Published in final edited form as:

Intravital. 2013 April 1; 2(2): e25294. doi:10.4161/intv.25294.

Intravital multiphoton imaging reveals multicellular streaming as a crucial component of in vivo cell migration in human breast tumors

Antonia Patsialou^{1,*}, Jose Javier Bravo-Cordero^{1,2}, Yarong Wang¹, David Entenberg², Huiping Liu³, Michael Clarke⁴, and John S. Condeelis^{1,2}

¹Department of Anatomy and Structural Biology; Albert Einstein College of Medicine; Bronx, NY USA

²Gruss Lipper Biophotonics Center; Albert Einstein College of Medicine; Bronx, NY USA

³The Ben May Department for Cancer Research; University of Chicago; Chicago, IL USA

⁴The Institute for Stem Cell Biology and Regenerative Medicine; Stanford University; Palo Alto, CA USA

Abstract

Metastasis is the main cause of death in breast cancer patients. Cell migration is an essential component of almost every step of the metastatic cascade, especially the early step of invasion inside the primary tumor. In this report, we have used intravital multiphoton microscopy to visualize the different migration patterns of human breast tumor cells in live primary tumors. We used xenograft tumors of MDA-MB-231 cells as well as a low passage xenograft tumor from orthotopically injected patient-derived breast tumor cells. Direct visualization of human tumor cells in vivo shows two patterns of high-speed migration inside primary tumors: a. single cells and b. multicellular streams (i.e., cells following each other in a single file but without cohesive cell junctions). Critically, we found that only streaming and not random migration of single cells was significantly correlated with proximity to vessels, with intravasation and with numbers of elevated circulating tumor cells in the bloodstream. Finally, although the two human tumors were derived from diverse genetic backgrounds, we found that their migratory tumor cells exhibited coordinated gene expression changes that led to the same end-phenotype of enhanced migration involving activating actin polymerization and myosin contraction. Our data are the first direct visualization and assessment of in vivo migration within a live patient-derived breast xenograft tumor.

Keywords

multiphoton imaging; breast cancer; invasion; migration; multicellular streaming

© 2013 Landes Bioscience

*Correspondence to: Antonia Patsialou; antonia.patsialou@einstein.yu.edu.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/intravital/article/25294

Introduction

Metastasis of adenocarcinomas involves the escape of epithelial cells from the primary tumor, intravasation into the blood vessels and lymphatics, extravasation out of the blood vessels and finally, homing to a secondary organ site.¹ For each of these steps of metastasis, tumor cells are believed to acquire unique behaviors and mechanisms that allow them to perform the necessary tasks involved. One particular example is that of cell migration, a molecular function essential for metastasis and especially important in the initial step of the metastatic cascade: invasion in the primary tumor microenvironment.^{2,3} A more in-depth understanding of the mechanisms and behaviors of tumor cell motility during this early metastatic step will enable cancer researchers to design better prognostics and treatments.

Intravital multiphoton imaging has been a valuable tool for the study of cell behaviors and cell signaling in solid tumors, providing enhanced high-resolution visualization at greater depths inside living tissues.⁴⁻⁹ In breast cancer specifically, intravital multiphoton imaging has greatly improved our comprehension of tumor heterogeneity, revealing that only a very small portion of tumor cells are capable of migration and invasion inside a living mammary tumor, even in mouse models of aggressive and metastatic breast cancer.¹⁰⁻¹³ Intravital imaging has also played a pivotal role in defining the specific microenvironments associated with invasion and intravasation in mammary tumors.¹⁴⁻¹⁹ Previous studies with rodent models of breast cancer have shown that tumor cells within primary tumors tend to move in solitary paths along extracellular matrix (ECM) fibers at velocities that are up to 10 times faster than their in vitro counterparts.¹¹ In addition, macrophages were shown to be an essential partner for tumor cell invasion in vivo, due to an EGF/CSF1 paracrine loop between the two cell types, where macrophages sense CSF1 and secrete EGF while tumor cells sense EGF and secrete CSF1.^{14,20} Gene expression profiling of the migratory tumor cells performed in parallel with the intravital multiphoton imaging revealed that conserved changes in mRNA expression occur uniquely in the invasive subpopulation of tumor cells that fall on the NWASP/Arp2/3 complex, and Mena/cofilin pathways,¹⁵ which are required for tumor cell migration and dissemination in vivo.²¹⁻²³

These previous studies that used intravital multiphoton microscopy to describe the primary breast tumor microenvironment were performed mainly in rodent models of breast cancer: xenografts or syngeneic grafts of rat MTLn3 mammary carcinoma cells, or transgenic mouse models of mammary tumors, such as the MMTV-PyMT mice. An open question remains about whether similar migration and invasion behaviors exist in tumors derived from human breast tumor cells. We report here the direct visualization, as well as the gene expression analysis, of migration and invasion in the primary tumor microenvironment of human breast tumor cells. In particular, this is the first intravital imaging report for a low-passage breast tumor derived directly from a patient. Our data demonstrate that human breast tumor cells, even from different genetic backgrounds, exhibit a conserved pattern of cell migration in vivo, namely multicellular streaming, and that this streaming migration correlates with hematogenous dissemination. Finally, coordinated gene expression changes in cell migration pathways occur in the invasive human breast tumor cells that lead to the same end-

phenotype of enhanced migration involving activated actin polymerization and myosin contraction.

Results

Characterization of migration in vivo in human cell-derived mammary tumors

We used in vivo multiphoton imaging to visualize migration in live tumors derived from human breast tumor cells grown orthotopically in mice. We used two types of human breast tumor cells in this study. The first, MDA-MB-231, is an established ATCC breast adenocarcinoma cell line that is widely used in the scientific community for its ability to grow highly invasive and metastatic orthotopic tumors in mice. The second, TN1 cells, was isolated from the pleural effusion of a patient with metastatic triple-negative breast cancer and has previously been shown by us to be invasive and metastatic in mice.²⁴ Both tumor cell types were genetically engineered to stably express GFP (green fluorescent protein).^{24,25} In order to generally evaluate the migratory and invasive properties of these primary tumors in vivo, we performed time-lapse imaging of random $512 \times 512 \mu\text{m}$ fields within the primary tumors spanning a depth of $100 \mu\text{m}$ from the tumor edge (areas with signs of necrosis or apoptosis were excluded from the analysis). Overall, MDA-MB-231 cells were found to be more motile than the TN1 cells, with approximately 93% of all random fields imaged containing at least one single motile cell vs. 70%, respectively (Fig. 1A). Upon further analysis of the 4D imaged tumor areas, we generally observed that both tumors showed two common patterns of migration: a. single cells moving in a random pattern that was uncorrelated with the motion of neighboring cells, and b. multicellular streaming (Fig. 1B and C; Vids. S1–S4). We define multicellular streaming as two or more tumor cells that co-migrate in an organized and directional pattern, following each other in a single file for the duration of the time-lapse movie. We further tracked the centroids of the motile cells over the duration of the 4D time-lapse using the custom image processing plugin ROI_tracker (described previously in ref. 26) and determined that the overall tracks of the streaming cells largely overlap, showing that these cells truly follow each other in the same path (Fig. 1B–C). In this pattern of migration, cell contact is not necessary and therefore gaps of up to two cell diameters can exist between the streaming cells. We have previously shown in MTLn3 rat breast carcinoma cells that expression of the invasion-specific isoform of the protein Mena (Mena^{INV}) leads to increased multicellular streaming in these rat xenograft tumors, and that multicellular streaming is overall a more efficient migration mode, leading to higher velocities and increased path lengths than that observed in randomly moving single cells.¹⁸ We quantified these motility parameters here as well for the streaming vs. the randomly moving single cells in both MDA-MB-231 and the TN1 tumors (Fig. 2A). We show quantitatively that streaming migration in both human breast tumors imaged in this study results in higher velocities and longer net path lengths compared with the randomly moving single cells (Fig. 2A). Directionality, turning frequency, average cell size and number of protrusions were similar between the two types of migration (Fig. 2A; Fig. S1). In addition, the occurrence of either migration pattern was not correlated with cell density or blood vessel density in the imaged tumor fields (Figs. S2–S3). Finally, moving cells show great plasticity and flexibility in their morphology during migration in vivo, with apparent rounds of contraction, at times constricting down to 30% of their average cell area,

then protrusion and expansion in order to propel themselves forward (Fig. 1B and C and quantification of mini-mum cell area in Fig. 2A and Fig. S4). Overall, we confirm in this report that multicellular streaming migration occurs in both MDA-MB-231 tumors and TN1 patient-derived breast tumors and that this migration modality is more efficient in terms of speed and distance covered than randomly moving single cells.

We went on to quantify the frequencies of the single cell and multicellular streaming migration patterns. Overall, approximately 56% of randomly imaged fields showed streaming migration in the MDA-MB-231 tumors vs. 46% of randomly imaged fields in TN1 tumors (again suggesting that TN1 cells are overall less motile than MDA-MB-231 cells) (Fig. 1A). However, when we calculated the number of cells that migrate in either a single cell or in a multicellular streaming pattern as a percentage of the total number of motile cells observed per mouse, then we found that multicellular streaming is a more frequent pattern of migration in both MDA-MB-231 and TN1 tumors. Interestingly, despite the fact that TN1 cells were generally less motile than MDA-MB-231 cells, in both MDA-MB-231 and TN1 tumors 44% of total motile cells migrated as single cells, while 56% migrated as organized multicellular streams (Fig. 2B). This observation suggests that although these two tumors were isolated from different patients and have diverse genetic backgrounds, they overall display very similar patterns of in vivo cell migration.

Stromal cells co-migrate with human breast tumor cells in the multicellular streams

In both of the human cell-derived breast tumors imaged, we observed that unlabeled cells within streams are seen as black shadows silhouetted against the GFP-tumor due to their lack of fluorescence expression. These shadow cells co-migrated with tumor cells in the multicellular streams (Fig. 1B and C). In order to investigate whether the origin of these shadow cells was tumor cells that had lost their GFP expression in vivo or stromal cells that come from the mouse host, we performed FACS analysis of the MDA-MB-231 and TN1 orthotopic tumors. We analyzed the proportion of the total primary tumor that is positive for GFP, as well as the proportion of the total primary tumor that consists of infiltrated mouse host cells (as sorted by pan-mouse surface antigen H-2K^d expression). As shown in Figure S5, less than 2% of TN1 human tumor cells and less than 10% of the MDA-MB-231 human tumor cells have lost their GFP expression in vivo at the time of imaging. If unlabeled tumor cells were part of multicellular streams, this would be a stochastic event and therefore their frequency in streams would be equal to their frequency in the tumors; i.e., 2% chance that unlabeled TN1 tumor cells were part of streams, and 10% chance that unlabeled MDA-MB-231 cells were part of streams. However, almost every multicellular stream we observed in vivo included a “shadow” cell in between the tumor cells. Therefore, we conclude that the likelihood that these shadow cells are unlabeled tumor cells is very small, and that they most likely are indeed stromal cells from the mouse host.

We have previously shown that similar host cells co-migrating with tumor cells in rat MTLn3 tumors and mouse transgenic MMTV-PyMT tumors are indeed tumor-associated macrophages,^{14,18} and that this interaction between the two cell types is dependent upon an EGF/CSF1 paracrine loop.²⁰ We also recently reported that TN1 cells have the ability to spontaneously pair with mouse macrophages in vitro, showing that the two cell types can

interact in a cell-autonomous manner.²⁷ We therefore investigated whether the host stromal cells involved in migration in the human breast tumors imaged in this study are indeed macrophages. First, we performed gene expression analysis of the tumor cells by PCR with human gene-specific primers and found that both MDA-MB-231 and TN1 have a gene expression pattern consistent with the EGF/CSF1 paracrine loop previously reported for rodent models:^{14,20} both human tumor cells express high levels of mRNA for the EGF receptor (EGFR), as well as high levels of mRNA for the CSF1 ligand (Fig. S6). Second, we isolated and characterized the migratory cell population out of the live primary breast tumors. We have previously shown that both MDA-MB-231 and TN1 tumors are invasive *in vivo*, as measured by their ability to chemotax to EGF in an *in vivo* invasion assay.^{24,25} The *in vivo* invasion assay can capture the migratory tumor cells out of a live primary tumor, by their ability to chemotax toward known growth factors or chemokines, invade through the tumor matrix and migrate over long distances to enter the collection microneedle.²⁸ By use of this assay, we collected the migratory cell subpopulation from MDA-MB-231 and TN1 tumors and used immunofluorescence staining *ex vivo* with cell type-specific antibodies in order to assay which cell types migrated in the device. We used an anti-pan-cytokeratin antibody to identify tumor cells and an anti-F4/80 antibody to identify macrophages.¹⁴ In both MDA-MB-231 and TN1 tumors, macrophages were identified in the collected migratory cell population (approximately 27% macrophages in TN1 and 6% in MDA-MB-231 tumors) (Fig. 2C), with MDA-MB-231 cells showing a smaller ratio of macrophages partly because of their additional CSF1R autocrine invasion capacity.²⁵ Despite the differences in the percentages of migrating cell types, macrophages were the second largest component after tumor cells of the migratory cell subpopulation in both human tumors. In addition, as shown in Figure 3B, macrophages were found to be the cell type between tumor cells in streams observed in FFPE tissue. The above data taken all together, strongly argue that host cells co-migrate with human breast tumor cells in multicellular streams *in vivo* and that these host cells are tumor-associated macrophages.

Multicellular streaming migration *in vivo* is significantly correlated with proximity to blood vessels

Because migration is a key phenotype of the invasive and metastatic cells in breast cancer, and presumably a key phenotype for intravasation and hematogenous dissemination, we were interested in how the migration patterns we observed *in vivo* in the human breast tumors imaged in this study correlated with blood vessels. To assess this, we used multiphoton intravital imaging, where the blood vessels were visualized by intravenous injection of the tumor-bearing mice with Texas-red dextran right before each imaging session. We then imaged 4D random fields containing flowing vessels and analyzed the migration patterns evident in these fields. We quantified the total number of motile cells observed, as well as the number of cells involved in each pattern of migration, in all the imaged fields in which blood vessels were visible in order to get exact data on the correlation of mode of cell migration relative to blood vessels. When migration was quantified overall as total motile cells (independent of the pattern of migration), no correlation with proximity to blood vessels was evident (Fig. 3A). However, if cell migration was separately measured as single cell migration vs. multicellular streaming migration, interestingly only multicellular streaming was significantly positively correlated

with proximity to blood vessels in both MDA-MB-231 and TN1 tumors (Fig. 3A; Vid. S5). In addition, single cell migration appeared anti-correlated with blood vessels, meaning that single cells were more probable to be observed away from blood vessel regions (result significant in MDA-MB-231 tumors, similar trend in TN1 tumors but not significant) (Fig. 3A). Because the imaging depth in our 4D imaged fields was limited to 100 μm , we cannot exclude the possible presence of blood vessels existing deeper than the 100 μm imaged. Their presence however, would only affect the interpretation for motile cells at the deepest slices (at $z = 100 \mu\text{m}$) and further, would lead to an undersampling of our data, making the correlation of streaming with proximity to blood vessels even more significant than we report here. Our data show that multicellular streaming *in vivo* in human breast tumors is significantly associated with a vascularized microenvironment.

In addition to intravital imaging and to further analyze the special correlation of tumor cells, macrophages and blood vessels in the primary tumors *in situ*, we performed immunohistochemistry of fixed and paraffin-embedded MDA-MB-231 tumors. We stained tumor sections with antibodies against pan-cytokeratin, endomucin and Iba1 to identify tumor cells, blood vessels and macrophages respectively. Overall, blood vessels could be seen throughout the tumor tissue, except for necrotic areas (which were excluded from the analysis). Macrophages were visible both around the blood vessels (perivascular macrophages) as well as scattered in between the human breast tumor cells throughout the tumor (Fig. 3B). Close evaluation of the stained sections showed areas where tumor cells and macrophages were involved in linear arrangements with each other and in close proximity to a blood vessel (Fig. 3B, inset). These arrangements resemble the actively motile streams migrating toward blood vessels that we observed by multiphoton imaging in the live tumors.

Macrophage function is required for multicellular streaming and intravasation *in vivo*

We showed that macrophages are present in the migratory cell populations of both MDA-MB-231 and TN1 tumors (Fig. 2C). We also showed that host stromal cells, most likely macrophages, form multicellular streams with tumor cells in both MDA-MB-231 and TN1 tumors and that these streams are significantly more frequent in proximity to blood vessels (Figs. 1B, 1C and 3). These data imply that macrophage function may be important for the formation of multicellular streaming *in vivo*. Additionally, since streaming correlates with blood vessels one could hypothesize that it may correlate with *in vivo* intravasation. We tested this hypothesis, by impairing macrophage function *in vivo* in tumor-bearing mice by treatment with clodronate-liposomes.²⁹ We used intra-vital multiphoton imaging to visualize *in vivo* migration in the clodronate- or control (PBS)-liposomes treated animals, and quantified total motility as well as cells that migrate in a single cell or multicellular streaming pattern. Upon clodronate treatment, we found that in both MDA-MB-231 and TN1 tumors, the percentage of tumor cells involved in multicellular streaming was significantly diminished, while the percentage of cells moving as single cells was now increased (Fig. 4A). These data suggest that macrophage function is important for the organization of motile cells into multicellular streams. We also tested intravasation in tumor-bearing mice treated with either clodronate or control liposomes, by counting the circulating tumor cells in their blood. We found that in MDA-MB-231 tumor-bearing mice, treatment

with clodronate almost completely eliminated the presence of circulating tumor cells in the blood circulation (Fig. 4B). For technical reasons we were unable to confirm this result in the TN1 tumors, due to counts of circulating tumor cells in these tumors close to background levels, most likely because this tumor is less motile and less metastatic than the MDA-MB-231 tumors. Overall, these data support the hypothesis that macrophages co-migrate with tumor cells in multicellular streams and that macrophage function is required for both multicellular streaming and intravasation in human breast tumors in vivo.

Gene expression changes in human migratory tumor cells in vivo are found in pathways involved in actin-polymerization regulated protrusion and cell migration

We have shown here that two genetically different human breast tumors, MDA-MB-231 and TN1, show very similar patterns of migration and invasion in vivo with multiphoton intravital imaging. We have also shown that the presence of these migratory cells may be correlated with hematogenous dissemination. In order to understand in vivo migration in more depth, we sought to determine whether the similar patterns of migration in these two human breast tumors were driven by similar gene expression changes in their cell motility pathways, thereby signifying that specific expression changes in motility pathway genes could predict the migratory and potentially metastatic potential of breast tumors. We isolated the migratory tumor cells from the MDA-MB-231 and TN1 tumors with the in vivo invasion assay and compared them to the tumor cells of the bulk primary tumor (isolated by FACS sorting for GFP-positive tumor cells). We used real-time PCR to assess mRNA expression of genes identified previously by studies in mouse and rat mammary tumors as key regulators of motility pathways.¹⁵ We also assayed the mRNA expression of additional motility genes postulated by a recent expression profiling study³⁰ to be involved in human migratory cells. In both the MDA-MB-231 and TN1 tumors, migratory tumor cells showed gene expression changes in multiple genes that are involved in motility pathways (Fig. 5A). For ease of presentation and interpretation, the gene expression changes for each tumor were superimposed on the known motility pathways in the schematics of Figure 5B (genes that were upregulated are shown in bold red font, downregulated in bold green font, and genes with no significant change in plain black font). From this analysis, it is evident that multiple genes are coordinately regulated in motility pathways in both of the human breast tumors during in vivo invasion. The genes *ENAH* (Mena), *MSN* (moesin), *CDC42*, *RAC1*, *CAP1*, *CAPZA2* (Capping protein α 2), *CNN1* (Calponin 1), *CNN3* (Calponin 3) and *MPRIP* (Myosin Phosphatase Rho Interacting Protein) are coordinately upregulated in both MDA-MB-231 and TN1 migratory cells, while the gene *LIMK1* (LIM domain kinase 1) is coordinately downregulated in both tumors (denoted in Fig. 4B by an asterisk next to the gene name). Interestingly, only subunits of the Arp2/3 complex were oppositely regulated in the mRNA expression between the two tumors. Further comparison of our results to previous studies of migratory tumor cells from rat and mouse mammary tumors¹⁵ show that only two genes from all the motility genes tested are consistently upregulated in all four tumors (MTLn3, MMTV-PyMT, MDA-MB-231 and TN1): *ENAH* invasion-specific isoform (Mena^{INV}) and *CDC42*. This could potentially suggest that these two genes may be main regulators of the migration phenotype in vivo, and therefore potential targets for prognostics or therapeutics. Overall, in many cases, both an inhibitor and an activator within the same pathway were found to be upregulated. While this may seem contradictory, such

coordinated regulation has been shown to lead to overall amplification of a feedback loop in a pathway in order to achieve sustained enhanced activity.^{15,31}

We went on to determine whether the pattern of gene expression in the motility pathways seen in migratory cells from MDA-MB-231 and TN1 tumors contributed to a similar end-phenotype. Protrusion formation is the initial response of tumor cells toward an EGF gradient.³² Protrusion formation is powered by actin polymerization from free actin filament barbed ends and mouse invasive tumor cells show increased barbed end formation upon EGF stimulation.^{15,21} We therefore measured the formation of EGF-induced barbed ends at lamellipodial protrusions of MDA-MB-231 and TN1 tumor cells as an end-phenotype of activity of the motility pathways and actin-cytoskeleton related migration. The number of barbed ends in migratory tumor cells isolated with the *in vivo* invasion assay from live primary tumors was compared with the number of barbed ends generated in response to EGF in the general tumor cell population from the same primary tumors. We found that EGF-induced actin barbed ends at the lamellipodium cell compartment were significantly increased in the migratory tumor cells in both MDA-MB-231 and TN1 tumors (Fig. 6). These data suggest that although gene expression changes in the motility pathway are not completely overlapping between the two tumors tested in this study, they achieve the same end-result, i.e., increased actin polymerization leading to protrusion and cell migration.

Discussion

In this report, we have used intravital multiphoton microscopy to characterize the *in vivo* migration properties of human breast tumor cells in live primary tumors. We used two different breast tumors, an orthotopic xenograft of the highly metastatic breast cancer cell line MDA-MB-231, and a low passage orthotopic xenograft of breast tumor cells isolated from a patient pleural effusion sample (TN1). High-resolution 4D imaging of these two human tumors *in vivo* showed that they both shared common patterns of high-speed migration: a. cells moving as single entities irrespective of their neighboring cells (i.e., random single cell migration), and b. multiple cells following each other in the same direction and in a single file but without cohesive cell junctions (i.e., multicellular streaming). We found that although the two tumors were derived from diverse genetic backgrounds, they exhibited almost identical proportions of either single or streaming cells, suggesting our observations may be generally applied to human breast cancer *in vivo* cell migration. Critically, we found that only multicellular streaming and not random migration of single cells was significantly associated with proximity to blood vessels. Our observations suggest that macrophages were co-migrating with the tumor cells in the multicellular streams in both human tumors analyzed. Functional inhibition of macrophages *in vivo* by clodronate liposomes significantly decreased the frequency of multicellular streams as well as intravasation. Finally, we analyzed the gene expression profile of the migratory tumor cells from both the MDA-MB-231 and the TN1 tumors and found that they both exhibited multiple expression changes in genes regulating cell motility. These gene expression changes were largely common in the two tumors and coordinated to regulate the basic actin motility machinery, leading to the same end phenotype of activated actin polymerization and enhanced migration. To our knowledge, this is the first direct visualization and analysis of migration and invasion *in vivo* of patient-derived human breast tumor cells by multiphoton

intravital imaging. Previous reports have imaged patient-derived tumor cells only as explants in collagen or matrigel lattices in the absence of physiologically relevant chemotactic gradients or tumor microenvironment (e.g.,³³ for breast tumors and³⁴ for melanoma).

Multicellular streaming migration has been described in the past in chemotaxis of *Dictyostelium discoideum*³⁵ and in migration of neural crest cells during development.³⁶ In breast cancer, our group has previously observed multicellular streaming during in vivo invasion in xenografts of MTLn3 rat breast carcinoma cells that overexpress an invasion specific isoform of the cytoskeletal regulator protein Mena (Mena^{INV}).¹⁸ Here we report that multicellular streaming occurs spontaneously in two different human breast tumors grown in mice, one of them derived directly from a patient. Of interest, we found that Mena^{INV} was one of the two genes consistently upregulated in the migratory tumor cells in all four mammary tumor studied in our previous and current report (MTLn3, MMTV-PyMT, MDA-MB-231 and TN1).

We define multicellular streaming as two or more tumor cells that co-migrate in an organized and directional pattern, following each other in a single file. In this migration modality, cell contact, which may temporarily occur, is not necessary. We have also shown that this migration modality is more efficient than randomly moving single cells, achieving higher velocities and longer net path lengths of migration. Multicellular streaming differs from collective migration mainly in two aspects: (1) multi-cellular streaming cells move following each other in a single file while collective migrating cells move as whole clusters or sheets of multiple cell diameters in width, and (2) multicellular streaming cells move without apparent cell-cell contacts or junctions between them while collective migrating cells retain their junctions over prolonged periods of time while migrating (collective migration reviewed recently in ref. 37). Interestingly, we did not observe collective tumor cell invasion in either of the tumors we used in our study. This could be either a property of the two tumor types analyzed in this study, or due to the fact that we collected 4D images of short intervals in order to evaluate high-speed tumor cell movements and therefore, we may not have been able to observe cell migration of much slower speeds reported for collective invasion. Further studies with long interval time-lapse imaging may be needed to address whether the lack of collective invasion in the tumors analyzed in this study is a true property of these tumors, or is due to our short interval time-lapse imaging.

Interestingly, we found that macrophage function was required for the multicellular streaming migration, and functional blocking of macrophages in vivo significantly reduced this pattern of migration. However, single cell migration was increased in these same clodronate liposome treated tumors, which signifies that inhibition of macrophages does not reduce the general capacity of the tumor cells to migrate. Macrophage function thus most likely contributes toward organizing and directing the migrating tumor cells in the observed multicellular streams. One can imagine that when macrophage function is blocked, tumor cells are “lost” and without direction and therefore migrate more in what appears to be an unorganized random pattern. Indeed, we have previously shown that tumor cells and macrophages can spontaneously form pairs in vitro on 1D micropatterned collagen surfaces in a cell autonomous manner.²⁷ All the above suggests a model for in vivo migration where

either gene expression changes, mutagenesis or microenvironmental signals induce the general motile ability of the tumor cells in the primary tumor (i.e., single motile cells), but a second event, such as signaling with the tumor-associated macrophages, gives them directionality to form organized directed streams (multicellular streaming). More detailed future studies will be needed in order to unequivocally test this model.

One of the most novel and significant results of this study is the observation that only multicellular streaming migration, and not migration of single cells, is significantly correlated with a vascularized microenvironment. Strikingly, functional inhibition of macrophages *in vivo* almost completely abolished intravasation. Since we showed that macrophage inhibition reduced streaming migration while enhancing single cell migration, one can hypothesize that it is the multicellular streaming migration that contributes to intravasation, and thus metastatic dissemination. Overall, our data suggest that the presence of blood vessels or other stromal cells associated with blood vessels are actively contributing to the organization of the motile cells into streams that lead to intravasation.

Immunohistochemistry of fixed tumors shows that macrophages are very abundant throughout the tissue, and that they are located both adjacent to endothelial cells in blood vessels (perivascular macrophages), as well as in between tumor cells. One possible hypothesis is therefore that these perivascular macrophages may provide the initiating chemotactic signal guiding the streams toward the vessels. Alternatively, perivascular macrophages could facilitate chemotactic streaming of cells toward blood vessels and/or intravasation by regulating vascular permeability. We have previously shown by immunohistochemistry staining in patient tumor samples that the frequency of a physical tripartite arrangement of a Mena-overexpressing tumor cell, a macrophage and an endothelial cell (arrangement termed as TMEM or Tumor Microenvironment of Metastasis) can significantly predict systemic hematogenous metastasis in breast cancer patients.³⁸ These data underscore the potential application of markers developed from studies of intravital multiphoton imaging with parallel expression profiling in the development of prognostic tools for metastatic breast cancer. Further studies, aiming to analyze the exact mechanisms controlling chemoattraction of tumor cells to blood vessels and active intravasation, are currently underway.

Materials and Methods

Cell culture

MDA-MB-231-GFP cells were generated as previously described,²⁵ and maintained in DMEM (Invitrogen, cat # 11965–092) with 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂ incubator. Cells were never kept in culture for more than one month, at which point a new vial of low passage cells was revived.

Animal models

All procedures were conducted in accordance with the National Institutes of Health regulations and approved by the Albert Einstein College of Medicine animal use committee. A total of 2×10^6 MDA-MB-231-GFP cells per animal were suspended in sterile PBS with

20% collagen I (BD Biosciences, cat # 354249) and injected into the lower left mammary fat pad of severe combined immunodeficiency mice (SCID) (NCI).

TN1 cells were isolated from a pleural effusion sample from a patient with triple negative breast cancer (ER-/PR-/Her2-). Purified tumor cells were stably labeled to express GFP by infection with high-titer lentivirus while in suspension for 3 to 4 h, then the cells were washed before being injected into mice (described in detail in ref. 24). TN1 cells are a primary sample and therefore cannot be passaged in culture; instead TN1 cells were only passaged in vivo in NOD.SCID mice (Jackson Laboratories) by orthotopic injection of 10^6 cells mixed with 50% matrigel (BD Biosciences, cat # 354234) directly into the lower left mammary fat pad.

All experiments were performed on tumors that had reached 0.7–1 cm in diameter, as we have determined that this is the optimal time window for intravital imaging for the human cell-derived orthotopic tumors. MDA-MB-231 tumors of < 0.5 cm diameter are not yet disseminated nor metastatic (we measured intravasation by counting the number of circulating tumor cells in the peripheral blood of tumor-bearing mice and spontaneous lung metastasis by counting GFP-expressing tumor cells in the lungs by microscopy) (data not shown). Tumors bigger than 1.5 cm become necrotic, which results in technical complications when exposing the primary tumor and subsequent artifacts in the imaging (e.g., necrotic tumor areas with abnormally increased immune infiltration, collapse of the collagen matrix surrounding the tumor etc.).

For the functional impairment of macrophages, tumor-bearing animals were injected in the lateral tail-vein with 200 μ l of either PBS liposomes or clodronate liposomes at 48 and 24 h before experiments. Liposomes were prepared as previously described using a clodronate concentration of 2.5 g/10 ml of PBS.²⁹ Clodronate was a gift of Roche Diagnostics (Mannheim). Phosphatidylcholine (Lipoid E PC) was obtained from Lipoid (Ludwigshafen). Cholesterol was purchased from Sigma. Clodronate liposomes have been previously shown to effectively deplete macrophage function (example in ref. 39).

Intravital imaging

Intravital multiphoton imaging of the MDA-MB-231 and TN1 tumor-bearing mice was performed with methods similar to previous studies^{18,40,41} using an Olympus FV1000-MPE microscope with a 25 $\times$, 1.05 NA water immersion objective with correction collar. The laser-light source consists of a standard femtosecond-pulsed laser system (Mai Tai HP with DeepSee, Newport/Spectra-Physics) used for excitation of fluorophores in the range of 740–950 nm (e.g., CFP, GFP, YFP). The fluorescence and second-harmonic signals generated were collected via a dichroic mirror and sent to three photomultiplier-tube (PMT) detectors to allow detection of second harmonic generation (SHG), GFP and RFP. 70 kDa Texas Red 2 dextran (Invitrogen, cat # D1830) was used to mark the blood vasculature. To generally characterize in vivo migration, we imaged random fields of $512 \times 512 \mu\text{m}$ at 512×512 pixels for a depth of 100 μm (21 slices at steps of 5 μm) beginning at the edge of the tumor. The edge of the tumor was defined as the interface of the GFP labeled tumor cells with the absence of GFP signal. The second harmonic signal was excited at 880 nm and imaged through a filter with a 410–440 nm bandpass. Tumor areas with any signs of necrosis or

apoptosis were excluded from the analysis. In both tumors, images were taken at 2 min intervals for a total of 30 min. Both MDA-MB-231 and TN1 cells analyzed in this study express GFP and were visualized based on their fluorescence expression. Host stromal cells were visualized by their appearance as shadows on the GFP-expressing tumor due to light scattering. Blood vessels were visualized by direct injection of Texas red 70 kDa dextran in the blood circulation through the lateral tail-veins of the mice, exactly prior to an imaging session.

Image analysis

Images were reconstructed in 3D and through time using ImageJ. Cell motility was defined as translocation of a cell centroid by 1 cell diameter (15–20 μm). Single cell migration was defined as the motility of a solitary cell independent of surrounding cells. Multicellular streaming was defined as the organized motility of two or more tumor cells that follow each other in the same path and with the same direction. For quantification of motility, we only quantified images 15 μm apart (although we took images at 5 μm steps) in order to avoid quantifying the same cell more than once. For Figure 1A, we define motility per field as the sum of motile cells in all slices of the same 100 μm -deep optical field. We used a custom ImageJ plugin (ROI_tracker, described in ref. 26) to track the centroid of the motile cells for the duration of the time-lapse movies. Velocity, net path length, directionality, average turning frequency and average cell area were calculated using ROI_tracker, as described previously in reference 18. Directionality is defined as the ratio of net to total path length traveled by the cell during the time lapse (described in ref. 42). Turning frequency is defined as the angle between the trajectory that a cell was projected to follow and the actual path it followed in two consecutive frames (described in detail in ref. 42). Cell density within each imaged field was calculated by using the area threshold tool in ImageJ to segment and measure the area of GFP-positive tumor cells, and then dividing this by the average cell area. We calculated blood vessel density by counting the flowing, texas-red dextran labeled vessels in each imaged field. Motility was defined as positive for proximity to blood vessels if at least one tumor cell of a stream or a single cell was within 25 μm in all three dimensions of the wall of a blood vessel. At least 6–8 areas were imaged from each tumor for better representation. Only fields with visibly labeled vessels were analyzed for the results of Figure 3. Dextran can leak from blood vessels over time during the imaging sessions, so, depending on the timepoint of a specific field being imaged, vessels can be observed as either filled with flowing dextran, or as an empty lumen with only dextran left around the vessel wall (e.g., image in Fig. 3; Vid. S5). Leaked dextran is then taken up by macrophages around vessels (usually at approximately 4 h after intravenous injection).

In vivo invasion assay

Cell collection into needles placed into live anesthetized animals was performed as described previously.^{25,28,30} Cells can only enter the needles by active migration since a block is used to prevent passive collection of cells and tissue during insertion of the needle into the tissue. Cell migration has been demonstrated to be required for cell collection.¹⁴ Human recombinant EGF (Invitrogen, cat # PHG0311) was used as a chemoattractant in the microneedles at final concentration of 25 nM. After 4 h, the needles were removed and the

total number of cells collected was determined by 4',6-diamidino-2-phenylindole (DAPI) staining.

Determination of cell types collected

Typing of the collected cells was done as described previously,¹⁴ using the cell type-specific antibodies rabbit anti-pancytokeratin (Santa Cruz Biotechnology Inc., cat # sc-15367) for carcinoma cells and rat anti-F4/80⁴³ for macrophages. DAPI was used for counting total cells.

FACS analysis and sorting of primary tumors

FACS analysis of the MDA-MB-231 and TN1 tumors was performed in order to determine the proportion of tumor cells that remain labeled with GFP in vivo within the primary tumor. FACS sorting was also performed in both MDA-MB-231 and TN1 for GFP+ tumor cells for the gene expression and barbed end analysis of Figures 5 and 6. Tumors were excised and necrotic areas were trimmed and excluded from the preparation. The tumor pieces were then mechanically dissociated with scalpels and filtered into single cell suspension in PBS/2% BSA on ice. Red blood cells were lysed by resuspending the sample in ACK buffer for 5 min on ice (Invitrogen, cat # A10492-01). Cells were subsequently washed three times with PBS/2% BSA and DAPI was added for viability scoring right before FACS. For labeling of total mouse stromal cells, samples were immunostained with a PE-conjugated anti-H-2K^d antibody (major histocompatibility complex class I antigen, BD Biosciences cat # 553566), as reported previously.²⁴ Samples were finally sorted for DAPI-negative, PE-negative, GFP-positive tumor cells, using a Becton Dickinson FACS Aria high-speed flow cytometer. Analysis of the data was performed using FlowJo.

RNA extraction and real-time PCR

For the comparison of the average primary tumor cells with the migratory tumor cells, amplified total cDNA was used as input in the real-time PCR. The detailed protocol and validation of the technique has been published elsewhere.^{30,44} Briefly, for the bulk tumor cells, tumors were excised, mechanically dissociated into single cell suspensions on ice, sorted for the GFP-positive cells (> 95% purity GFP+ tumor cells) and lysed with the RNeasy Micro kit (Qiagen, cat # 74004). For the migratory tumor cells, cells collected with the in vivo invasion assay were extruded from the microneedles in PBS buffer, span down and then lysed with the RNeasy Micro kit. The contents of the microneedles were individually examined using a microscope in order to exclude samples that would be derived from potential necrotic tumor areas where cells could enter the microneedles because of passive interstitial flow and not active chemotactic migration. Total RNA from the tumor samples was converted to cDNA and amplified with the SMART amplification kit (BD Clontech, cat # 635001). The final amplified cDNA was purified with MinElute columns (Qiagen, cat # 28004). Two ng of total cDNA was used per real-time PCR reaction with gene-specific primers (see Table S1 for primer sequences). Quantitative PCR analysis was performed as described previously,³⁰ using the Power SYBR Green PCR Core Reagents system (Applied Biosystems). Each PCR reaction was performed in triplicate, and the mean threshold cycle (Ct) values were used for analysis. All the genes tested were compared with

levels of the housekeeping gene *GAPDH*. Results were evaluated with the ABI Prism SDS 2.1 software.

Barbed ends assay

MDA-MB-231 or TN1 cells collected with the in vivo invasion assay and bulk tumor cells from the same tumors were plated on gelatin matrices on complete media. Cells were allowed to adhere for 2 h after plating, and then the cells were starved in 0.8% BSA, 0.5% FCS in DMEM for 12–16 h. The next day, the media was changed to L-15/0.35% BSA exactly prior to the experiment. Cells were stimulated with 2.5 nM EGF (Invitrogen, cat # PHG0311). The barbed end assay was performed with MDA-MB-231 and TN1 cells using biotin–actin as described previously.⁴⁵ Briefly, after stimulation for 3 min with EGF, cells were permeabilized with permeabilization buffer (20 mM HEPES pH 7.5, 138 mM KCl, 4 mM MgCl₂, 3 mM EGTA, 0.2 mg/ml saponin, 1 mM ATP, 1% BSA) containing 0.4 μM biotin–actin (Cytoskeleton, cat #AB07-C) for 1 min at 37°C. Cells were then fixed in 3.7% formaldehyde for 5 min, washed once with 0.1 M Glycine for 10 min and blocked with PBS containing 1% FBS, 1% BSA and 3 μM phalloidin (Invitrogen, cat # R415). Cells were stained with Cy5 anti-biotin (Jackson Immuno, cat # 200-172-096) to visualize barbed ends and Arp2 (Santa Cruz Biotechnology, cat#H-84). Images were acquired on a wide-field microscope (Inverted Olympus IX70) by use of a cooled CCD camera (Sensicam QE cooled CCD camera) with a 60× 1.4 NA oil immersion objective and IP Laboratory 4.0 software. For barbed ends quantification at the leading edge, cells were traced and analyzed with a custom designed macro that automates the collection of pixel intensities in the perimeter of the cell, and the averaged fluorescence intensity of the first 1 μm inward from the cell membrane (i.e., the leading edge) was used to calculate the barbed ends intensity.

Immunohistochemistry

Tumors were excised, fixed in formalin overnight, and then embedded in paraffin blocks. Samples for immunohistochemistry (IHC) were sectioned at 5 μm, and deparaffinized in xylene followed by graded alcohols. Antigen retrieval was performed in 10 mM sodium citrate buffer at pH 6.0, heated to 96°C, for 20 min. Endogenous peroxidase activity was quenched by using 3% hydrogen peroxide in PBS for 10 min. Blocking was performed by incubating sections in 5% normal donkey serum with 2% BSA for 1 h. The primary antibody to Iba1, (Wako Chemicals USA, cat # 019-19741) was used at 1:500 for 1 h at room temperature. Primary species (rabbit IgG) was substituted for the primary antibody to serve as a negative control. The sections were stained by routine IHC methods, using HRP rabbit polymer conjugate (SuperPicture HRP Polymer Conjugate Rabbit Primary (DAB), Life Technologies, cat # 87-9263) for 15 min to localize the antibody bound to antigen, with diaminobenzidine as the final chromogen. After washing, the sections were then incubated with Dual Endogenous Enzyme Block (Dako, cat # S2003) for 5 min to quench endogenous alkaline phosphatase. The primary anti-body to endomucin (Santa Cruz, cat # sc-65495) was used at 1:50 for 1 h. Rat IgG2a served as negative control. The secondary antibody (biotinylated antirat IgG), was used at 1:400 for 30 min followed by ABC-AP for 30 min and developed with Vector Blue (Alkaline Phosphatase Substrate kit III, Vector Labs, cat # SK-5300) for 25 min. After washing, endogenous alkaline phosphatase was again quenched with Dual Endogenous Enzyme Block for 5 min. Finally, sections were incubated with

mouse anti-cytokeratin pan (mixture) antibody (Sigma, cat # 2562), used at 1:1000 at 4°C overnight. The sections were then incubated with rabbit/mouse link [Envision G/2 system/AP, Rabbit/Mouse (Permanent Red), Dako, cat # K5355] for 30 min, followed by 30 min incubation with the AP Enzyme Enhancer and developed with Permanent red for 10 min. After washing, the sections were lightly counterstained with light green SF yellowish (Polyscientific, cat # S232B) for 3 sec. Sections were mounted in Vecta mount (Vector, cat # H-5000). Images were acquired using a Nikon Coolscope. Necrotic areas at the center of the tumor were excluded from the analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Dr E. Richard Stanley for the generous gift of anti-F4/80 antibody, and members of the Condeelis laboratory for helpful discussions and advice. For technical help at Albert Einstein College of Medicine, we wish to thank the Analytical Imaging Facility, the Genomics Core facility, the Institute of Animal Studies, the Flow Cytometry Facility (especially Drs Jinhang Zhang, Lydia Tesfa and Olisambu Uche, supported by NCI P30 CA 013330) and the Histotechnology and Comparative Pathology Facility (especially Drs Rani Sellers, Barbara Cannella and Hong Zhang). This work was supported by: NIH RO1 CA 164468 and 100324 and NCI K99 CA160638-01 (HL).

Abbreviations

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
CSF1	colony stimulating factor 1
CSF1R	colony stimulating factor 1 receptor
MMTV-PyMT	mouse mammary tumor virus – Polyoma Middle T antigen
ECM	extracellular matrix
GFP	green fluorescent protein
PBS	phosphate buffer saline
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum
SCID	severe combined immunodeficient
NOD.SCID	nonobese diabetic.severe combined immunodeficient
DAPI	4', 6-diamidino-2-phenylindole
CNN	calponin
MSN	moesin
CAP1	adenylate cyclase-associated protein 1
CDC42	cell division cycle 42

RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
CAPZA2	capping protein (actin filament) alpha 2
MPRIIP	myosin phosphatase Rho interacting protein
LIMK1	LIM domain kinase 1
FFPE	formalin-fixed, paraffin-embedded

References

1. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell*. 2011; 147:275–292. PMID:22000009; <http://dx.doi.org/10.1016/j.cell.2011.09.024>. [PubMed: 22000009]
2. Bravo-Cordero JJ, Hodgson L, Condeelis J. Directed cell invasion and migration during metastasis. *Curr Opin Cell Biol*. 2012; 24:277–283. PMID:22209238; <http://dx.doi.org/10.1016/j.ceb.2011.12.004>. [PubMed: 22209238]
3. Roussos ET, Condeelis JS, Patsialou A. Chemotaxis in cancer. *Nat Rev Cancer*. 2011; 11:573–587. PMID:21779009; <http://dx.doi.org/10.1038/nrc3078>. [PubMed: 21779009]
4. Condeelis J, Weissleder R. In vivo imaging in cancer. *Cold Spring Harb Perspect Biol*. 2010; 2:a003848. PMID:20861158; <http://dx.doi.org/10.1101/cshper-spect.a003848>. [PubMed: 20861158]
5. Hoffman RM. Imaging cancer dynamics in vivo at the tumor and cellular level with fluorescent proteins. *Clin Exp Metastasis*. 2009; 26:345–355. PMID:18787963; <http://dx.doi.org/10.1007/s10585-008-9205-z>. [PubMed: 18787963]
6. Beerling E, Ritsma L, Vrisekoop N, Derksen PW, van Rheenen J. Intravital microscopy: new insights into metastasis of tumors. *J Cell Sci*. 2011; 124:299–310. PMID:21242309; <http://dx.doi.org/10.1242/jcs.072728>. [PubMed: 21242309]
7. Sahai E. Illuminating the metastatic process. *Nat Rev Cancer*. 2007; 7:737–749. PMID:17891189; <http://dx.doi.org/10.1038/nrc2229>. [PubMed: 17891189]
8. Friedl P, Alexander S. Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell*. 2011; 147:992–1009. PMID:22118458; <http://dx.doi.org/10.1016/j.cell.2011.11.016>. [PubMed: 22118458]
9. Timpson P, McGhee EJ, Anderson KI. Imaging molecular dynamics in vivo—from cell biology to animal models. *J Cell Sci*. 2011; 124:2877–2890. PMID:21878495; <http://dx.doi.org/10.1242/jcs.085191>. [PubMed: 21878495]
10. Farina KL, Wyckoff JB, Rivera J, Lee H, Segall JE, Condeelis JS, et al. Cell motility of tumor cells visualized in living intact primary tumors using green fluorescent protein. *Cancer Res*. 1998; 58:2528–2532. PMID:9635573. [PubMed: 9635573]
11. Sidani M, Wyckoff J, Xue C, Segall JE, Condeelis J. Probing the microenvironment of mammary tumors using multiphoton microscopy. *J Mammary Gland Biol Neoplasia*. 2006; 11:151–163. PMID:17106644; <http://dx.doi.org/10.1007/s10911-006-9021-5>. [PubMed: 17106644]
12. Sahai E, Wyckoff J, Philippart U, Segall JE, Gertler F, Condeelis J. Simultaneous imaging of GFP, CFP and collagen in tumors in vivo using multiphoton microscopy. *BMC Biotechnol*. 2005; 5:14. PMID:15910685; <http://dx.doi.org/10.1186/1472-6750-5-14>. [PubMed: 15910685]
13. Pinner S, Sahai E. Imaging amoeboid cancer cell motility in vivo. *J Microsc*. 2008; 231:441–445. PMID:18754999; <http://dx.doi.org/10.1111/j.1365-2818.2008.02056.x>. [PubMed: 18754999]
14. Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res*. 2004; 64:7022–7029. PMID:15466195; <http://dx.doi.org/10.1158/0008-5472.CAN-04-1449>. [PubMed: 15466195]

15. Wang W, Wyckoff JB, Goswami S, Wang Y, Sidani M, Segall JE, et al. Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors. *Cancer Res.* 2007; 67:3505–3511. PMID:17440055; <http://dx.doi.org/10.1158/0008-5472.CAN-06-3714>. [PubMed: 17440055]
16. Kedrin D, Gligorijevic B, Wyckoff J, Verkhusha VV, Condeelis J, Segall JE, et al. Intravital imaging of metastatic behavior through a mammary imaging window. *Nat Methods.* 2008; 5:1019–1021. PMID:18997781; <http://dx.doi.org/10.1038/nmeth.1269>. [PubMed: 18997781]
17. Giampieri S, Manning C, Hooper S, Jones L, Hill CS, Sahai E. Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. *Nat Cell Biol.* 2009; 11:1287–1296. PMID:19838175; <http://dx.doi.org/10.1038/ncb1973>. [PubMed: 19838175]
18. Roussos ET, Balsamo M, Alford SK, Wyckoff JB, Gligorijevic B, Wang Y, et al. Mena invasive (MenaINV) promotes multicellular streaming motility and transendothelial migration in a mouse model of breast cancer. *J Cell Sci.* 2011; 124:2120–2131. PMID:21670198; <http://dx.doi.org/10.1242/jcs.086231>. [PubMed: 21670198]
19. Kedrin D, Wyckoff J, Boimel PJ, Coniglio SJ, Hynes NE, Arteaga CL, et al. ERBB1 and ERBB2 have distinct functions in tumor cell invasion and intravasation. *Clin Cancer Res.* 2009; 15:3733–3739. PMID:19458057; <http://dx.doi.org/10.1158/1078-0432.CCR-08-2163>. [PubMed: 19458057]
20. Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, et al. Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer Res.* 2005; 65:5278–5283. PMID:15958574; <http://dx.doi.org/10.1158/0008-5472.CAN-04-1853>. [PubMed: 15958574]
21. Wang W, Mouneimne G, Sidani M, Wyckoff J, Chen X, Makris A, et al. The activity status of cofilin is directly related to invasion, intravasation, and metastasis of mammary tumors. *J Cell Biol.* 2006; 173:395–404. PMID:16651380; <http://dx.doi.org/10.1083/jcb.200510115>. [PubMed: 16651380]
22. Roussos ET, Wang Y, Wyckoff JB, Sellers RS, Wang W, Li J, et al. Mena deficiency delays tumor progression and decreases metastasis in polyoma middle-T transgenic mouse mammary tumors. *Breast Cancer Res.* 2010; 12:R101. PMID:21108830; <http://dx.doi.org/10.1186/bcr2784>. [PubMed: 21108830]
23. Gligorijevic B, Wyckoff J, Yamaguchi H, Wang Y, Roussos ET, Condeelis J. N-WASP-mediated invadopodium formation is involved in intravasation and lung metastasis of mammary tumors. *J Cell Sci.* 2012; 125:724–734. PMID:22389406; <http://dx.doi.org/10.1242/jcs.092726>. [PubMed: 22389406]
24. Liu H, Patel MR, Prescher JA, Patsialou A, Qian D, Lin J, et al. Cancer stem cells from human breast tumors are involved in spontaneous metastases in orthotopic mouse models. *Proc Natl Acad Sci U S A.* 2010; 107:18115–18120. PMID:20921380; <http://dx.doi.org/10.1073/pnas.1006732107>. [PubMed: 20921380]
25. Patsialou A, Wyckoff J, Wang Y, Goswami S, Stanley ER, Condeelis JS. Invasion of human breast cancer cells in vivo requires both paracrine and autocrine loops involving the colony-stimulating factor-1 receptor. *Cancer Res.* 2009; 69:9498–9506. PMID:19934330; <http://dx.doi.org/10.1158/0008-5472.CAN-09-1868>. [PubMed: 19934330]
26. Entenberg D, Wyckoff J, Gligorijevic B, Roussos ET, Verkhusha VV, Pollard JW, et al. Setup and use of a two-laser multiphoton microscope for multichannel intravital fluorescence imaging. *Nat Protoc.* 2011; 6:1500–1520. PMID:21959234; <http://dx.doi.org/10.1038/nprot.2011.376>. [PubMed: 21959234]
27. Sharma VP, Beaty BB, Patsialou A, Liu H, Clarke M, Cox D, et al. Reconstitution of in vivo macrophage-tumor cell pairing and streaming motility on one-dimensional micro-patterned substrates. *IntraVital.* 2012 [Epub ahead of print]; dx.doi.org/10.4161/intv.22054.
28. Wyckoff JB, Segall JE, Condeelis JS. The collection of the motile population of cells from a living tumor. *Cancer Res.* 2000; 60:5401–5404. PMID:11034079. [PubMed: 11034079]
29. van Rooijen N, van Kesteren-Hendrikx E. “In vivo” depletion of macrophages by liposome-mediated “suicide”. *Methods Enzymol.* 2003; 373:3–16. PMID:14714393; [http://dx.doi.org/10.1016/S0076-6879\(03\)73001-8](http://dx.doi.org/10.1016/S0076-6879(03)73001-8). [PubMed: 14714393]
30. Patsialou A, Wang Y, Lin J, Whitney K, Goswami S, Kenny PA, et al. Selective gene-expression profiling of migratory tumor cells in vivo predicts clinical outcome in breast cancer patients.

- Breast Cancer Res. 2012; 14:R139. PMID:23113900; <http://dx.doi.org/10.1186/bcr3344>. [PubMed: 23113900]
31. Tania N, Prosk E, Condeelis J, Edelstein-Keshet L. A temporal model of cofilin regulation and the early peak of actin barbed ends in invasive tumor cells. *Biophys J*. 2011; 100:1883–1892. PMID: 21504724; <http://dx.doi.org/10.1016/j.bpj.2011.02.036>. [PubMed: 21504724]
 32. Segall JE, Tyerach S, Boselli L, Masseling S, Helft J, Chan A, et al. EGF stimulates lamellipod extension in metastatic mammary adenocarcinoma cells by an actindependent mechanism. *Clin Exp Metastasis*. 1996; 14:61–72. PMID:8521618; <http://dx.doi.org/10.1007/BF00157687>. [PubMed: 8521618]
 33. Nguyen-Ngoc KV, Cheung KJ, Brenot A, Shamir ER, Gray RS, Hines WC, et al. ECM microenvironment regulates collective migration and local dissemination in normal and malignant mammary epithelium. *Proc Natl Acad Sci U S A*. 2012; 109:E2595–E2604. PMID:22923691; <http://dx.doi.org/10.1073/pnas.1212834109>. [PubMed: 22923691]
 34. Hegerfeldt Y, Tusch M, Bröcker EB, Friedl P. Collective cell movement in primary melanoma explants: plasticity of cell-cell interaction, beta1-integrin function, and migration strategies. *Cancer Res*. 2002; 62:2125–2130. PMID:11929834. [PubMed: 11929834]
 35. Kriebel PW, Barr VA, Parent CA. Adenylyl cyclase localization regulates streaming during chemotaxis. *Cell*. 2003; 112:549–560. PMID:12600317; [http://dx.doi.org/10.1016/S0092-8674\(03\)00081-3](http://dx.doi.org/10.1016/S0092-8674(03)00081-3). [PubMed: 12600317]
 36. McLennan R, Teddy JM, Kasemeier-Kulesa JC, Romine MH, Kulesa PM. Vascular endothelial growth factor (VEGF) regulates cranial neural crest migration in vivo. *Dev Biol*. 2010; 339:114–125. PMID:20036652; <http://dx.doi.org/10.1016/j.ydbio.2009.12.022>. [PubMed: 20036652]
 37. Friedl P, Locker J, Sahai E, Segall JE. Classifying collective cancer cell invasion. *Nat Cell Biol*. 2012; 14:777–783. PMID:22854810; <http://dx.doi.org/10.1038/ncb2548>. [PubMed: 22854810]
 38. Robinson BD, Sica GL, Liu YF, Rohan TE, Gertler FB, Condeelis JS, et al. Tumor microenvironment of metastasis in human breast carcinoma: a potential prognostic marker linked to hematogenous dissemination. *Clin Cancer Res*. 2009; 15:2433–2441. PMID:19318480; <http://dx.doi.org/10.1158/1078-0432.CCR-08-2179>. [PubMed: 19318480]
 39. Hernandez L, Smirnova T, Kedrin D, Wyckoff J, Zhu L, Stanley ER, et al. The EGF/CSF-1 paracrine invasion loop can be triggered by heregulin beta1 and CXCL12. *Cancer Res*. 2009; 69:3221–3227. PMID:19293185; <http://dx.doi.org/10.1158/0008-5472.CAN-08-2871>. [PubMed: 19293185]
 40. Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res*. 2007; 67:2649–2656. PMID:17363585; <http://dx.doi.org/10.1158/0008-5472.CAN-06-1823>. [PubMed: 17363585]
 41. Wyckoff J, Gligorijevic B, Entenberg D, Segall J, Condeelis J. High-resolution multiphoton imaging of tumors in vivo. *Cold Spring Harb Protoc*. 2011; 2011:1167–1184. PMID:21969629; <http://dx.doi.org/10.1101/pdb.top065904>. [PubMed: 21969629]
 42. Sidani M, Wessels D, Mouneimne G, Ghosh M, Goswami S, Sarmiento C, et al. Cofilin determines the migration behavior and turning frequency of metastatic cancer cells. *J Cell Biol*. 2007; 179:777–791. PMID:18025308; <http://dx.doi.org/10.1083/jcb.200707009>. [PubMed: 18025308]
 43. Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol*. 1981; 11:805–815. PMID:7308288; <http://dx.doi.org/10.1002/eji.1830111013>. [PubMed: 7308288]
 44. Wang W, Wyckoff JB, Wang Y, Bottinger EP, Segall JE, Condeelis JS. Gene expression analysis on small numbers of invasive cells collected by chemotaxis from primary mammary tumors of the mouse. *BMC Biotechnol*. 2003; 3:13. PMID:12914671; <http://dx.doi.org/10.1186/1472-6750-3-13>. [PubMed: 12914671]
 45. Bravo-Cordero JJ, Oser M, Chen X, Eddy R, Hodgson L, Condeelis J. A novel spatiotemporal RhoC activation pathway locally regulates cofilin activity at invadopodia. *Curr Biol*. 2011; 21:635–644. PMID:21474314; <http://dx.doi.org/10.1016/j.cub.2011.03.039>. [PubMed: 21474314]

Figure 1.

General characterization of in vivo migration in MDA-MB-231 and TN1 human breast tumors by intravital multiphoton microscopy. MDA-MB-231 and TN1 primary tumors were imaged by multiphoton intravital microscopy as described in Methods. (A) Quantification of general motility as average percentage of randomly imaged fields per mouse that contained either any type of motility (single cell or streaming), or streaming over total number of fields imaged. Error bars: SEM. (B) Representative images of cells migrating in either single or multicellular streaming patterns in MDA-MB-231 tumors. Still images shown here were

derived from Videos S1 and S2. (C) Representative images of cells migrating in either single or multicellular streaming patterns in TN1 tumors. Still images shown here were derived from Videos S3 and S4. $n = 4\text{--}6$ fields per mouse imaged, 9 different mice for MDA-MB-231 and 8 different mice for TN1 tumors. In the images: Migrating tumor cells (green) are outlined in white and numbered. Stromal cells (black) co-migrating with the tumor cells in the streams are outlined in red and numbered. Collagen fibers (blue) are imaged by second harmonic generation. Yellow arrows in the leftmost panels denote direction of migration. Rightmost panels show a still image at $t = 30$ min with the cumulative centroid tracks of the motile cells overlaid. Scale bar: 20 μm .

Figure 2.

Quantification of in vivo migration patterns in MDA-MB-231 and TN1 human breast tumors. **(A)** Quantification of average cell velocity, net path length, directionality, average turning frequency, average cell area and minimum cell area for single or streaming moving cells in both MDA-MB-231 and TN1 tumors. Error Bars: SEM, $n = 20$ cells per group from at least 4 different mice, * $p < 0.05$, ** $p < 0.01$ (by Student's t-test). **(B)** Quantification of migration patterns as average percentage of total motile cells per mouse that were involved in either single cell migration or multicellular streaming migration. Error Bars: SEM, $n = 4-6$ fields per mouse imaged, 9 different mice for MDA-MB-231 and 8 different mice for TN1 tumors. **(C)** Immunostaining with cell type-specific antibodies (anti-pan-cytokeratin for tumor cells and anti-F4/8 for macrophages) was performed in migratory cells as collected from MDA-MB-231 and TN1 tumors with the in vivo invasion assay. Results are reported as average percentage for each cell type over the total number of cells collected per mouse. Total cells were counted by nuclear counterstain using DAPI. Data shown for MDA-

MB-231 were re-analyzed from previously reported experiments.²⁵ Data for TN1 were performed in this study for the first time. Error Bars: SEM, n = 3 mice per group.

Figure 3.

Multicellular streaming migration is significantly associated with proximity to blood vessels.

(A) Migration was quantified in vivo in MDA-MB-231 and TN1 tumors by intravital multiphoton microscopy, in tumor-bearing mice where blood vessels were labeled by intravenous injection of fluorescent dextrans. A representative image of a field with multicellular streaming directed close to a blood vessel is shown (still image was derived from Vid. S5). In this image, tumor cells are green, blood vessels are red, and collagen I fibers are blue. Migrating cells are outlined in white and numbered for ease of reference. The yellow arrow indicates the direction of migration. Quantification is shown as average percentage of total migrating cells per mouse either close to blood vessels (vascularized microenvironment) or away from blood vessels (avascular microenvironment). This quantification was repeated separately for the singly migrating cells and the cells migrating in multicellular streams. Error Bars: SEM, $n = 4-6$ fields per mouse imaged, 4 different mice for each MDA-MB-231 and TN1 tumors, $*p < 0.05$, $***p < 0.001$. Scale bar: 20 μ m.

(B) Immunohistochemistry of fixed tissue sections from MDA-MB-231 tumors was performed against pan-cytokeratin for all tumor cells (pink), endomucin for blood vessels (blue), and Iba1 for macrophages (brown), in order to identify the spatial correlation between these three cell populations in tumors in situ. A representative image is shown at

20× magnification at the left panel. The right panel is a magnification of the inset, where a potential multicellular stream involving both tumor cells and macrophages can be seen in close proximity to a blood vessel. BV, blood vessel (blue arrow); TC, tumor cell (pink arrows); M, macrophage (brown arrows). Scale bar: 50 μ m.

Figure 4.

Macrophage function is required for multicellular streaming migration and intravasation in vivo. MDA-MB-231 and TN1 tumor-bearing mice were treated with either control (PBS) liposomes or clodronate liposomes, in order to functionally impair macrophages. **(A)** Quantification of total migrating cells in the treated tumors that follow either a single cell or multicellular streaming pattern. Shown in the graph is the average percentage of cells migrating in either pattern over the total number of migrating cells per mouse. Error Bars: SEM, $n = 4-6$ fields per mouse imaged, 5 different mice for each MDA-MB-231 and TN1 tumors, ** $p < 0.01$, *** $p < 0.001$ (by Student's t-test). **(B)** Intravasation was measured in treated MDA-MB-231 tumor-bearing mice as count of circulating tumor cells in the blood of the mice. Shown is the average number of circulating tumor cells per ml of blood per mouse. Error Bars: SEM, $n = 5$ mice per condition, ** $p < 0.01$ (by Student's t-test).

Figure 5.

Coordinated gene expression changes in the migratory cells from MDA-MB-231 and TN1 primary tumors fall into pathways that initiate protrusive force and chemotaxis. (A) mRNA expression for genes in known motility pathways was quantified in the migratory tumor cells from MDA-MB-231 and TN1 tumors, as isolated with the *in vivo* invasion assay. Results are shown here as relative mRNA expression compared with the bulk primary tumor cells, isolated from the same primary tumors (shown in a log₂ scale for ease of presentation). Error bars: SEM, n = 4 different mice per group, all results shown in this graph are

significant with $p < 0.05$. **(B)** Gene expression changes from the real-time PCR results of panel **A** were superimposed in motility pathway protein maps, for ease of comparison. All genes that are present on the map were assayed by real-time PCR, and if change was not significant the gene is denoted in plain black font. Upregulated genes are in bold red font, downregulated genes are in bold green font, and genes that coordinately regulated in both the MDA-MB-231 and TN1 tumors are denoted by an asterisk next to the gene name. The fold change in expression is shown next to each gene.

Figure 6.

In vivo migratory cells from human breast tumors show increased barbed end activity. The EGF-induced barbed ends at the leading edge were measured in the migratory tumor cells (isolated with the in vivo invasion assay) and the bulk primary tumor cells population (isolated from the same tumors by sorting all GFP-positive tumor cells), in both the (A) MDA-MB-231 tumors and (B) TN1 tumors. Shown are representative images of the immunofluorescence staining of the cells for the actin barbed ends, as well as Arp2 protein for the visualization of the leading edge (traced by dotted white lines). Graphs show

quantification of three independent experiments per tumor, as average intensity of barbed ends in the cell leading edge. Error bars: SEM, n = 20 cells from 3 different mice for each tumor, *p < 0.05, ***p < 0.001 (by Student's t-test). Scale bar: 10 μ m.