Caught in the act: revealing the metastatic process by live imaging

Miriam R. Fein\textsuperscript{1,2} and Mikala Egeblad\textsuperscript{1,*}

The prognosis of metastatic cancer in patients is poor. Interfering with metastatic spread is therefore important for achieving better survival from cancer. Metastatic disease is established through a series of steps, including breaching of the basement membrane, intravasation and survival in lymphatic or blood vessels, extravasation, and growth at distant sites. Yet, although we know the steps involved in metastasis, the cellular and molecular mechanisms of dissemination and colonization of distant organs are incompletely understood. Here, we review the important insights into the metastatic process that have been gained specifically through the use of imaging technologies in murine, chicken embryo and zebrafish model systems, including high-resolution two-photon microscopy and bioluminescence. We further discuss how imaging technologies are beginning to allow researchers to address the role of regional activation of specific molecular pathways in the metastatic process. These technologies are shedding light, literally, on almost every step of the metastatic process, particularly with regards to the dynamics and plasticity of the disseminating cancer cells and the active participation of the microenvironment in the processes.

Introduction

The prognosis for cancer patients is generally poor once cancer cells have metastasized (American Cancer Society, 2011), and metastasis is therefore a major clinical problem. Nevertheless, studies in mouse models suggest that it is an inefficient process, because the majority of disseminating cancer cells never successfully invade a distant organ but die in the vasculature (Wong et al., 2001; Chambers et al., 2002; Hunter et al., 2008; Kourou-Mehr et al., 2008). Genetic changes in the cancer cells and contributions from the microenvironment are both crucial for the establishment of metastatic lesions (Bos et al., 2009; Joyce and Pollard, 2009). The microenvironment plays a role via, for example, the production of growth factors, cytokines and proteases, and both primary and metastatic tumors can recruit host cells, such as bone-marrow-derived macrophages, to promote angiogenesis (the formation of new blood vessels from pre-existing vessels) and invasion (Joyce and Pollard, 2009).

Traditionally, studies on metastasis have relied on measurements made at the end-point of the process: the establishment of micro- or macrometastasis. However, such methods do not address the dynamic aspect of the metastatic process: cells exit the primary tumor, invade the local tissue, enter blood or lymphatic vessels (a process known as ‘intravasation’), and are passively transported to the secondary site where the cells exit the vessels (a process known as ‘extravasation’) and enter the tissue (Fig. 1) (Fidler, 2003; Kim et al., 2009a). To gain insight into the dynamics of metastasis, different types of imaging technologies have been applied to animal models (Box 1). Bioluminescence imaging has been used to track luciferase-expressing cancer cells and thus to determine their ability to metastasize at the organism level. Live microscopy with expression of fluorescent proteins [e.g. green and red fluorescent proteins (GFP and RFP, respectively)] by cancer and stromal cells has provided insights into what types of behavior and cell-cell interactions differentiate metastatic and non-metastatic cancer cells (Fig. 1). Here, we review some of the most important biological insights into the process of metastasis that have been gained through different types of imaging technologies. These insights include how the cancer cells interact with their surrounding microenvironment, the stroma, which refers to all connective tissue within an organ, such as the extracellular matrix (ECM), the vasculature, the fibroblasts and the immune cells.

Leaving the primary site

The first step in the metastatic process is for the cancer cells to leave the primary tumor site. Live imaging has in particular provided insights into how the dynamics of the tumor-stroma interactions at the primary site contribute to metastasis.

Moving through the tissue

Cancer cells initiate dissemination when they breach the basement membrane (an ECM barrier consisting of e.g. laminin, type IV collagen and proteoglycans that surrounds epithelial, endothelial, muscle, fat and nerve cells). Once the cancer cell breaches the basement membrane, it comes into contact with the interstitial ECM, which is rich in fibrillar collagens. Second harmonic generation (SHG) imaging allows for visualization of fibrillar ECM proteins, primarily collagens. Because of their molecular structure, these proteins have the capacity to combine photons from light
Live imaging of the metastatic process

Fig. 1. Live imaging of the metastatic process. Central cartoon summarizes the metastatic stages detailed in A–F. (A) Top: blood (green) and lymphatic (red) vessels in an ear with a transplanted T241 fibrosarcoma cell line overexpressing the growth factor VEGF-C were visualized with simultaneous angiography and lymphangiography (using FITC- and TRITC-conjugated dextrans). Tumor blood and lymphatic vessels were dilated compared with normal ear (not shown). Scale bar: 850 μm. Bottom: GFP-positive T241 fibrosarcoma cells (green) in a lymphatic vessel (arrowheads; red; tetramethyl-rhodamine lymphangiography), traveling from the primary tumor to the cervical lymph node. Scale bar: 100 μm. Reprinted with permission (Hoshida et al., 2006). (B) Angiogenesis imaged in a dorsal skinfold window chamber by fluorescence microscopy after transplantation of GFP-labeled 4T1 mammary carcinoma cells (green) in a BALB/c mouse. Red arrow in the day 2 panel indicates an elongated cancer cell. In the day 8 panel, purple arrows point to microvessels within the tumor (localized in the marked circle), and red arrows point to dilated vasculature outside the tumor. Scale bars: 200 μm. Reprinted with permission (Li et al., 2000). (C) GFP-labeled MTLn3 mammary carcinoma cells (green) move along collagen fibers (purple), visualized by second-harmonic generation (SHG) imaging. Arrows are pointing to carcinoma cells, and arrowheads are pointing to cell-matrix interactions. Scale bar: 25 μm. Reprinted with permission (Condeelis and Segall, 2003). (D) Intravasation of GFP-labeled MTLn3 mammary carcinoma cells (green) into a dextran-labeled blood vessel (red) imaged by multiphoton microscopy. Three cells that have crossed into the blood vessel are yellow, indicated by arrows. Scale bar: 25 μm. Reprinted with permission (Condeelis and Segall, 2003). (E) Imaging extravasation in transgenic zebrafish embryos. Confocal image of a non-extravasating, wild-type (expressing CFP, colored blue) or extravasating and twist overexpressing (co-expressing RFP and colored red) MDA-MB-231 human breast cancer cell. Cells are shown near the intersegmental vessels (ISV) of a zebrafish embryo, inside (in) or extravasated (out) from the vessel lumen. Scale bar: 200 μm. Reprinted with permission (Stoletov et al., 2010). (F) The establishment of a micrometastasis (‘colonization’) in the liver of a BALB/c mouse. Mouse C26 colorectal carcinoma cells (cyan, expressing fluorescent Dendra2) shown by repeated imaging through an abdominal imaging window. Fibrillar collagens (purple) are detected by SHG imaging. Scale bars: 20 μm. Reprinted with permission (Ritsma et al., 2012).
intravasate. The directional migration probably involves the release of chemoattractants from vascular endothelial cells or their supporting cells (Condeelis and Segall, 2003).

Imaging studies have further revealed at least two major types of cellular movement through the ECM: protease-dependent and -independent migration (Madsen and Sahai, 2010) (Fig. 2). Protease-dependent migration relies mainly on membrane-anchored matrix metalloproteinase (MMP) activity (Rowe and Weiss, 2008). There are 23 MMP family members expressed in humans and they play a role in regulating tumor angiogenesis, invasiveness and metastasis by cleaving a diverse group of substrates (Kessenbrock et al., 2010). One MMP in particular, MMP14 [also known as membrane-type 1 matrix metalloproteinase (MT1-MMP)], has been shown to be crucial for cell invasion and morphogenesis in three-dimensional type I collagen matrices used to mimic the interstitial ECM (Hotary et al., 2000; Sabeh et al., 2004). Although expression of MMP14 can confer epithelial cancer cells the ability to invade, animal experiments using tissue recombination between MMP14-proficient and -deficient cancer and stromal cells strongly suggest that MMP14 expressed by stromal cells mediates cancer cell metastasis (Szabova et al., 2008). The mechanism by which stromal MMP14 promotes invasion and metastasis is not clear, but it is likely to involve degradation of fibrillar collagen in the interstitial matrix.

MMP14 is a cell-membrane-anchored MMP, but most MMPs are secreted, commonly by stromal cells (reviewed by Kessenbrock et al., 2010). Several of these MMPs are also important for invasive behavior, either through degradation of structural components of the ECM or through activation of chemokine and growth factors that stimulate invasion (Kessenbrock et al., 2010). Nevertheless, when synthetic MMP inhibitors were tested in the clinical setting, they had no effect on patient survival (Cousens et al., 2002). One likely contributing factor to this failure was that most clinical studies tested the effect of MMP inhibitors on end-stage cancer, after the establishment of metastatic disease. Imaging experiments, however, have offered an alternative, intriguing explanation for the lack of clinical results: when proteases are blocked, cancer cells can compensate for the loss of protease activity by using amoeboid- or mesenchymal-type movement to disseminate into the adjacent tissue as individual cells (Friedl and Wolf, 2003; Wolf et al., 2003; Wolf et al., 2007). Thus, in the absence of proteolytic ECM breakdown, the cells instead change their shape (Fig. 2) and temporarily deform the ECM (not shown) (Friedl and Alexander, 2011; Wolf and Friedl, 2011).

To date, most work on the function of MMPs in invasion has been performed in culture on two-dimensional or three-dimensional matrices, which cannot faithfully recapitulate the full complexity of the ECM or of the different cell types in tumors. However, fluorogenic MMP substrates can now be used to assess the proteolytic activity of MMPs, as well as the efficacy of their inhibition, in vivo, in tumors (Weissleder et al., 1999; Bremer et al., 2001; Littlepage et al., 2010). Future studies will therefore probably give additional insights into the importance, or lack thereof, of specific MMPs in vivo.

Even in tumors generated from an established metastatic rodent mammary carcinoma cell line, only a small proportion (1-5%) of cancer cells are highly motile, and the type of motility (cohesive versus single-cell amoeboid motility) within the tumors is heterogeneous (Giampieri et al., 2009). The heterogeneity in
Live imaging of the metastatic process

motility might reflect heterogeneity within the cancer cell population. However, another possible explanation is that cancer cell motility is influenced by factors from the microenvironment that are unevenly present within the tumor. Transforming growth factor beta (TGFβ) could be one such factor; it is present in the microenvironment and influences migratory behavior of cells. Using fluorescent reporter constructs, it was shown that cells with transient and local activation of TGFβ signaling pathways disseminate as single cells and spread through blood vessels (see supplementary material Movies 2, 3). In contrast, collective migration of carcinoma cells does not rely on TGFβ, and such cells, which maintain contact with each other, move much slower and spread through the lymphatic system (Giampieri et al., 2009) (see supplementary material Movie 4). Thus, these studies used live imaging to directly link activation of a signaling pathway with specific cellular and metastatic behaviors (Giampieri et al., 2009; Giampieri et al., 2010) (Fig. 3).

Interaction with the vasculature and lymphatics

Metastatic spread occurs through the blood and the lymphatic vasculature, and the structure and organization of these vessels therefore impact the rate of metastasis (Fig. 1A,B). The tumor vasculature has an abnormal organization, structure and function compared with the vasculature of normal tissues (Jain, 1988). Live imaging has revealed that tumor blood vessels often are leaky, in part due to incomplete coverage with pericytes, which are specialized cells that wrap around endothelial structures (Jain, 2003; Fukumura and Jain, 2008). In addition, lymphatic vessels inside tumors have a compromised ability to transport fluid or macromolecules, leading to a high interstitial fluid pressure inside tumors (Fukumura and Jain, 2008; Fukumura et al., 2010). Imaging of tumors after anti-angiogenic therapy using optical frequency domain imaging (OFDI; see below) elegantly shows how tumor vessel structure is normalized after treatment (Vakoc et al., 2009) (see supplementary material Movie 5).

One experiment highlighting the importance of vasculature for the metastatic process utilized an imaging window that was surgically inserted over a mouse mammary carcinoma (Kedrin et al., 2008). This allowed for consecutive imaging over several days and was used together with cancer cells engineered to express the photoswitchable fluorescent protein Dendra2. Dendra2 is derived from octocoral Dendronephthya sp. and it photoswitches from a green to red fluorescent state in response to either visible blue or UV-violet light (e.g. 405 nm) (Gurskaya et al., 2006). When the same areas were imaged at 0, 6 and 24 hours after photoswitching, there was a marked decrease over time in the number of red,
photoswitched cancer cells in well-vascularized regions, suggesting that the vascular environment supported the disappearance of the cancer cells through intravasation. In contrast, there were limited changes in the number and location of the photoswitched cells in regions without detectable vessels (Kedrin et al., 2008) (Fig. 4A). Using this photoswitch assay, neural Wiskott-Aldrich syndrome protein (N-WASP), a protein that regulates the polymerization of the actin cytoskeleton, was shown to be necessary for intravasation of cancer cells (Gligorijevic et al., 2012). N-WASP is essential for the formation of invadopodia, which are cell-membrane protrusions with proteolytic activity, and the data therefore suggest that localized proteolysis is necessary for intravasation.

Live imaging has shown that the cancer cells that intravasate are in close proximity to perivascular tumor-associated macrophages (TAMs), suggesting that these cells facilitate the breaching of the barrier provided by either the basement membrane or the cell-cell interactions between endothelial cells (Wyckoff et al., 2004; Wyckoff et al., 2007). The cooperation between macrophages and cancer cells is clinically relevant because a higher number of foci of interacting cancer cells, macrophages and endothelial cells were found in primary breast tumors of patients that developed metastatic disease than in tumors of patients that did not (Robinson et al., 2009). Confocal imaging in an in vitro three-dimensional microfluidic model for intravasation suggests that macrophages

Fig. 3. Signaling pathways in metastasis: the role of TGFβ. Model illustrating how combinations of signals within a primary tumor can direct the mode of cell motility. (A) Non-motile, cohesively packed cells in the primary tumor (no signal). (Bi) Cells receiving pro-motility cues (such as EGF; red circles), but not TGFβ, move cohesively and collectively via the lymphatic route (Bii), and metastasize to the lymph nodes (Biii), where the signal is lost and cells become non-motile. (Biv) Image of an MTLn3E lymph node metastasis constitutively expressing myristoylated Cherry (red) and CFP (cyan) from a SMAD-dependent promoter (not expressed by cells in the metastasis); collagen second harmonic signal is in blue. (C) High TGFβ signals (blue circles), without pro-motility cues, cause loss of cell-cell cohesion. (Di) Pro-motility cues with TGFβ together promote single-cell motility, entry into the blood (Dii), and lung metastases (Diii). (Div) Image of an MTLn3E lung metastasis constitutively expressing myristoylated Cherry (red) and CFP (cyan) from a SMAD-dependent promoter (not expressed by cells in the metastasis); collagen second harmonic signal is in blue. (E) A primary tumor originating from MTLn3E mammary carcinoma cells constitutively expressing myristoylated Cherry (red) and CFP (cyan) from a SMAD-dependent promoter; collagen second harmonic signal is in blue. White and yellow arrows indicate motile cells. The marked area is shown at higher magnification at 0, 4.5 and 9 minutes. Panels Biv, Div and E are reprinted with permission (Giampieri et al., 2009), and cartoons are adapted with permission from the American Association for Cancer Research (Giampieri et al., 2010).
Fig. 4. Advances in imaging technologies. (A) Photoswitching using a mammary imaging window. Tumor cells are labeled with the photoswitchable protein Dendra2. Non-photoswitched cells (green) and photoswitched cells (red) are shown at 0, 6 and 24 hours after the photoswitch in vascular microenvironments. White dotted lines indicate the vessel. The photoswitched cells adjacent to the vessel disappear quickly over time, suggesting that they leave the primary tumor through intravasation. Scale bar: 30 μm. Reprinted with permission (Kedrin et al., 2008). (B) Three-dimensional microscopy using optical frequency domain imaging (OFDI) compared with multiphoton microscopy. Imaging of a murine mammary adenocarcinoma tumor in a dorsal skinfold chamber using OFDI is shown (a,c,e), compared with multiphoton microscopy (b,d,f). c-f are higher-magnification views of the corresponding areas outlined in white on panels a and b. OFDI is superior to multiphoton for visualizing vessels in deeper regions (c,d), whereas multiphoton microscopy has better resolution of finer structures in more superficial regions (e,f). Depth is denoted by color: yellow (superficial) to red (deep). Scale bars: 250 μm. Reprinted with permission (Vakoc et al., 2009). (C) Fluorescence recovery after photobleaching (FRAP). Images of FRAP experiments of GFP–E-cadherin performed at the front of a wound heal assay. Red arrows indicate cell protrusions, and white arrows point to the areas photobleached in the middle panel. Reprinted with permission (Timpson et al., 2009). (D) Fluorescence lifetime microscopy (FLIM) used to monitor uptake of doxorubicin, a chemotherapeutic drug, at the invasive front of a spheroid consisting of mouse mammary MMT-DC cells. Fluorescent lifetime is visualized before (left panel) and after (middle and right panels) treatment and shows a stepwise reduction of fluorescence lifetime. False-color range: 0 to 3.5 ns. Scale bar: 20 μm. Reprinted with permission (Bakker et al., 2012). (E) Use of the gradient index (GRIN) lens with a multiphoton microscope. One end of the GRIN lens is positioned close to the focal plane of the objective lens of a standard multiphoton laser-scanning microscope, with the opposite end inserted inside the animal. A piezoelectric focus control performs fine focusing without moving the GRIN lens. Reprinted with permission (Levene et al., 2004).
increase the permeability of the endothelial barrier through secretion of tumor necrosis factor-α (TNFα) (Zervantonakis et al., 2012).

Lymphatic vessels transport antigens, immune cells and fluids from tissues to the lymph nodes and back to the vascular system. They lack a continuous basement membrane and this contributes to the high permeability of these vessels and probably also to the metastatic spread of cells through lymphatic vessels, a first step in dissemination in many human cancers (Detmar and Hirakawa, 2002; Oliver and Detmar, 2002). Lymph nodes activated by inflammation or tumor growth can be imaged with immunopositrion emission tomography (PET) using radiolabeled antibodies against lymphatic endothelial cell surface markers, possibly allowing for detection of lymphangiogenesis as an early indicator of metastasis (Mumprecht et al., 2010). When fluorescent dextran is injected into tissues and tumors, the lymphatic drainage can be visualized. This has enabled imaging of cancer cells within the lymphatics and of structural changes of tumor-draining lymphatic vessels. Furthermore, by combining fluorescent dextran labeling with fluorescence photobleaching imaging, it was shown that the velocity of the lymphatic fluid that drains tumors was reduced compared with that of normal tissues, but total fluid volume was increased, because of an enlarged vascular diameter (Hoshida et al., 2006). A mouse model allowing dual fluorescent and bioluminescence imaging of the lymphatic vessels, through expression of reporters under the control of the promoter for lymphatic endothelial-specific Vegfr3 (vascular endothelial growth factor receptor 3) (Martinez-Corral et al., 2012), is likely to contribute to further insights into lymphatic metastatic spread.

**Importance of angiogenesis for supporting the metastatic process**

Because dissemination often occurs via blood vessels, angiogenesis supports metastasis (Weidner et al., 1991). Insights into the biology of angiogenesis therefore contribute to our understanding of metastasis. It was previously thought that angiogenesis only started after a tumor mass reached 1 mm in diameter and hypoxia occurred. Using dorsal skinfold window chambers in rats or mice, the dynamics of tumor angiogenesis can be monitored for weeks by repeated intravenous injections with fluorescein isothiocyanate (FITC)-labeled dextran, allowing for assessments of how blood vessel diameter, surface area and branching patterns change with tumor progression (Zhou et al., 2011). This method was used to determine that modification of the normal vasculature occurs already when the tumor consists of only 60-80 cancer cells, and that functional, new blood vessels are established as the tumor reaches about 100-300 cells (Li et al., 2000) (Fig. 1B).

Tumor hypoxia can result in activation of angiogenesis (Harris, 2002). Interestingly, the ability to respond to hypoxia by the induction of angiogenesis might be associated with metastasis specifically to the lungs. The levels of hypoxia were tracked in tumors from two subclones of the MDA-MB-231 mammary carcinoma cell line using firefly luciferase as a reporter for the activity of hypoxia inducible factor-1α (HIF-1α; an indicator of hypoxia levels). Tumors from the cell line that metastasized to the lungs had a strong angiogenic response, and hypoxia levels were reduced as the tumors grew. In contrast, tumors from the cell line that metastasized to the bone had limited angiogenesis, and hypoxia continued to increase as the tumors grew (Lu et al., 2010).

Another model system for analysis and imaging of angiogenesis is the engrafting of human carcinoma cells into the chorioallantoic membrane (CAM) of chick embryos before the full development of the immune system. The CAM is highly vascularized with a dense capillary network, and it rarely exceeds 100 μm in thickness, making it very amenable for the imaging of angiogenesis. Interestingly, the ability of human fibrosarcoma and prostate cancer cells to intravasate and disseminate in this model correlated positively with their capacity to induce angiogenesis and to recruit neutrophils (Bekes et al., 2011).

**Imaging at secondary sites**

Imaging at secondary sites where disseminated cancer cells extravasate and colonize can provide crucial insights into the mechanisms underlying these processes. However, this is inherently challenging because the organs involved (e.g. brain, lung, liver, bone marrow) are often difficult to access while maintaining survival of the animals. Furthermore, getting the timing right for monitoring the establishment of secondary lesions is difficult. However, new methods have recently opened up possibilities in real-time imaging of metastatic sites.

Live imaging of the liver – through repeated exteriorization and stabilization of the left liver lobe in the same mouse – has allowed visualization of the progression of angiogenesis during the establishment of colorectal liver metastases (Tanaka et al., 2012). Another, less invasive, technique was recently developed involving the insertion of an imaging window over abdominal organs, including the spleen, kidney, small intestine, pancreas or liver (Ritsma et al., 2012). Once inserted, the window allows for visualization of the same site for up to 1 month. Using this technology, the establishment of liver metastases was found to involve the extravasation of single cancer cells followed by a proliferative state with high cancer-cell motility in the micrometastatic lesions and reduced motility in the larger lesions (Fig. 1F). Intriguingly, imaging showed that, when the high cancer-cell motility in the small lesions was inhibited, metastatic burden was reduced (Ritsma et al., 2012).

Many mouse models of cancer develop metastasis in the lung, but this organ is particularly challenging to image for several reasons. The alveolar structure of the lung causes a high degree of light scattering and, in addition, the movement of the lung during cardiorespiratory cycles makes imaging difficult (Wagner, 1969). A thoracic window has been developed, which allows imaging for several hours, but not days or weeks, under physiological gas exchange and blood flow. The surface of the lung adheres to the window loosely with 20-25 mmHg reversible vacuum, allowing normal ventilation and physiological blood flow with limited tissue movement in the imaging field. This system has been used to image immune surveillance during lung injury in mice (Looney et al., 2011), and it should be possible to also apply it to study lung metastasis in vivo.

**Homing to distant organs**

It was reported as far back as 1889, by Stephen Paget, that metastases primarily develop in certain organs, and he therefore proposed that the organ environment influenced metastasis

---

*References*

Bekes, T., Liptay, A., Pusztai, A., and Oláh, Á. (2011). Neutrophils are essential for the migration of tumor cells through blood vessels and for the formation of tumor microvessels. Am. J. Physiol. Lung Cell Mol. Physiol. 301, L1106-L1115.

Bekes, T., Pusztai, A., Liptay, A., and Oláh, Á. (2011). Tumor dissemination to the bone marrow correlates with increased angiogenesis. Cancer Res. 71, 7705-7713.

Bekes, T., Liptay, A., Pusztai, A., and Oláh, Á. (2011). Neutrophils are essential for the migration of tumor cells through blood vessels and for the formation of tumor microvessels. Am. J. Physiol. Lung Cell Mol. Physiol. 301, L1106-L1115.

Bekes, T., Liptay, A., Pusztai, A., and Oláh, Á. (2011). Tumor dissemination to the bone marrow correlates with increased angiogenesis. Cancer Res. 71, 7705-7713.

Bekes, T., Liptay, A., Pusztai, A., and Oláh, Á. (2011). Neutrophils are essential for the migration of tumor cells through blood vessels and for the formation of tumor microvessels. Am. J. Physiol. Lung Cell Mol. Physiol. 301, L1106-L1115.

Bekes, T., Pusztai, A., Liptay, A., and Oláh, Á. (2011). Tumor dissemination to the bone marrow correlates with increased angiogenesis. Cancer Res. 71, 7705-7713.

Bekes, T., Liptay, A., Pusztai, A., and Oláh, Á. (2011). Neutrophils are essential for the migration of tumor cells through blood vessels and for the formation of tumor microvessels. Am. J. Physiol. Lung Cell Mol. Physiol. 301, L1106-L1115.

Bekes, T., Liptay, A., Pusztai, A., and Oláh, Á. (2011). Tumor dissemination to the bone marrow correlates with increased angiogenesis. Cancer Res. 71, 7705-7713.

Bekes, T., Liptay, A., Pusztai, A., and Oláh, Á. (2011). Neutrophils are essential for the migration of tumor cells through blood vessels and for the formation of tumor microvessels. Am. J. Physiol. Lung Cell Mol. Physiol. 301, L1106-L1115.

Bekes, T., Liptay, A., Pusztai, A., and Oláh, Á. (2011). Tumor dissemination to the bone marrow correlates with increased angiogenesis. Cancer Res. 71, 7705-7713.
[reprinted in 1989 (Paget, 1989)], the so-called ‘seed and soil’ hypothesis. However, the question of how this preference is established is still not fully understood, although factors intrinsic to the cancer cells as well as factors contributed by the specific microenvironment of different tissues have been identified (Nguyen et al., 2009; Chaffer and Weinberg, 2011). A microenvironmental homing factor in the bone marrow is stromal cell derived factor 1 (SDF-1; also known as CXCL12), a potent chemoattractant for cells expressing the CXCR4 chemokine receptor. Several cancer cell lines were imaged homing to specific regions of the bone marrow endothelium, and disruption of the interaction between SDF-1 and CXCR4 blocked homing of cancer cells to these regions (Sipkins et al., 2005).

In vivo studies in mouse models have shown that VEGFR1-expressing bone-marrow-derived hematopoietic progenitor cells home to sites of future metastases before the arrival of cancer cells (Kaplan et al., 2005). One potential function of such cells could be to enhance the survival of the disseminating cells. Bioluminescence imaging has shown that expression of vascular cell adhesion molecule 1 (VCAM-1) in breast cancer increases their ability to colonize the lung. The VCAM-1-expressing cancer cells were found to interact with macrophages through α4 integrins and this in turn activated a phosphoinositide-3-kinase-dependent survival pathway (Chen et al., 2011a).

A role for another myeloid-derived cell type, the neutrophils, in determining the site of metastasis was suggested in a dissemination model in zebrafish. Zebrafish embryos are optically transparent with an immature immune system that allows for xenotransplantation of fluorescently labeled human cancer cells. Human cells can also be transplanted to adult, chemically immunosuppressed animals (Stoletov et al., 2007; Stoletov and Klemke, 2008). Intravital imaging showed that, although cancer cells disseminated to multiple locations within the embryo, they were only able to extravasate and establish micrometastatic lesions at sites of physiologically high neutrophil transmigration into the tissue (He et al., 2012). Imaging further suggested that the neutrophils aided the invasive process into the tissue by modulating the collagen matrix (He et al., 2012). Intriguingly, the observation in the zebrafish embryo model contrasts with findings from mice that propose that metastasis to the lung is inhibited by neutrophils, as shown by bioluminescent imaging (Granot et al., 2011). These conflicting results could reflect species differences. However, it is also possible that pro- and anti-metastatic effects of neutrophils represent different activation stages of the cell (Fridlender and Albelda, 2012), because neutrophils can promote intravasation in mice (Bekes et al., 2011).

Extravasation
Zebrafish embryos have also been useful for high-resolution imaging of the process of extravasation. It was found that cancer cells do not damage or induce vascular leakage at the site of extravasation, as previously thought, but instead arrest in the capillaries and induce local vessel remodeling. The ability of cancer cells to successfully extravasate is associated with their ability to adhere to the endothelium and migrate along the vascular wall. Time-lapse imaging has shown an increased number of endothelial cells and a thickening of the vascular wall surrounding the arrested cancer cells, as well as remodeling of the cell junctions at the site of extravasation. The expression of Twist, a gene involved in epithelial-to-mesenchymal transition (EMT), increased the intravascular migration and extravasation through the vessel wall of the cancer cells (Stoletov et al., 2010) (Fig. 1E; see supplementary material Movie 6).

Myeloid-derived cells have been linked to increased metastasis and extravasation. Using an ex vivo intact whole lung imaging approach, in which the lung is dissected and placed in a specially designed chamber with artificial ventilation, it was shown that cancer cell extravasation is reduced when monocyte recruitment is blocked with a chemokine (C-C motif) ligand 2 (CCL2) neutralizing antibody (Qian et al., 2011).

Dormancy and establishment of metastatic lesions
Cancer can recur years after initial treatment or removal of the primary tumor, providing clinical evidence that some metastatic cancer cells are dormant at distant sites for long periods of time (Aguirre-Ghiso, 2007; McGowan et al., 2009). However, it is not clear what factors regulate whether the cells stay dormant or eventually start proliferating. The primary tumor might participate in the regulation of distant dormancy: an acceleration of growth of the metastatic lesions after resection of the primary tumor has been reported in mouse models of both hepatic and lung cancer (Qadri et al., 2005; Kollmar et al., 2006). It is possible that this is a reflection of the ability of disseminating cancer cells to home back to the primary tumor (so-called ‘self-seeding’) (Comen et al., 2011): when the primary tumor is removed it no longer acts as a ‘sink’ for disseminating cells, which might instead start to grow elsewhere. Nevertheless, retrospective studies of human patients with end-stage breast cancer show that long-term survival is improved by resection of the primary tumor as compared with those who did not undergo surgery (Fields et al., 2007; Zhang and Yang, 2009).

Imaging can contribute to the understanding of the process of dormancy and reactivation. Mammary carcinoma cells, which occasionally form liver metastases after a long latency, were loaded with fluorescent nanospheres to trace the dilution of the dye, which occurs with cell division. Solitary cells with strong nanosphere signal were found up to 11 weeks after injection, indicating the survival of non-dividing, dormant cells (Naumov et al., 2002). Dormancy of cancer cells that have disseminated to the brain has been suggested by the use of a highly sensitive magnetic resonance imaging (MRI) technique to track cancer cells labeled with micron-sized iron oxide particles. In this model, most of the cancer cells that arrive at the brain disappear, but a small subset (1.5%) go on to form metastases, and a slightly larger proportion (4.5%) persist as undivided, single cells, which might represent a population of dormant cells (Heyn et al., 2006). These types of studies using long-lasting labeling have the potential to uncover the types of cells or environmental factors involved in reactivation after dormancy.

Long-term fluorescent imaging of the brain can be performed in rats and mice by using cranial imaging windows (Yuan et al., 1994). Using such windows, the fate of individual metastasizing cancer cells was followed from minutes to months after intraarterial injection of the cells. Four crucial steps of metastasis formation in the brain were identified: arrest at blood vessels, extravasation, the requirement for the cells to be in a perivascular position in order to advance to micro- or macrometastases and,
Finally, angiogenesis or co-option of preexisting microvasculature. Interestingly, treatment with the angiogenesis-inhibiting antibody to VEGF (bevacizumab) induced long-term dormancy of human lung carcinoma cells, highlighting that angiogenesis is also important at the secondary site (Kienast et al., 2010).

Bioluminescent imaging is commonly used for non-invasive monitoring of the establishment of metastatic lesions. For example, the importance of Src activity for late-onset experimental bone metastasis of breast cancer cells was shown by comparing the outgrowth of bone lesions arising from cancer cells stably expressing luciferase and either Src shRNA or a control shRNA construct (Zhang et al., 2009). Bioluminescent imaging was also used to show that increased expression of vascular cell adhesion molecule 1 (VCAM-1) is important for expansion of dormant micrometastases to overt bone metastasis (Lu et al., 2011). Metastatic lesions, as identified by bioluminescence imaging, were further subjected to two-photon imaging after preparation for ex vivo bone explant cultures. VCAM-1 can bind to integrin αvβ3 on osteoclast progenitors, and imaging of the explant cultures showed that the velocity of cells of the monocytic lineage, probably osteoclast progenitors, was increased when the cultures were treated with an antibody against VCAM-1, probably owing to the inhibition of VCAM-1-mediated cell-cell adhesion. This suggested that recruitment of osteoclasts to dormant micrometastases through upregulated VCAM-1 expression had taken place in vivo and triggered the expansion of the lesions (Lu et al., 2011).

Current limitations

Live imaging in mice, rats, chick embryos and zebrafish has provided insights into cancer metastasis at almost every stage of the process. Yet, there are limitations to these technologies, as discussed below, some of which could be addressed through further technical developments.

Imaging of the metastatic process has mostly been performed for tumors of superficial organs (e.g. skin and breast), and it is not clear whether this is representative of the process in organs that are less accessible to imaging, such as pancreas, colon and ovary. Although there have been promising studies on imaging tumors in pancreas and liver (McElroy et al., 2008; Ritsma et al., 2012; Tanaka et al., 2012), as described above, additional protocols are needed for imaging internal organs, and particularly to image deeper in the tumors. In the brain, this has been achieved using gradient index (GRIN) lenses for deep brain imaging, up to about 1.5 mm below the surface of the cortex. GRIN lenses have a negative gradient in the refractive index of glass from the center of the lens to the outside edge to bend and focus light, and they are capable of resolving optical sections relatively far from the surface of the lens (Levene et al., 2004) (Fig. 4E). Imaging with such lenses might, however, lead to compression of the tissue in front of the lens, resulting in bleeding and tissue edema. Nevertheless, the use of GRIN lenses in imaging probes seems to be a very promising approach for invital imaging of internal organs (Kim et al., 2012). Other approaches are to use a small-diameter (e.g. 1.2 mm) microprobe ‘stick’ objective lens, which has been used to image ovarian cancer within the abdominal cavity in a mouse model (Williams et al., 2010), or to use fiber optic microprobes instead of objective lenses (e.g. Lin et al., 2008). For Imaging of internal organs, tissue motion due to respiration and heartbeat can be very limiting. Techniques to reduce motion artifacts include putting pressure on the organ’s surface (Toiyama et al., 2010), or using a microstage device to stabilize the organ (Cao et al., 2012).

The use of live imaging to gain insight into the biological processes of metastasis has exclusively been done in model organisms. However, it is unclear how well these models mimic the metastatic process in humans. In vivo microscopy in humans is still largely in the early stages, but various microscopic techniques have clinical potential, e.g. for the identification of suspicious tissue regions for biopsy collection (Liu et al., 2011). Confocal endomicroscopes have been used in the clinical setting with injection of non-targeted fluorescein to identify areas of mucosal dysplasia in the esophagus (Johnson et al., 2012). Targeted imaging using an integrin-aβ3-directed near-infrared (NIR) fluorescent probe could potentially be used to guide surgical resection of liver metastatic lesions in colon cancer, because colon cancer cells and tumor vasculature express integrin aβ3, whereas normal liver tissue does not. However, so far this method has only been validated in rodent cancer models (Hutteman et al., 2011). It might also be feasible to use imaging without contrast probes to identify pathological tissue changes by using fiber optic probes with Fourier transform infrared spectroscopy, a technology that can detect vibrations in chemical bonds (Mackanos and Contag, 2010). This technology can potentially distinguish normal from diseased tissue because the signal will differ between regions with different molecular compositions.

New directions

Tracking cancer cells using live imaging has been valuable for determining how cancer cells move throughout the metastatic process. Now, new technologies are set to push the field beyond the observation of movement of cells with, for example, approaches that allow for the manipulation of signaling pathways in cells in real-time in the live tissue using techniques such as photoactivation (reviewed by Timpson et al., 2011).

Primary tumors are heterogeneous, but it is generally believed that metastases originate from a single cell of the primary tumor, as suggested by single-cell sequencing of a primary breast tumor and its liver metastasis (Navin et al., 2011). Indeed, in experimental models, metastatic lesions have a clonal origin, as based on analysis of karyotypes (Fidler and Kripke, 1977; Fidler and Talmadge, 1986), or lentiviral integration sites (Winslow et al., 2011). Heterogeneity in cellular invasive behavior in primary tumors is often reported (e.g. Giampieri et al., 2009; Pinner et al., 2009). Combining live imaging with methods to trace cancer cell lineage, for example using fluorescent-based lineage-tracking techniques (Snippert et al., 2010; Schepers et al., 2012), might enable a deeper understanding of how heterogeneity contributes to metastasis.

EMT is a highly conserved cellular program known to play a role in processes such as mesoderm formation, neural crest development and heart valve development. During embryogenesis, the process is reversible and mesenchymal cells can revert back to an epithelial cell state, for example during the formation of the nephron in the developing kidney (Yang and Weinberg, 2008). It has been suggested that, in order for cancer cells to metastasize, they must undergo EMT and acquire mesenchymal traits (Hardy et al., 2010; Wells et al., 2011). It has also been proposed that a reverse EMT, mesenchymal-to-epithelial transition (MET), is...
necessary to enable metastatic colonization, which might require re-expression of epithelial genes and downregulation of mesenchymal genes (Chao et al., 2011). Imaging could potentially be employed to address the dynamics of EMT and MET in vivo and determine the importance of cellular plasticity in metastasis. One study has taken advantage of the correlation between pigmentation and differentiation of melanoma cells: less differentiated cells with a low level of pigmentation (resembling an EMT-like state) were migratory at the primary site and they intravasated, whereas differentiated and pigmented cells did not. Interestingly, the cells that had established themselves at the secondary site had high levels of pigmentation, suggesting re-differentiation (an MET-like process) (Pinner et al., 2009). Phototoxicity might have contributed to the reduced cell migration of the pigmented cells, because pigmented cells absorb pulsed laser light more strongly than non-pigmented cells. Nevertheless, the study illustrates how imaging can be employed to understand the link between the differentiation state of cancer cells and invasive behavior.

Intravital imaging has been combined with technologies for genomic level analysis of the metastatic pathway only to a limited degree. Genes involved in invasive behavior were identified by live imaging of cells migrating towards a microneedle containing chemoattractants (including EGF) followed by analysis of gene expression profiles of cells collected in the needles (Wyckoff et al., 2000; Wang et al., 2003; Wang et al., 2005). Such approaches could now be further developed to link cellular behavior in vivo with a molecular level analysis, because single-cell genomic level analysis has become feasible (Navin et al., 2011).

The use of imaging techniques to determine the status of signaling activities within single cells is well established in cell culture settings and has great potential for in vivo imaging, although it is at this point still largely in its infancy. Adaptation of these techniques for in vivo imaging will be useful to gain insights into the dynamics of signaling activity and its relationship with metastatic cellular behavior. However, this has so far been challenging because the ratio of signal-to-noise often is reduced in vivo, in part due to scattering and absorption of light in the live tissue (e.g. Handmaker, 1975; Cuccia et al., 2009). Furthermore, maintaining tissue viability and stability is also more difficult than for cell culture systems, where more optimal imaging conditions can easily be achieved.

Techniques for molecular level imaging that have been adapted from the cell culture setting and applied in vivo include fluorescence recovery after photobleaching (FRAP). With this technique, fluorescently tagged fusion proteins are used to track changes in protein mobility after photobleaching, the photochemical destruction of fluorophores (Fig. 4C). In vivo, this was used to show that the mobility of E-cadherin in cell membranes is lower in migratory cells than in stationary cells, and that inhibition of migration, through inhibition of Src, FAK or β1-integrin, reduces collective cell movement and increases E-cadherin membrane mobility (Serrels et al., 2009; Canel et al., 2010). These studies show the potential for using FRAP to understand how activation of specific signaling pathways influences cell migration, an important component of the metastatic process, in vivo.

To monitor the activation stage of signaling molecules in real time, including the activation of Rho-family GTPases, fluorescence resonance energy transfer (FRET) is a valuable technique. FRET is the transfer of emission energy from one fluorophore to another, resulting in excitation of the latter. FRET only occurs when the two molecules are in close proximity and it is therefore useful for studying molecular interactions in live cells (Timpson et al., 2009). Through imaging of brain slice cultures, FRET biosensors for Rho-family GTPase activity were used to show that glioblastoma cells that invade along blood vessels with a single pseudopodium at the leading edge of the cell have high levels of Rac1 and Cdc42 and low levels of RhoA activities. In contrast, slower moving glioblastoma cells within the brain parenchyma had multiple pseudopodia, and low Rac1 and Cdc42 and high RhoA activities (Hirata et al., 2012). Many other FRET biosensors have been developed, including those that label protein conformational changes, post-translational modifications and second messengers (Sabouri-Ghomi et al., 2008), but these have not been used to any large extent in studies on metastasis.

Fluorophores are not just characterized by their excitation and emission spectra, but also by their unique lifetime. Fluorescent lifetime imaging microscopy (FLIM) measures the exponential decay in emission after the excitation of a fluorescence probe. The factors affecting the fluorescence lifetime include: changes in pH, temperature, ion intensity, hydrophobic properties, oxygen concentration, molecular binding and molecular interaction by energy transfer when two proteins approach each other (Ishikawa-Ankerhold et al., 2012). FLIM can therefore be used to provide insights into the microenvironment surrounding a fluorescence probe. FLIM has recently been employed in vivo in tumors for detection of both FRET and naturally fluorescent metabolites such as FAD and NADH (Provenzano et al., 2009; McGinty et al., 2011; Bakker et al., 2012) (Fig. 4D). Because detection of FAD and NADH does not require the addition of exogenous labels, it has potential for use in human studies.

Nanoparticles have also been adapted for live imaging studies. They are often defined as particles ranging from 1 to 100 nm in diameter, but broadly can encompass particles up to 1000 nm in diameter (Bharali et al., 2009). Integrin lymphocyte function-associated antigen-1 (LFA-1) is an important adhesion molecule for the interactions occurring between leukocytes and the endothelium of inflamed tissues and tumors. Super paramagnetic iron oxide nanoparticles labeled with fluorescent dyes and conjugated to the I domain of LFA-1 have been used for fluorescent and MRI detection of the vasculature of tumors (Chen et al., 2011b). The design of the nanoparticles allowed visualization of sites of inflamed vasculature by these two different techniques.

A triple-modality nanoparticle has enabled detection of mouse glioblastoma with MRI and intraoperative detection of tumor margins with photoacoustic and Raman imaging, resulting in accurate tumor resection (Kircher et al., 2012). Both photoacoustic and Raman imaging are technologies with great potential for live animal imaging. Photoacoustic imaging uses light pulses to excite molecular imaging agents, causing thermal expansion that produces ultrasound waves recorded by an ultrasound transducer (Yang et al., 2012). An advantage is that it can provide images of biological tissue at a greater depth than conventional optical imaging. Raman imaging relies on detecting the so-called Raman scattering effect and allows for highly specific and sensitive detection when used in vivo, in part due to scattering and absorption of light in the live tissue.
Disease Models & Mechanisms

Review

other means. Thus, insights from live imaging could ultimately shed light on various stages of metastasis could not have been revealed by any other means. They have been used to track the delivery of microspheres and migration of bone-marrow-derived cells to tumors (Stroh et al., 2005). They have also been used to label tumor vessels and to visualize vascular leakage (Stroh et al., 2005; Kim et al., 2009b). Quantum dots conjugated to RGB peptides, which bind to integrin \( \alpha_5\beta_3 \), have been used to demonstrate heterogeneity in both vascularity and extravasation between tumor models (Smith et al., 2010).

Optical frequency domain imaging (OFDI) is another emerging imaging technique for live animal imaging. It is based on optical coherence tomography (OCT), which is analogous to ultrasound but measures the echo time delay and magnitude of light rather than sound (Fujimoto, 2003). A focused laser beam scans the tissue sample while the reflected optical signals are recorded and compiled in real-time. Vessels can be detected without the use of any exogenous contrast agents because contrast is derived from circulating red blood cells. Post-processing imaging analysis is used to provide three-dimensional images and quantitative analysis (Vakoc et al., 2005; Vakoc et al., 2009). OFDI reportedly has a penetration depth of up to 2-3 mm into tissue, which exceeds that of multi-photon microscopy. It enables imaging of wide fields of view and has been used to visualize vascular morphology over multiple sessions within tumors (Vakoc et al., 2009) (Fig. 4B). OFDI was recently combined with near-infrared imaging for atherosclerotic plaque microstructure imaging using an intra-arterial catheter. This approach could detect microscopic changes associated with plaque formation in live rabbits and is under development for use in human patients (Yoo et al., 2011).

Conclusions

Visualizing cancer and stromal cells in real-time has been crucial for understanding cellular mechanisms of metastasis: how cells move from primary to secondary sites, seed and proliferate in their new environment. In vivo studies using confocal or two-photon microscopy have shown that: (1) cancer cells migrate towards chemoattractants; (2) cancer cells can migrate as single cells, in single-cell files or collectively; (3) both protease-dependent and independent cancer cell migration can lead to dissemination; (4) the vasculature undergoes dynamic changes; (5) macrophages and invadopodia promote intravasation; (6) extravasation of cancer cells is related to expression of EMT-regulating transcription factors; and (7) specific microenvironmental factors are necessary for homing of cancer cells. Two overall themes have emerged from imaging experiments: they have pointed to the crucial role of the tumor microenvironment (e.g. ECM, vasculature, macrophages, fibroblasts and neutrophils) in the metastatic process, and they have helped to visualize the dynamics of cellular movements as well as the signaling pathways involved in these processes. The dynamics of tumor-stroma interactions and cellular behavior required for the various stages of metastasis could not have been revealed by any other means. Thus, insights from live imaging could ultimately shed light on how to effectively treat cancer metastasis.

ACKNOWLEDGEMENTS

We thank Charles Camarda for generating the cartoons in Fig. 1, Elvin Wagenblast for critical comments on the manuscript, and the authors and journals that contributed images and movies to this review.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

M.R.F. and M.E. wrote the text. M.R.F. designed the figures.

FUNDING

This work was supported by funds (to M.E.) from the National Cancer Institute (U01 CA141451), Long Island 2 Day Walk to Fight Breast Cancer, Manhasset Women’s Coalition Against Breast Cancer, Islip Breast Cancer Coalition, and Glen Cove Cares.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.009282/-/DC1

REFERENCES

Aguirre-Ghioto, J. A. (2007). Models, mechanisms and clinical evidence for cancer dormancy. Nat. Rev. Cancer 7, 834-846.

American Cancer Society (2011). Cancer Facts and Figures 2011. Atlanta, GA: American Cancer Society.

Bakker, G. J., Andresen, V., Hoffman, R. M. and Friedl, P. (2012). Fluorescence lifetime microscopy of tumor cell invasion, drug delivery, and cytotoxicity. Methods Enzymol. 504, 109-125.

Beerling, E., Ritsma, L., Vrisekoop, N., Derksen, P. W. and van Rheenen, J. (2011). Intravital microscopy: new insights into metastasis of tumors. J. Cell Sci. 124, 299-310.

Bokes, E. M., Schweighofer, B., Kupriyanova, T. A., Zajac, E., Ardi, V. C., Quigley, J. P. and Deryugina, E. I. (2011). Tumor-recruited neutrophils and neutrophil TIMP-free MMP-9 regulate coordinate levels of the tumor angiogenesis and efficiency of malignant cell intravasation. Am. J. Pathol. 179, 1455-1470.

Bharali, D. J., Khalil, M., Gurbuz, M., Simone, T. M. and Mousa, S. A. (2009). Nanoparticles and cancer therapy: a concise review with emphasis on dendrimers. Int. J. Nanomedicine 4, 1-7.

Bow, P. D., Zhang, X. H., Nadal, C., Shu, W., Gomis, R. R., Nguyen, D. X., Minn, A. J., van de Vijver, M. J., Gerald, W. L., Foekens, J. A. et al. (2009). Genes that mediate breast cancer metastasis to the brain. Nature 459, 1005-1009.

Bremmer, C., Tung, C. H. and Weissleder, R. (2001). In vivo molecular target assessment of matrix metalloproteinase inhibition. Nat. Med. 7, 743-748.

Campagnola, P. J. and Loew, L. M. (2003). Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms. Nat. Biotechnol. 21, 1356-1360.

Canel, M., Serrels, A., Miller, D., Timpson, P., Serrels, B., Frame, M. C. and Brunton, V. G. (2010). Quantitative in vivo imaging of the effects of inhibiting integrin signaling via Src and FAK on cancer cell movement: effects on E-cadherin dynamics. Cancer Res. 70, 9431-9432.

Cao, L., Kobayakawa, S., Yoshiaki, A. and Abe, K. (2012). High resolution intravital imaging of subcellular structures of mouse abdominal organs using a microstage device. PloS ONE 7, e33876.

Chaffer, C. L. and Weinberg, R. A. (2011). A perspective on cancer cell metastasis. Science 331, 1559-1564.

Chambers, A. F., Groom, A. C. and MacDonald, I. C. (2002). Dissemination and growth of cancer cells in metastatic sites. Nat. Rev. Cancer 2, 563-572.

Chen, Q., Zhang, X. H. and Massagué, J. (2011a). Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. Cancer Cell 20, 538-549.

Chen, X., Wang, R., Khalidov, I., Wang, A. Y., Leelawattanachai, J., Wang, Y. and Jin, M. M. (2011b). Inflamed leukocyte-mimetic nanoparticles for molecular imaging of inflammation. Biomaterials 32, 7651-7661.

Comen, E., Norton, L. and Massague, J. (2011). Clinical implications of cancer self-seeding. Nat. Rev. Clin. Oncol. 8, 369-377.

Condeelis, J. and Segall, J. E. (2003). Intravital imaging of cell movement in tumours. Nat. Rev. Cancer 3, 921-930.

Condeelis, J. and Weissleder, R. (2010). In vivo imaging in cancer. Cold Spring Harb. Perspect. Biol. 2, a003848.

Courtois, L. M., Fingleton, B. and Matrisian, L. M. (2002). Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science 295, 2387-2392.

590
Cuccia, D. J., Bevilacqua, F., Durkin, A. J., Ayers, F. R. and Tromberg, B. J. (2009). Quantitation and mapping of tissue optical properties using modulated imaging. J. Biomed. Opt. 14, 024012.

Detmar, M. and Hirakawa, S. (2002). The formation of lymphatic vessels and its importance in the setting of malignancy. J. Exp. Med. 196, 713-718.

Fidler, I. J. (2003). The pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited. Nat. Rev. Cancer 3, 453-458.

Fidler, I. J. and Talmadge, J. E. (1986). Evidence that intravenously derived murine pulmonary metastases can originate from the expansion of a single tumor cell. Cancer Res. 46, 5167-5171.

Fields, R. C., Jeffe, D. B., Trinkaus, K., Zhang, Q., Arthur, C., Aft, R., Dietz, J. R., Eberlein, T. J., Gillanders, W. E. and Margenthaler, J. A. (2007). Surgical resection of the primary tumor is associated with increased long-term survival in patients with stage IV breast cancer after controlling for site of metastasis. Ann. Surg. Oncol. 14, 3345-3351.

Fridlender, Z. G. and Albeida, S. M. (2012). Tumor-associated neutrophils: friend or foe? Carcinogenesis 33, 949-955.

Friedl, P. and Alexander, S. (2011). Cancer invasion and the microenvironment: plasticity and reciprocity. Cell 147, 992-1009.

Friedl, P. and Wolf, K. (2003). Tumor-cell invasion and migration: diversity and escape mechanisms. Nat. Rev. Cancer 3, 362-374.

Fujimoto, J. W. (2009). Optical coherence tomography for ultrahigh resolution in vivo imaging. Nat. Biotechnol. 21, 1361-1367.

Fukumura, D. and Jain, R. K. (2008). Imaging angiogenesis and the microenvironment. APMIS 116, 695-715.

Fukumura, D., Duda, D. G., Munn, L. L. and Jain, R. K. (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141, 52-67.

Friedl, P. and Wolf, K. (2003). Tumor invasion and migration: the ‘seed and soil’ hypothesis revisited. Nat. Rev. Cancer 3, 858-863.

Fridlender, Z. G. and Albelda, S. M. (2012). Tumor-associated neutrophils: friend or foe? Carcinogenesis 33, 949-955.

Friedl, P. and Alexander, S. (2011). Cancer invasion and the microenvironment: plasticity and reciprocity. Cell 147, 992-1009.

Friedl, P. and Wolf, K. (2003). Tumor-cell invasion and migration: diversity and escape mechanisms. Nat. Rev. Cancer 3, 362-374.

Fukumura, D., Padera, T. P. and Jain, R. K. (2010). Tumor-associated neutrophils inhibit seeding in the premetastatic lung. J. Pathol. 223, 414-419.

Fukumura, D., Duda, D. G., Munn, L. L. and Jain, R. K. (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141, 52-67.

Fridlender, Z. G. and Albelda, S. M. (2012). Tumor-associated neutrophils: friend or foe? Carcinogenesis 33, 949-955.
Disease Models & Mechanisms

Wells, A., Chao, Y. L., Grahovac, J., Wu, Q. and Lauffenburger, D. A. (2011). Epithelial and mesenchymal phenotypic switchings modulate cell motility in metastasis. Front. Biosci. 16, 815-837.

Williams, R. M., Flesken-Nikitin, A., Ellenson, L. H., Connolly, D. C., Hamilton, T. C., Nikitin, A. Y. and Zipfel, W. R. (2010). Strategies for high-resolution imaging of epithelial ovarian cancer by laparoscopic nonlinear microscopy. Transl. Oncol. 3, 181-194.

Winslow, M. M., Dayton, T. L., Verhaak, R. G., Kim-Kiselak, C., Snyder, E. L., Feldser, D. M., Hubbard, D. D., DuPage, M. J., Whittaker, C. A., Hoersch, S. et al. (2011). Suppression of lung adenocarcinoma progression by Nkx2-1. Nature 473, 101-104.

Wolf, K. and Friedl, P. (2011). Extracellular matrix determinants of proteolytic and non-proteolytic cell migration. Trends Cell Biol. 21, 736-744.

Wolf, K., Mazo, I., Leung, H., Engelke, K., von Andrian, U. H., Deryugina, E. I., Strongin, A. Y., Bröcker, E. B. and Friedl, P. (2003). Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. J. Cell Biol. 160, 267-277.

Wolf, K., Wu, Y. I., Liu, Y., Geiger, J., Tam, E., Overall, C., Stack, M. S. and Friedl, P. (2007). Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. Nat. Cell Biol. 9, 893-904.

Wong, C. W., Lee, A., Shientag, L., Yu, J., Dong, Y., Kao, G., Al-Mehdi, A. B., Bernhard, E. J. and Muschel, R. J. (2001). Apoptosis: an early event in metastatic inefficiency. Cancer Res. 61, 333-338.

Wyckoff, J. B., Jones, J. G., Condeelis, J. S. and Segall, J. E. (2000). A critical step in metastasis: in vivo analysis of intravasation at the primary tumor. Cancer Res. 60, 2504-2511.

Wyckoff, J., Wang, W., Lin, E. Y., Wang, Y., Pixley, F., Stanley, E. R., Graf, T., Pollard, J. W., Segall, J. and Condeelis, J. (2004). A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res. 64, 7022-7029.

Wyckoff, J. B., Wang, Y., Lin, E. Y., Li, J. F., Goswami, S., Stanley, E. R., Segall, J. E., Pollard, J. W. and Condeelis, J. (2007). Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res. 67, 2649-2656.

Yang, J. and Weinberg, R. A. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev. Cell 14, 818-829.

Yang, J. M., Favazza, C., Chen, R., Yao, J., Cai, X., Maslov, K., Zhou, Q., Shung, K. K. and Wang, L. V. (2012). Simultaneous functional photoacoustic and ultrasonic endoscopy of internal organs in vivo. Nat. Med. 18, 1297-1302.

Yoo, H., Kim, J. W., Shishkov, M., Namati, E., Morse, T., Shubochkin, R., McCarthy, J. R., Ntziotakis, V., Bouna, B. E., Jaffer, F. A. et al. (2011). Intra-arterial catheter for simultaneous microstructural and molecular imaging in vivo. Nat. Med. 17, 1680-1684.

Yuan, F., Salehi, H. A., Boucher, Y., Vasthare, U. S., Tuma, R. F. and Jain, R. K. (1994). Vascular permeability and microcirculation of gliomas and mammary carcinomas transplanted in rat and mouse cranial windows. Cancer Res. 54, 4564-4568.

Zervantonakis, I. K., Hughes-Alford, S. K., Charest, J. L., Condeelis, J. S., Gertler, F. B. and Kamm, R. D. (2012). Three-dimensional microfluidic model for tumor cell intravasation and endothelial barrier function. Proc. Natl. Acad. Sci. USA 109, 13515-13520.

Zhang, N. and Yang, Q. (2009). Primary tumor resection may improve prognosis for nonoperable advanced breast cancer. Med. Hypotheses 73, 1058-1059.

Zhang, X. H., Wang, Q., Gerald, W., Hudis, C. A., Norton, L., Smid, M., Foekens, J. A. and Massagué, J. (2009). Latent bone metastasis in breast cancer tied to Src-dependent survival signals. Cancer Cell 16, 67-78.

Zhou, Z. N., Boimel, P. J. and Segall, J. E. (2011). Tumor-stroma: In vivo assays and intravital imaging to study cell migration and metastasis. Drug Discov. Today Dis. Models 8, 95-102.