Emergence of HGF/SF-Induced Coordinated Cellular Motility

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Abstract

Collective cell migration plays a major role in embryonic morphogenesis, tissue remodeling, wound repair and cancer invasion. Despite many decades of extensive investigations, only few analytical tools have been developed to enhance the biological understanding of this important phenomenon. Here we present a novel quantitative approach to analyze long term kinetics of bright field time-lapse wound healing. Fully-automated spatiotemporal measures and visualization of cells’ motility and implicit morphology were proven to be sound, repetitive and highly informative compared to single-cell tracking analysis. We study cellular collective migration induced by tyrosine kinase-growth factor signaling (Met-Hepatocyte Growth Factor/Scatter Factor (HGF/SF)). Our quantitative approach is applied to demonstrate that collective migration of the adenocarcinoma cell lines is characterized by simple morpho-kinetics. HGF/SF induces complex morpho-kinetic coordinated collective migration: cells at the front move faster and are more spread than those further away from the wound edge. As the wound heals, distant cells gradually accelerate and enhance spread and elongation –resembling the epithelial to mesenchymal transition (EMT), and then the cells become more spread and maintain higher velocity than cells located closer to the wound. Finally, upon wound closure, front cells halt, shrink and round up (resembling mesenchymal to epithelial transition (MET) phenotype) while distant cells undergo the same process gradually. Met inhibition experiments further validate that Met signaling dramatically alters the morpho-kinetic dynamics of the healing wound. Machine-learning classification was applied to demonstrate the generalization of our findings, revealing even subtle changes in motility patterns induced by Met-inhibition. It is concluded that activation of Met-signaling induces an elaborated model in which cells lead a coordinated increased motility along with gradual differentiation-based collective cell motility dynamics. Our quantitative phenotypes may guide future investigation on the molecular and cellular mechanisms of tyrosine kinase-induced coordinate cell motility and morphogenesis in metastasis.

Introduction

Collective cell migration is prevalent in many physiological phenomena and is the most common motility pattern in living organisms [1]. In morphogenesis, large clusters of cells travel long distances to reach their ultimate biological destination. In tissue repair, sheets of cells move coordinately to repair damaged tissue. In cancer, cells invade the extracellular matrix and traverse across normal tissue with extreme efficiency to form metastases.

Extensive research has been carried out for many years in various experimental model systems to investigate, describe, analyze, model and simulate collective cell migration. There are several theories concerning the mechanisms behind collective motility [2,3]. A relatively common one regarding the physical interactions is "Follow the Leader" [4], were cells at the leading edge are assumed to produce force to pull passive followers from cells located further away from the front [5,6,7,8,9]. For example, Inaki et al. [9] recently demonstrated that directionality can be encoded within a group of cells by the constituents attaining different signaling levels.

However, accumulating evidence implies that the behavior is more complex. Modern microscopy [10] revealed that distant cells extend in what is referred to as ‘cryptic’ lamellipodia against the substratum beneath their preceding cells, evidence that the cells further behind the leading edge do not simply act as naïve followers. Recent measurements of distributions of traction- and intercellular-forces within the monolayer also suggest that the "follow the leader" paradigm is too simplistic [11,12,13,14] and argue that cells further away from the front are also self-propelled in the collective motility process. Several mathematical models have been devised to describe collective migration based on single cell motility and cell-cell interactions [15,16,17].

Another theory assumes that cell proliferation expands the colony and thereby generates pressures that cause the leading cells to move [5,10,18,19,20], but earlier studies demonstrated that cell migration in mucosal healing is largely independent of pro-
liferation [20]. Moreover, Poujade et al. [5] showed that proliferation can occur almost exclusively in the void regions and hence cannot provide complete explanation for the general phenomenon.

In the standard in-vitro wound healing assay, collective migration of cells toward the wound is induced by a sudden injury created by removal of a sheet of cells from the monolayer [21]. Traditionally, the assay is applied to measure the change in healing rate caused by chemicals, other environmental conditions or cell types.

The epithelial to mesenchymal transition (EMT) activated by alternations in gene expression regulates epithelial plasticity during morphogenesis, tissue repair and cancer invasion [22]. During EMT, epithelial cells become motile and invasive, a process that is characterized by an elongated and more spread morphology throughout [23]. Cancer metastasis consists of a sequential series of events, and the EMT and mesenchymal-epithelial transition (MET) are recognized as critical events for metastasis of carcinomas [24]. A current area of focus is the histopathological similarity between primary and metastatic tumors, and MET at sites of metastases has been postulated as part of the process of metastatic tumor formation [24].

Understanding collective cell motility and how it may lead to metastatic formation is an important task since the vast majority of cancer deaths result of progression from a localized lesion to distant metastases [25]. In vivo collective migration is common in breast cancer, as well as in many other cancer types [26]. Several signal transduction pathways and proteins that are related to collective processes in morphogenesis contribute to cancer progression, but their molecular action mechanisms remain mostly unknown [1]. Many efforts are invested in targeting the tyrosine kinase growth factor receptor Met and its ligand HGF/SF, the master regulators of cell motility in normal and malignant processes [27,28,29,30].

Here, we investigate the link between cells' morpho-kinetic dynamics and collective migration of tumor cells using mammary adenocarcinoma cells expressing high levels of Met, image them using a time-lapse microscopy wound healing assay, and study the effect of HGF/SF-Met signaling on morphology and collective motility patterns.

We devised a novel approach to analyze wound healing in vitro using bright field, time-lapse microscopy, based on the combination of a fully automated algorithm that extracts motility measurements from all cells in the monolayer with indirect cellular morphology measures using image-texture descriptors, and single cells' morphology measurements extracted semi-manually. Recording these as a function of location over prolonged periods allows a coherent and concise depiction of the essence behind three collective motility modes of cancerous cells, and to reveal that Met-activation by HGF/SF induces elaborated collective cell motility that is correlative to the EMT-MET morphological transition.

Materials and Methods

Cell Lines/Growth

DA3 cells, derived from the mouse mammary adenocarcinoma cell line D1-DMBA-3, induced in BALB/C mice by dimethyl-benzanthracene [31] were grown in DMEM supplemented with 10% heat-inactivated FCS (Gibco ± BRL).

Wound Healing Assay

DA3 cells expressing the fluorescent protein mCherry were grown to 90% confluence in wells of 2 cm² diameter and treated with or without the Met inhibitor PHA665752 [32] (2.5 μM) for 2 hours. A scratch of approximately 300 μm in width was generated using a 200 μl tip, and the cells were incubated with or without HGF/SF (80 ng ml⁻¹) and subjected to time lapse confocal laser scanning microscopy (CLSM-510, Zeiss, Germany) for approximately 26 hours, at frequency of once every 14.5 minutes. The position of each scratch was predefined, and a macro that repetitively positions the microscope on each point was executed. The acquired differential interference contrast (DIC) channel of the time-lapse sequence was used for the multi-cellular analysis; the red fluorescence channel was exploited for single cell tracking.

Phase in the healing process. Three phases were defined (Figure 1). Phase 1, from the scratch formation until first contact between cells from opposing wound edges; Phase 2, from first contact until full closure of the wound - the wound area is completely covered by one layer of cells; Phase 3, post wound closure. The effect of HGF/SF on cellular motility patterns, morphology and multi-cellular texture were evaluated according to the three healing phases. Dividing the healing process to phases is important since the duration of each phase varies between the treatments.

Velocity Magnitude Map – Fully Automated, Objective, Multi-Cellular Motility Visualization and Representation

Motility measurements were extracted via a fully-automated algorithm that quantifies local motion estimation from the time-lapse bright field (DIC) channel. The algorithm includes segmentation to partition a DIC image to multi-cellular- and background-regions, followed by local-motion estimation and quantification of the extracted motion fields' magnitude (which resembles cells' local speed) at different distances from the wound edge. Continuous quantitative description of cells' velocity magnitude as a function of distance from the wound throughout the healing process is achieved by constructing a “velocity magnitude map”.

Given two consecutive DIC frames t, t+1 from the time-lapse sequence.

- Partition the current image (at time t) to a grid of sub-cellular sized local patches.
- Apply motion estimation to retrieve velocity fields estimations for each patch (Figure 2, top rows).
- Segment the image to cellular and background regions, and use the segmented image to define strips, mask containing all pixels at a given distance from the wound edge (Figure 2, bottom rows).
- For a given distance d from the wound edge, calculate the speed of the “average” cell located at d by averaging the velocity magnitude of all pixels in the corresponding strip. This step is repeated for every d.

Examples of two representative velocity magnitude maps, of HGF/SF-treated and untreated cells are presented in Figure 3a; the two vertical lines define the partition to the three healing phases. Detailed description of each step in velocity magnitude map’s construction is found in the Methods S1.

Multi-Cellular Texture Representation

Similarly to the velocity map, each image is represented by the histogram of texture-descriptors in its cellular regions (defined by the segmented image). The Local-Binary Patterns (LBP), known to perform well in face-recognition [33], were applied as the texture descriptor. It is a gray-scale invariant texture measure: for every pixel, a code is generated based on the number and location of

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neighboring pixels with higher and lower intensities than that pixel. There are ten possible codes, and their histogram over all cellular pixels is used to describe the image’s texture (Figure S1), an implicit measure for cells’ morphology that is similar to the one for cell scattering [34]. Thus, a time-lapse experiment is again represented by a two-dimensional map: the x-axis represents time, whereas the y-axis is the LBP histogram.

**Treatment Prediction for Wound Healing Time-Lapse Experiments**

To enable automatic prediction whether a full DIC time lapse wound healing experiment was or was not treated with HGF/SF, the 3-dimensional time-lapse (image space and time) was "compressed" to a 2-dimensional representation, the velocity magnitude map described above. This compact description was further represented by a one-dimensional descriptor vector as follows: distances from the wound were partitioned to 6 intervals, and the average speed of all cells in any given interval during the 3 phases in the healing process were used to define a vector representation of a time lapse wound healing experiment (Figure S2a). To cancel out the effect of the general increased motility induced by HGF/SF, a second representation was achieved similarly, by normalizing these vectors to be of norm 1. Similar predictions were performed with multi-cellular texture representation (Figure S2b), using the average texture-descriptor (described above) from first contact between cells from opposing edges of the wound until full closure is achieved (Phase 2).

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**Results**

**Motility Analysis**

Qualitative comparison of the velocity magnitude maps visualization between untreated and treated cells revealed the unique motility patterns induced by HGF/SF (Figure 3a). Front layers of untreated DA3 cells move faster than those located behind, demonstrating a homogeneous motility pattern during the wound healing process (Phases 1 and 2). During post wound closure (Phase 3), all cells decelerate regardless of their position. HGF/SF treatment leads to emergence of dramatic different cell motility patterns: at the beginning, front cells move faster than distant cells. Throughout Phase 1, distant cells gradually join the rapid motion by accelerating layer by layer. This gradual acceleration continues during Phase 2, where distant cells maintain a significantly higher motility toward the wound than cells located closer to the wound edge (data not shown). Finally, post wound closure (Phase 3), front cells halt, while distant cells gradually decelerate. These results demonstrate that Met-activation via HGF/SF induces complex motility patterns indicating cell-cell coordination and dynamic signaling that generates micro-differentiation in the healing wound.

To further demonstrate these phenomena, an alternative visualization is presented in Figure 4a–b, taking into account the direction perpendicular to (toward) the scratch. "Average" cells (calculated as indicated in Materials & Methods) at several locations (25–335 μm from the wound edge) were selected and "tracked" throughout the healing process. The distance that an "average" cell travels in each frame (retrieved from the velocity...
fields’ estimation) was accumulated to define its displacement as a function of time. Indeed, this measure is not exactly the actual displacement, as it contains algorithmic “noise”, cells deformations and proliferation. Nevertheless, since the errors and noise occur in all “directions”, we assume that this measure is an approximated representation of the true dynamics. Figure 4a–b displays this
displacement measure (denoted R) as a function of time until full closure (Phases 1 and 2). It is shown (Figure 4a) that untreated cells express a "fan-like" dynamics, where front cells expand a physical gap from cells behind, a gap that grows steadily during healing. As for treated cells (Figure 4b): during Phase 1, a gap is formed between front and distant cells, but cells from behind progressively accelerate so that this displacement-gap shrinks or at least remains constant during Phase 2 for cells located about 10 cell-layers behind the leading edge. Figure 4c–d plots the average velocity component toward the wound over time. Untreated cells exhibit roughly constant velocity, front cells being faster than farther cells (Figure 4c). The gradual acceleration of distant HGF/SF-treated cells throughout Phase 1, and the higher velocity maintained by distant cells compared to front cells is displayed in Figure 4d. These conclusions referring to an estimation of the "average" cells' velocity over time are supported by single-cell tracking experiments as shown in Figure 3c.

To validate the local motion-estimation, which is a fundamental component in our analysis, we compared manually-validated fluorescence-based semi-automated single cells tracking to fully-automated single-cell trajectory estimation extracted using these local motion-fields (as described in Methods S1). These trajectories are highly correlative to the manually-validated trajectories (Figure 5a). Moreover, examinations of corresponding multicellular versus single-cell based velocity maps (Figure 3a-b) clearly demonstrate a significant qualitative advantage of the former approach: using noisy estimation of all cells (Figure 5b) enables an enhanced and a more coherent representation of the true nature of the process.

Single-Cell Morphology Analysis

Figure 6a illustrates the average cell’s area as function of distance from the wound over the different healing phases for untreated (left) and treated (right) cells (as described in Methods...
In addition to the predefined three healing phases, a fourth time point was added, which represents the last frame in the time-lapse and is used to demonstrate the final stages of the healing process. The x-axis consists of 4 different distance intervals from the wound edge, the y-axis is the average cells' area at a given phase and distance-interval. Until full closure (Phases 1 and 2), untreated cells that are close to the wound are larger than distant cells. Front cells shrink upon wound closure, and after the wound has healed all cells in the monolayer shrink to maintain approximately the same size independently of their location. Similarly to its effect on cells' speed, HGF/SF treatment induces dramatic changes in the dynamics of cellular morphology. At the initial stage, close cells are larger than distant cells. During Phase 2, front cells begin to shrink while farther cells grow. In Phase 3, only the most distant cells continue to grow while the rest shrink. After the wound has healed, all cells have shrunk to approximately the same size. A bar graph that compares treated and untreated cells' area for every distance interval over time demonstrates that the main differences occur in Phases 2 and 3, when treated cells that are located far from the leading edge grow dramatically in a progressive manner (Figure S3a). Similar patterns of morphology alteration are depicted using cell eccentricity. Untreated cells that are close to the wound are more elliptical than distant cells throughout the healing, while front treated cells begin as more elliptical than distant cells, that in turn, during the later stages, become more elliptical than these front cells (Figure S4).

**Cells Density and Motility**

Next, the relation between cell motility and density was examined. Cells density was estimated based on cell size measurements as detailed in Methods S1. Throughout the healing process, the cell density increased, especially in the treated samples. The automated trajectories extracted from local DIC-based motion estimation are highly correlated to the manually-validated trajectories. Examples of untreated (left pane) and HGF/SF-treated (right pane) are illustrated. Figure 5a demonstrates the advantage in using all cells' information in comparison to part of the cells. Green trajectories are the manually-validated trajectories, red are trajectories extracted by our method. Left pane - untreated cells, right pane - treated cells.

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process, untreated cells that are close to the wound's edge (<248 μm) are consistently spread sparsely compared to distance cells (>248 μm) (Figure S3b, left). Treated cells maintain similar location-dependent characteristics to those described for untreated cells during Phase 1. However, upon Phase 2, treated cells "switch" - distant cells become sparsely distributed compared to front cells (Figure S3b, right). Investigating cells' density and motility reveals that, as expected, sparser regions are highly correlated with faster...
Effect of Met Inhibition on Velocity Magnitude Patterns

To investigate the molecular mechanism underlying collective motility and to examine the robustness of our measures, we examine the effect of Met-inhibition on the quantitative measure described above. Velocity magnitude maps of cells treated with the Met inhibitor and HGF/SF were extracted (e.g., Figure 7c), and two SVM-classifiers were trained and tested using "leave-one-out" validation to separate between (6 repeats of) cells treated with the Met inhibitor + HGF/SF and (1) untreated or (2) HGF/SF-treated cells. Since some of the experiments treated with the Met inhibitor + HGF/SF did not achieve full-closure (Phase 2 was not completed), only the first two healing phases were considered (descriptor vector of length 12 per experiment). To exclude the global healing speed and to examine the effect of Met-inhibition on the quantitative patterns for treated and untreated cells (Figure 7a), two SVM-classifiers were trained and tested using "leave one out" validation (p<0.0043, the same a-parametric test). Velocity magnitude maps are 2-dimensional continuous and compact representation of the local motion estimation vector fields during the entire time lapse wound healing experiment. Driscoll et al. [38] recently presented a similar visualization for the spatiotemporal evolution of a cell’s boundary curvature. This concise and coherent visualization demonstrate the alteration of collective motility patterns induced by HGF/SF; we hypothesize that the treatment stimulates cells distant from the leading edge to become self-propelled in an organized and coordinated manner. A major qualitative and quantitative utilization of velocity magnitude maps is the generalization of the change in collective motility patterns as a consequence of adding HGF/SF to the medium with or without Met inhibition. This phenomenon was validated qualitatively, by visual inspection of the velocity magnitude maps, and quantitatively by applying classification, treating them as plain images and extracting appropriate image-features. This measure allows perfect classification based solely on the motility patterns; the relative-role that cells take in collective migration as a function of their location. This means that given a full time lapse wound healing experiment, and two time points representing the three healing-phases, it is possible to predict with

Discussion

Collective cell migration mechanisms are important for normal and pathological biological processes. We propose a quantitative hybrid measure that incorporates fully automated cellular spatiotemporal motility and indirect morphological measures together with semi-automated direct morphological measures to describe the kinetics of collective cells migration. Applying this analysis, we demonstrate that HGF/SF dramatically alters the morpho-kinetic dynamics of the healing wound: from a simple model in which the front cells lead the healing at constant speed; to a more elaborate model in which cells lead a coordinated increased motility along with spatiotemporal phenotypic EMT-MET-like collective cell motility dynamics (Figure 8). Met-inhibition experiments demonstrating inhibition of cell motility, validated the important role of HGF/SF-induced Met activation in breast cancer metastasis.

Traditionally, velocity fields are extracted by tracking individual cells during a time-lapse experiment [35]. Practically, single cell tracking in a monolayer requires considerable labor and can be usually performed only for a small number of cells, providing limited statistical coherency. Our method does not require single cell tracking nor fluorescent-based imaging and is fully-automated. The proposed collective cell migration morpho-kinetic analysis is based on local motion estimation, an approach well suited for DIC images, where inner cellular regions maintain high textural information enabling accurate motion-estimation at the patch level without further processing [8]. The main motivation behind it is the ability to process all cells within the monolayer; the dynamics of collective motility is complex, understanding the individual cell in more detail does not necessarily explain the collective kinetics of a monolayer of cells [36]. Another important advantage is the ability to be performed in high-throughput settings, such as suggested by Yarrow et al. [37]. Velocity magnitude maps are 2-dimensional continuous and compact representation of the local motion estimation vector fields during the entire time lapse wound healing experiment. Driscoll et al. [38] recently presented a similar visualization for the spatiotemporal evolution of a cell’s boundary curvature. This concise and coherent visualization demonstrate the alteration of collective motility patterns induced by HGF/SF; we hypothesize that the treatment stimulates cells distant from the leading edge to become self-propelled in an organized and coordinated manner. A major qualitative and quantitative utilization of velocity magnitude maps is the generalization of the change in collective motility patterns as a consequence of adding HGF/SF to the medium with or without Met inhibition. This phenomenon was validated qualitatively, by visual inspection of the velocity magnitude maps, and quantitatively by applying classification, treating them as plain images and extracting appropriate image-features. This measure allows perfect classification based solely on the motility patterns; the relative-role that cells take in collective migration as a function of their location. This means that given a full time lapse wound healing experiment, and two time points representing the three healing-phases, it is possible to predict with
high accuracy the treatment applied to cells. This ability is a substantial improvement over the standard measures, usually only showing correlation between treatment and phenotype. Thus, we address Tambe et al.’s call [12], "…our understanding of collective cellular migration lacks predictive power and remains largely descriptive".

It is demonstrated that marking a small number of cells within a monolayer can be sufficient to reveal cellular morphology-dynamics. This analysis was applied to demonstrate the high

Figure 7. Generalization: multi-cellular speed- and texture-based classification. (a) Velocity magnitude-based vector-representation of a full time lapse sequence. Each column represents a single experiment. The vector values were calculated as the average velocity magnitude of all cells at a given distance-interval from the wound, at a given phase. The analysis demonstrates that the first six experiments (untreated) are very different from the last five (HGF/SF-treated). (b) Texture-based vector-representation of a full time lapse sequence. The LBP image-texture descriptor normalized histogram is averaged over all time frames from Phase 2, when most morphological changes occur. Each column is the LBP histogram extracted from a single experiment. It was demonstrated that there exists a clear discrimination between any pair of the three conditions: untreated, HGF/SF, and Met inhibition + HGF/SF. (c) Example of a velocity magnitude map of cells treated with Met inhibition and HGF/SF. (d) Collective motility patterns of full time lapse experiments. Each column represents the normalized spatio-temporal velocity magnitude of a single experiment. It was demonstrated that there exists a clear discrimination in collective motility patterns between any pair of the three conditions: untreated, HGF/SF, and Met inhibition + HGF/SF, as Met-signaling becomes more active, the ratio between motility of distant cells and close cells decreases which implies that cells located farther from the wound become more active by Met-signaling activation.

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cells located closer to the wound edge. Finally, post wound closure (Phase 3), front cells shrink, round up and halt, while distant cells gradually decelerate, and change morphology in a similar manner. It is hypothesized that accelerated proliferation at the leading edge is the answer for the untreated cells gap mystery presented in the text. It is hypothesized that in treated cells proliferation occurs more intensively, but is spread approximately equally throughout the monolayer.

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It was demonstrated here that texture of a single image from time lapse sequence, captured by the LBP descriptor, is sufficient to predict the treatment. The image-texture of a monolayer of cells can thus be used to implicitly measure cells morphological characteristics, as in measuring cell scattering [34]. We hypothesize that when considering large numbers of cells in a monolayer, relations between neighboring pixels' intensities represent indirectly morphological characteristics of these cells. Indeed, the average texture descriptor of image frames from Phase 2 discriminate between cells untreated, HGF/SF-treated and treated with MET inhibitor and HGF/SF together, concordant with the data indicating that the morphological changes mostly occur during this phase. Thus, using image-texture as an indirect multi-cellular morphology descriptor can be exploited as a treatment-predictor. Further investigation should try to find a direct connection between cells' morphology and texture.

The combination of image texture and cellular velocity magnitude descriptors may serve as fully automated quantitative measures to represent morpho-kinetic dynamics, to enable in principle high throughput analyses without human intervention.

It is noteworthy that the in vitro model of tumor cells moving collectively studied herein does not take into account important parameters that maintain a crucial role in biological processes that include collective motility such as 3D motility and the tissue's microenvironment (e.g., extracellular matrix resistance [39,40,41]). These parameters have prominent effects on collective cell migration in embryogenesis [1], tumor invasion [1,42], and tubulogenesis [39,40,43]. However, important benefits in using in vitro models to study cellular and molecular mechanisms are controlling starting-point definition and ability to perform high-throughput screening and analysis [1].

The uneven velocity of DA3 cells in collective migration toward the wound in vitro is not associated with formation of finger-like structures [44] as in collective migration of MDCK cells [5]. The displacement gap formed between front and distant cells during untreated DA3 experiments, visualized by the fan-like dynamics (Figure 4a), contradicts the fact that the monolayer is kept continuous with no visible gaps throughout the healing process. Morphology transitions alone cannot account for this phenomenon, since cells' growth is insignificant in the gap formed by non, since cells' growth is insignificant in the gap formed by another indication for being self-propelled. We hypothesize that the lower (although still significant) morphology-motility correlation of treated cells is explained by their self-propelled nature, causing lower dependence on their surroundings.

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Figure 8. Model for HGF/SF-induced collective motility patterns during the healing process. HGF/SF dramatically alters the morpho-dynamics of the healing wound: from a simple model, in which the front cells lead the healing in constant acceleration, to a more elaborate model in which cells in different distances from the wound lead a coordinated increased motility along with spatio-temporal phenotypic EMT-MET based collective cell motility dynamics. Untreated DA3 front layers cells are larger, more elliptical and move faster (marked by wider arrows) than cells located behind demonstrating a homogeneous motility pattern during the wound healing process (Phases 1 and 2). During post wound closure (Phase 3), all cells decelerate, shrink and round up regardless of their position. HGF/SF treatment leads to the emergence of dramatic different cell motility patterns: at the beginning, front cells are larger, more elliptical and move faster than distant cells. Throughout Phase 1, distant cells become larger, more elliptical and gradually join the rapid motion by accelerating layer by layer. These morphology changes and gradual acceleration continues during Phase 2, were distant cells maintain a higher velocity toward the wound than
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Poujade et al. [3]. Since HGF/SF induces accelerated proliferation [45], we believe that it is spread approximately equally throughout the monolayer under treatment. This hypothesis, complementing the motility pattern description is also illustrated in Figure 6.

EMT is a process that changes proliferating cells from an applanatic state to a motile state [46,47], which allows cancer cells to leave the primary tumor and metastasize. The dramatic changes in cell morphology and behavior here is reminiscent of the EMT. It is thus likely that HGF/SF treatment leads to a more pronounced and accelerated morphological EMT, followed by an accelerated phenotypic MET post wound closure, which validates similar results regarding Met-induced EMT [48].

Analyses of Met-inhibited experiments indicate that endogeneously-activated over-expressed Met plays an important role in collective cell motility and further validates involvement of Met-signaling pathway in this process. These results are coherent with recent findings by Loerke et al [49], connecting cell speed to cell-cell adhesion upon HGF/SF-induced Met-signaling.

Matsubayashi et al. have demonstrated that a "wave" of increasing velocities propagates back from the leading edge during monolayer wound healing of mIMCD3 mouse kidney epithelial cells [50]. Recently, Serra-Picamal et al. demonstrated a similar phenomenon for MDCK cells, also showing that stress forces gradually propagate from the leading edge backwards. They suggested that progressive cell mobilization away from the leading edge is a general response of cell collective motility [51]. We argue that this "wave" is induced by specific signal transduction; it is induced by HGF/SF, and reduced by Met-inhibition in breast cancer cells.

Collective motility should thus not be explained from a mechanical perspective alone; increased efforts should be invested in understanding the effects of various chemical signaling, which constitute a significant role in collective cell motility as demonstrated herein and in other studies (e.g., [9,35]). Revealing the effect of Met-signaling on collective morpho-kinetic patterns is crucial to understand the molecular and cellular mechanisms behind metastasis. The ability to predict that a group of cells maintain a dynamical metastatic signature can have therapeutic implications in the long run; this may turn to be a first step in that exciting avenue.

Supporting Information

Figure S1 Multi-cellular texture-based classification. Local Binary Patterns (LBP) applied as an image texture descriptor. For every pixel in the image, a code is generated based on the intensities of neighboring pixels with relation to it. There are ten possible codes, and their histogram over all cellular pixels is used to describe the image’s texture which is used as an indirect descriptor of cells morphology.

(TIF)

Figure S2 Compact representations of a wound healing experiment. (a) Velocity-magnitude based vector representation of a wound healing experiment. Distances from the wound were partitioned to 6 intervals. The average motility of all cells in any given interval during each healing phase was recorded to define a length 18 vector representation. These values are calculated by taking the average intensities of the corresponding rectangular regions in the velocity magnitude map. (b) Texture-based vector representation of a wound healing experiment as an implicit measure for cells’ morphology. LBP normalized histogram is extracted for every image in the time-lapse sequence. All histograms of frames in Phase 2, where most morphological changes occur, are averaged to define the combined texture descriptor. (c) First two components of the principal component analysis (PCA) performed on the normalized velocity-magnitude based vector representation was not sufficient.

(TIF)

Figure S3 Single cell morphology (area) as function of time and distance from the wound. (a) “Average” cell’s area at different distances over time. Same data as presented in Figure 4, shown with different visualization. It can be seen that most morphological alterations occurs for HGF/SF-treated cells far from the wound at the later stages of healing. (b) Estimated density as function of time for close (<248 μm, red markers) and far (>248 μm, green markers) cells. Throughout the healing process, untreated cells that are close to the wound's edges are consistently spread sparsely compared to distance cells. During Phase 1 treated cells maintain similar location-dependent characteristics to those described for untreated cells. In Phase 2, treated cells "switch" - distant cells become sparsely distributed compared to front cells.

(TIF)

Methods S1 In this document we provide a detailed description of the following methods: (1) velocity magnitude map, (2) fully-automated single cell trajectory-estimation from DIC-based motion vector fields, (3) semi-automated single cell tracking, (4) velocity magnitude map based on semi-automated single cell tracking, and (5) single-cell morphology measures.

(OCX)

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Author Contributions

Conceived and designed the experiments: SN IT AZ. Performed the experiments: SN. Analyzed the data: AZ. Wrote the paper: AZ EBJ IT.
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