and low calcium conditions (n=1,454 cells).

Figure 2. Three parameters to describe physical phases of scattering

(a and b) **Motility parameter.** Upper panel – color-coded tails depict the cell positions in 6 previous movie frames, thus indicating the instantaneous cell velocity. Scalebar 50 μ m. **b** Histogram of velocities, before and 12 hours after stimulation. (c and d) **Spatial dispersal parameter.** Ripley's K-function measures the number of neighbors within a radially expanding distance d (c). L-function before and 12 hours after stimulation (d). The spatial dispersal parameter is defined as the integral between d_1 and d_2 as explained in note S3. (e and f) **Adhesion loss parameter.** The adhesion loss parameter of a cell at one time point is derived from the correlation of the displacement vector of the cell with those of its immediately neighboring cells (e). Histogram of correlation values before and 12 hours after stimulation (f). The images and graphs in (a–f) are from a single experiment with >YY cells, representative of all ($N > 20$) unstimulated and HGF stimulated samples used throughout this study. **g–i** Normalized time courses (7-frame rolling average) of motility, spatial dispersal, and adhesion loss parameters for unstimulated (dashed), HGF-stimulated (solid), and HGF-stimulated and drug treated (bold dashed; PD98059) cells.

Figure 3. Adhesion loss parameter reports the state of attachment of the cell population
a–d Effects of pre-dissociation of cell-cell junctions (by α -catenin knock-down) on the different scattering parameters. **a** The motility parameter increases more quickly for pre-dissociated cells (by α -catenin knock-down) than for attached cells. **b** The adhesion loss parameter is high in pre-dissociated cells. **c** Histograms of cell velocities (normalized to bin with maximal occurrence) before and 12h after stimulation to define partitioning of ‘slow’ and ‘fast’ cells that is used in d. **d** Average motion correlation of ‘slow’ and ‘fast’ cells. HGF-stimulated cells: $n=1,915$; control (unstimulated) cells: $n=945$; HGF-stimulated α -catenin-KD cells (pre-dissociated cells): $n=647$. Unstimulated α -catenin-KD cells: $n=761$ (n =number of cells)

Figure 4. A small molecule screen for inhibition of HGF-induced cell-scattering

Small molecule inhibitors were used in a live cell imaging based screen of MDCK cell scattering. The resulting timelapse image series (at least 3 per condition) was analyzed by the cell tracking software using the three parameters defined in Fig. 2. Color bars represent the inhibition efficiencies over time for all drugs and concentrations used in the screen (see legend). The inhibition efficiency in one parameter is defined as the time-resolved ratio of the normalized parameter values of the drug-treated condition compared to the reference

sample. Drugs, targets and specificity are summarized in Supplementary Table S1. Drug concentrations are indicated on the leftmost colorbar in white (units are μM).

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Figure 5. Classification of small molecules into motility-, adhesion- or unspecific inhibitors of scattering

a Hierarchical clustering of high-efficiency inhibitors (defined as those conditions with at least 66% inhibition efficiency in one parameter 12 hrs after HGF stimulation) from Supplementary Figure 5. Drug concentration is noted between brackets, unit is μM . Clustering was performed based on the Euclidean distance in a 3D feature space, in which each small molecule is characterized by the three normalized inhibition efficiencies defined in Fig. 4 12 hrs after HGF stimulation (note S5). The clustering generates three groups representing motility-specific high-efficiency (red), adhesion-specific high-efficiency

(green), and non-specific high-efficiency inhibitors (brown). The significance and robustness of the groups was validated by several tests illustrated in Fig. S6 (see also note S5). **b** Significance of the velocity difference between isolated and clustered cells (as defined by the image segmentation algorithm in the time lapse image series) for the members of the adhesion-specific and non-specific groups in (a). The p-values of a t-test comparing the velocities of isolated versus clustered cells are plotted on a logarithmic scale. **c** Disruption of cell-cell adhesion to test adhesion specificity. The effect of selected adhesion-specific, migration-specific, or non-specific drugs on HGF-induced cell migration illustrated by the ratio between the average inhibition efficiencies on α -catenin-restored compared to α -catenin-depleted cells was plotted. **d** Representative images of E-cadherin immunofluorescence after HGF stimulation. Drugs are indicated with the color code of their cluster association in (a). **e** Upper: Time course of the relative E-cadherin immunofluorescent signal in junctions (error bars indicate SE, $n > 50$ junction fragments). Lower: non-normalized values in 6hr HGF-treated samples. Center line is median, boxes are quartile, bars are full data range, individual squares are unaccounted outliers. **f** Average number of junctions per cell (6 images for each condition, 10–12 cells/image, error bars indicate SE).

Figure 6. The inhibition efficiency of MDCK-effective drugs on DU145 cell scattering
The average inhibition efficiency of the motility response in early (0–3 hrs post HGF) and late phases (3–9 hrs post HGF) of DU145 cell scattering is depicted for 18 efficient inhibitors of MDCK cell scattering, including the cMET inhibitor PHA-665752 as a positive control.

Figure 7. Validation of putative drug targets by RNAi

a Left panel: The velocity profile of HGF-stimulated DU145 cells transfected with scrambled siRNA duplexes or siRNAs targeting CDK1 or CDK5 as indicated. **b** Right panel: Immunoblots for protein abundance in samples taken at the end of the scattering experiment. **c** number of cells detected in each image as a measure of cell proliferation. **d** number of cell velocity time courses with a substantial drop in velocity between 3 and 6 hrs after HGF stimulation (which is indicative of cells in mitosis) compared to the total number of cell tracks in the same period. **e** Single cell velocity time courses of a CDK1-depleted cell that goes through mitosis compared to a cell treated with scrambled siRNA. The data in (a–e) were taken from a single representative experiment that was repeated 4 times. The velocity graphs are an average of the analysis of 3 time-lapse image sequences for each condition from this experiment. **f** Left panel: The velocity profile of HGF-stimulated MDCK cells

transfected with a non-targeting shRNA construct or shRNA constructs targeting E-cadherin or COX-2 as indicated. Right panel: Immunoblots for protein abundance in parallel samples taken at the indicated time points during this scattering experiment. These data are from a single representative experiment that was repeated 6 times. The velocity profile is an average of the analysis of 3 time-lapse image sequences for each condition from this experiment. **g** Left panel: The velocity profile of HGF-stimulated DU145 cells transfected with scrambled siRNA duplexes or siRNAs targeting RSK1 or RSK2 or both as indicated. Right panel: Immunoblots for protein abundance and phosphorylation of the 2 indicated sites in parallel samples taken at the indicated timepoints during this scattering experiment. These data are from a single representative experiment that was repeated 3 times. The velocity profile is an average of the analysis of 3 time-lapse image sequences for each condition from this experiment.