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MRI of metastasis-permissive microenvironments

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Abstract

One of the earliest documented observations of the importance of the microenvironment in metastasis was made by Stephen Paget in 1889. More than a century later, the metastatic cascade remains a major cause of mortality from cancer. Cancer meets the criterion of a successful organization that is able to survive by adapting to changing environments. In fact, the tumor microenvironment and stroma are co-opted and shaped by cancer cells to derive a survival advantage. Cohesive strategies integrating advances in molecular biology and chemistry, with noninvasive multimodality imaging, provide new insights into the role of the tumor microenvironment in promoting metastasis from primary tumors as well as insights into environments that attract and permit cancer cells to establish colonies in distant organs. This article provides an overview of molecular and functional imaging characterization of microenvironments that can promote or permit cancer cells to metastasize and the microenvironmental characteristics of distant metastases.

Keywords

imaging; metastasis; microenvironment

From all its hallmarks, the unsurpassed ability of cancer cells to adapt and survive during growth and following treatment, coupled with the ability to disseminate to distant sites, and the lack of effective treatments for metastases, have created major barriers in the successful treatment of cancer. A solid tumor is a complex organization that is populated not only by cancer cells, but also by networks of blood and lymphatic vessels, stromal cells and an extracellular matrix (ECM) that, excluding cancer cells, is collectively termed the tumor microenvironment (TME). A wide range of stromal cells that include cancer-associated fibroblasts (CAFs), adipocytes, pericytes, multiple immune cells, such as tumor-associated macrophages, and vascular and lymphatic endothelial cells (LECs), can be found within the tumor landscape. Chaotic vascular and lymphatic networks, as well as the aberrant metabolism of cancer cells impose additional micro-environmental conditions, such as

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hypoxia, high interstitial pressure and acidic micro-environments, which significantly contribute to promoting invasion and metastasis [1,2]. Clear similarities, such as hypoxia, high lactate, secretion of proinflammatory molecules and low extracellular pH (pHe), exist between tumors and injured tissue and more than a decade ago Dvorak postulated that tumors are 'wounds that do not heal' [3].

For metastasis to occur, the cancer cell has to escape from its primary site by disrupting cell-to-cell attachment. This is followed by invasion of underlying connective tissue, initial invasion of the vessel, arrest of the circulating tumor cells and adhesion to the blood vessel, extravasation from the vessel, and finally, further invasion at the metastatic site and growth of the metastatic nodule (Figure 1). The lymphatics and vasculature are recognized as the major routes for metastatic dissemination with links between the routes occurring owing to lymphaticovenous communication. While hematogenous metastasis requires the invasion of the vascular basement membrane, tumor cells enter lymphatics through gaps in the endothelium [4] and arrive in the marginal sinus of the lymph nodes as embolizations from the primary tumor [4]. In later stages of metastasis, lymphatics and lymph nodes become clogged with tumor cells and the lymph node cannot filter the cells. Tumor dissemination is thought to occur synchronously via lymphatics and blood capillaries, and not first via lymphatics and subsequently through the blood stream once the regional lymph nodes are overwhelmed. However, there does appear to be a pattern of preferential nodal metastasis, especially for breast cancer, and the destruction of tumor cells in the lymph node environment does not appear to be as successful as the destruction of tumor cells in blood vessels [5].

Multimodal molecular and functional imaging can report on several processes in the metastatic cascade. Optical microscopy and bioluminescence imaging permit the tracking of single cells in living organisms, but the scope of clinical translation is limited. MRI, magnetic resonance spectroscopy (MRS) and nuclear imaging are clinically translatable imaging modalities that span bench-to-bedside applications. MRI and MRS techniques can identify potential physiological and metabolic biomarkers that can be used to identify metastasis-permissive environments in tumors that are likely to metastasize [6] and metastatic lesions. PET imaging of radio-labeled probes can report on glucose metabolism, receptors and antigens to detect and target metastatic cells. Here, we have described the applications of multimodality imaging, with a focus on MRI and MRS to understand the role of the TME in the metastatic cascade. Within this context, imaging can be used to characterize physiological environments that are likely to promote invasion and metastasis and to study interactions between cancer cells and stromal cells in the TME. In this article we have presented examples within these broad areas to demonstrate the use of molecular and functional imaging in understanding these aspects of the metastatic cascade.

Imaging cancer cell–stromal cell interactions & invasion in cell culture systems

The initial escape of the cancer cell requires the ability to attach to, degrade and invade the primary tumor basement membrane and vascular basement membrane. Invasion is therefore a key component of the metastatic cascade. Cancer cells invade surrounding tissues to grow and disseminate by secreting proteolytic enzymes such as aspartic, cysteine and serine proteases and matrix-degrading metalloproteinases (MMPs) [7]. Basement membranes are thin sheets of ECM that separate parenchymal cells from interstitial stroma and present a major barrier to metastatic spread [8]; they are composed of characteristic collagens, glycoproteins and proteoglycans. Interactions with stromal cells and the physiological environment, such as hypoxia and acidic pHe, can influence cancer cell invasion [9,10].

Imaging can be applied to study the interaction between cancer cells and other stromal cells or different ECM components. MRI assays can be used to nondestructively and temporally observe the effects of paracrine factors secreted by cancer cells, on human vascular endothelial cell motility, network formation and invasion as shown in Figure 2 [11]. Cell labeling with superparamagnetic iron oxide, which generates contrast by loss of signal in magnetic resonance (MR) images, combined with high-resolution MRI, can be used for noninvasive and dynamic 3D characterization of endothelial cell networks formed in a 3D ECM gel – in this case Matrigel. This assay can be expanded to study cancer cell–endothelial cell interactions in response to single perturbations such as hypoxia, a chemokine or a cytokine. By acquiring high-resolution 3D MR images, further insights into the remodeling of basement membranes can be obtained. Moreover, the response to various factors and conditions, such as upregulation of growth factors and receptor activity, can be determined.

In addition, the assay can be used in longitudinal studies to assess the efficacy of treatments, such as proangiogenic and antiangiogenic drugs, and can be extended to study LEC motility, network formation and invasion. By using such an assay, it was possible to detect increased invasion and migration of LECs in the presence of breast cancer cells, which further increased in the presence of hypoxia. These studies identified hypoxia as an important factor in LEC invasion and migration through ECM in the presence of breast cancer cells [12].

The invasive potential of cancer cells can be characterized by quantifying the penetration of cells into reconstituted basement membrane gel (Matrigel) or other ECMs [8]. However, the metabolic status of cancer cells cannot be evaluated, nor can invasion be assessed in the same sample dynamically under controlled environmental condition or following interventions. An MRI and MR spectroscopic imaging (MRSI) compatible invasion assay system has been developed to dynamically track the invasion of cancer cells and simultaneously characterize oxygen tensions, and physiological and metabolic parameters (Figure 3A) [9,13]. Incorporation of perfluorotripropylamine-doped alginate beads in the MR tube allows direct measurement of oxygen tensions in the sample. The assay can be used to derive quantitative indices of invasion, and determine changes in the invasion index and metabolism in response to different physiological conditions, or from the downregulation or overexpression of specific genes, or to study the interaction between cancer cells and stromal cells. It is possible to use a layer of stromal cells on top of the Matrigel layer (Figure 3A) and determine their effect on cancer cell invasion under different physiological conditions (Figure 3B). ^{31}P , ^{13}C and ^1H MR spectra can be obtained to study cell metabolism, intracellular pH (pHi), pHe and glycolysis. The system maintains viable cells for up to 4 days and can also be used to study the effects of therapeutic agents without the complexities associated with *in vivo* systems.

***In vivo* preclinical & clinical studies Stromal cell–cancer cell interactions & the extracellular matrix**

As mentioned earlier, stromal and immune cells, such as fibroblasts, lymphocytes, dendritic cells and macrophages, populate cancers and can facilitate metastasis [14]. Imaging can provide insights into these interactions to develop new strategies to prevent or attenuate metastasis. CAFs have been shown to stimulate cancer growth, inflammation, angiogenesis and invasion, and are involved in the synthesis of ECM components [15]. In preclinical studies, color-coded optical imaging was used to visualize the recruitment of stromal cells in metastatic models [16]. Noncolored human colon cancer cells were injected into the spleen of green fluorescent protein (GFP) nude mice, resulting in the formation of metastatic colonies in the liver. Recruitment of GFP-expressing stromal cells, including desmin-positive CAFs, by the metastatic noncolored tumors was observed [16].

Intrinsic second harmonic generation (SHG) microscopy is increasingly being used to understand the remodeling of the collagen matrix in intact tissue [17]. SHG signal is produced from a molecule without a center of symmetry that can be used to image endogenous structural proteins such as collagen. Fibrillar collagen I (Col1) [18,19] is a major component of the tumor ECM, and fiber structure and volume can be determined in 3D with micrometer resolution with this approach. SHG microscopy studies have revealed that invading cancer cells align along collagen fibers while migrating [20] and denser fibrillar collagen is associated with increased metastasis [21]. By combining SHG microscopy to visualize Col1 fibers, with fluorescent microscopy to observe GFP-labeled CAFs, the association between stromal cells and Col1 fibers was tracked *in vivo* over several days [22].

The recruitment of fibroblasts labeled with biotin-BSA-gadolinium-diethylenetriamine-pentaacetate (GdDTPA) can also be visualized noninvasively with MRI, as shown by Granot *et al.* in a subcutaneous ovarian carcinoma tumor model [23]. In these preclinical studies recruited labeled fibroblasts colocalized with the neovasculature at the tumor rim, implicating a role for these stromal cells in tumor angiogenesis [23].

It is also possible to characterize the delivery, as well as interstitial transport, of the macromolecular contrast agent albumin-GdDTPA in human breast cancer models *in vivo*, using MRI [24,25]. Transport parameters that are derived include macromolecular fluid exudate volume, draining and pooling voxels. Numbers of draining and pooling voxels were compared between an invasive and metastatic MDA-MB-231 breast tumor and a less invasive and poorly metastatic MCF-7 breast tumor. Consistent with a decrease of ECM integrity, the invasive tumor demonstrated significantly higher number of draining voxels, which allowed the macromolecular contrast agent to drain through the ECM [25].

The immune system is another important component of the TME that is being exploited in cell-based therapies to treat cancer and to target metastatic lesions. MRI has been used to image different immune cells, such as lymphocytes, dendritic cells and natural killer cells, in both preclinical and clinical studies. T-cell infiltration can be monitored noninvasively *in vivo* by labeling activated T cells with MR contrast agents such as citrated anionic iron oxide-based nanoparticles [26]. Since dendritic cells mediate the immune response, the injection of tumor antigen-loaded dendritic cells is being used to enhance the endogenous immune response against tumor cells. Imaging can be used to detect these dendritic cells as was shown in T_2^* -weighted images that were acquired to track superpara-magnetic iron oxide-labeled dendritic cells in melanoma patients after intranodal injection [27]. In another example, genetically engineered natural killer cells directed against Her2/neu receptors were labeled with iron oxide-based contrast agents and imaged with MRI in pre-clinical studies [28]. In both cases, accumulation of iron oxide-labeled cells was detected by a decreased signal in T_2^* -weighted images of the tumor.

Proteolytic enzymes

The expression of proteases by tumor cells has been associated with the degradation of the ECM, tumor invasion and metastasis. Significant advances have been made in imaging proteolytic enzyme activity associated with tumor invasiveness. Preclinical studies with MRI have demonstrated the feasibility of detecting transglutaminase and hyaluronidase activities *in vivo* [29,30]. Transglutaminases mediate tissue remodeling by catalyzing covalent cross-links between proteins of the ECM [29] and hyaluronidases degrade hyaluronan, a major component of the ECM, producing factors that stimulate endothelial cell proliferation, promote neovascularization and contribute to invasiveness [30]. To detect transglutaminase activity with MRI, a low-molecular-weight reporter was developed consisting of a peptide with a specific recognition sequence for the enzyme linked to

GdDTPA. Enzyme activity generated MR contrast visible in T₁-weighted images [29]. A similar mechanism was exploited to detect hyaluronidase activity in ES-2 and OVCAR-3 human epithelial ovarian carcinoma cells and in an ES-2 ovarian cancer xenograft model with a T₂-contrast agent [30].

Matrix metalloproteinases are zinc-dependent endopeptidases that degrade ECM proteins to mediate tumor invasion [31]. Fluorescent (or Förster) resonance energy transfer is an optical imaging technique characterized by the transfer of electronic excitation energy of a donor chromophore to an acceptor molecule in close proximity, with the efficacy of the transfer dependent on the distance between the fluorophores [32,33]. This technique has been employed to detect proteolytic enzyme activity in living animals and tissues [33]. MMP-2 activity was assessed *in vivo* by attaching fluorophores to a linear copolymer with a peptide containing a proteolytic cleavage site, as shown in Figure 4 [34]. The proximity of the fluorophores quenched the fluorescent signal, but cleavage of the peptide by the enzyme resulted in fluorescence [34]. The efficacy of prinomastat that induces a decrease in MMP-2 activity was determined in a human fibrosarcoma tumor model using this approach (Figure 4). Near infrared fluorescent signal in the tumor significantly decreased in treated mice compared with controls following 2 days of treatment [34]. The peptide sequences can be modified to detect the activity of different MMPs [33].

Lysosomes contain several of the degradative enzymes that cancer cells use to invade and metastasize. By optical imaging of a dansylated glucosamine compound that is incorporated in the lysosomal-associated membrane protein, pericellular translocation of lysosomes from the perinuclear region was observed in intact cancer cells in response to an acidic pH [35]. An analogous lysosome-specific near-infrared fluorescent probe has been developed [36] that was found to accumulate in lysosomes in human tumor xenografts *in vivo*. Noninvasive imaging of lysosomes is useful to understand the role of lysosomes, within the context of the TME in invasion and metastasis, and to evaluate future lysosome-targeted anticancer therapies.

Vascularization & lymphatics

The most frequent routes of dissemination to distant target tissue are through the blood stream, and to a much lesser extent, by the lymphatic circulatory system. The final stage involves extravasation and establishment of micrometastases. The subsequent development of clinically detectable metastases is dependent upon acquisition of neovasculature by the process of angiogenesis. Thus, it is apparent that two critical steps in the metastatic cascade – dissemination and metastatic growth – are highly dependent upon the primary tumor vasculature and the ability to establish vasculature at the metastatic site. Vascular density, as evaluated in excised histological specimens, has been shown to correlate significantly with the subsequent formation of metastases in several cancers [37,38]. Consistent with these observations, increased levels of VEGF have also been associated with increased metastasis [39]. MRI can provide functional characterization of tumor vasculature in xenograft models as well as clinically with dynamic contrast-enhanced (DCE)-MRI [40,41]. Studies to date indicate that more aggressive tumors are characterized by increased vascular volume and permeability [42], and that the ability to invade is necessary but not sufficient for metastasis to occur.

Angiogenesis imaging can be performed *in vivo* by SPECT or PET imaging of radio-labeled probes targeting the VEGF receptor or $\alpha v\beta 3$ integrin [43]. Similarly, MRI can be used to visualize angiogenesis *in vivo* by using nanoparticles loaded with either gadolinium or iron oxide particles that are targeted to $\alpha v\beta 3$ integrin by using the arginine-glycine-aspartic acid (RGD) peptide or monoclonal antibodies as targeting moieties [44,45].

The effects of antiangiogenic agents can also be detected with imaging [46]. Antiangiogenic treatments have been previously linked to a decrease of metastases as demonstrated by Rasteli *et al.* using a peptide designed to bind the VEGF receptor [47]. Treatment with the peptide decreased tumor growth and metastases *in vivo* in a preclinical model of melanoma owing to its antiangiogenic effect and the ability to block VEGF-mediated activity [47].

Recently, Muldoon *et al.* compared the effects of two different monoclonal antibodies, intetumumab and bevacizumab, which target αv integrin and VEGF, respectively, on brain tumor vasculature in a rat model using dynamic MRI [48]. Anatomical MRI was used to assess the tumor volume, DCE-MRI to measure the tumor vascular permeability and dynamic susceptibility-contrast MRI using iron oxide nanoparticles to determine relative cerebral blood volume. In these studies, the treatments were found to have dissimilar effects on tumor vasculature. An increase in permeability and blood volume was detected following treatment with intetumumab, whereas treatment with bevacizumab resulted in a decrease in these parameters [48]. These studies highlight the importance of noninvasive imaging in evaluating changes in vasculature following antiangiogenic treatments.

Li *et al.* have identified imaging methods to assess the metastatic potential of primary tumors [2]. DCE-MRI, $T_1\rho$ -MRI and NADH/flavoprotein (Fp) fluorescence imaging (redox scanning) could differentiate highly metastatic from less metastatic indolent melanoma models [2]. Figure 5 shows representative Fp redox ratio images of tissue sections of several melanoma cell lines and their corresponding histograms, from the most invasive (Figure 5A), to the least invasive (Figure 5E). A nearly homogeneous low Fp redox ratio was observed within the entire indolent A375P tumor (Figure 5E). For the more metastatic tumor lines, two distinct peaks were observed in the histograms; the peak on the right corresponded to the more oxidized tumor core and that on the left to the tumor rim (Figure 5A–D). Highly metastatic lesions were characterized by more vascular structures but fewer functional blood vessels resulting in lower K_{trans} values in the core of metastatic tumors. Moreover, $T_1\rho$ values and the mitochondrial redox ratio increased with the invasive potential measured in cells in culture [2].

The expansion of lymphatic vasculature in tumor-draining lymph nodes has been observed to precede the onset of metastasis and is associated with distant metastases [49]. Imaging tumor-induced lymph node lymphangiogenesis may be used for predicting the likelihood of cancer metastases. Mumprecht *et al.* have established a method to image lymph node lymphangiogenesis *in vivo* with a radiolabeled antibody directed against a lymphatic-specific epitope [49]. Results from these preclinical studies indicate that, by using a ^{124}I -labeled antibody directed against lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), it is possible to visualize lymphatic vessel expansion in lymph nodes with metastases. Immuno-PET with lymphatic-specific antibodies could open up new avenues for the early detection of metastasis [49].

Hypoxia

Hypoxia has been closely associated with tumor aggressiveness, invasion and resistance to radiation and chemotherapy [50,51]. The existence of hypoxia in tumors and its implications for failure of radiation therapy has been documented since the 1950s and earlier [52]. Hypoxia and the hypoxia-inducible factors (HIF-1 and HIF-2) are being associated with the transcriptional activation of an ever-increasing number of genes that regulate several phenotypic characteristics of cancer, including invasion and metastasis [53]. Noninvasively identifying hypoxic tumor regions in tumors would be useful for understanding the interactions between hypoxia, vascularization, metabolism and the stroma in basic research studies, and for image-guided targeting of hypoxic environments in the clinic to eliminate lethal subpopulations. Such an approach was recently described by Flynn *et al.*

using $^{61}\text{Cu}(\text{II})$ -diacetyl-bis(N4-methylthio-semicarbazone) (^{61}Cu -ASTM) PET imaging to boost radiation dose using intensity modulated x-ray or intensity-modulated proton therapy in a head and neck squamous cell carcinoma patient [54].

Nitroimidazole compounds have been developed to detect hypoxia *in vivo* since these compounds are reduced by intracellular reductases in an oxygen-dependent manner and accumulate in viable hypoxic cells by binding covalently to the thiol groups of intracellular proteins [55]. Among these, ^{18}F -fluoromisonodazole is being used as a PET probe to detect hypoxia in tumors with PET imaging [55]. Tumor oxygen tensions can also be measured *in vivo* using ^{19}F and ^1H MRS [56,57] to detect these nitroimidazole-based compounds. The spatial distribution of the probe can be visualized with ^{19}F MR spectroscopic images overlaid with anatomic images [58]. Phase I studies of SR-4554, a fluorinated 2-nitroimidazole, demonstrated that ^{19}F MR spectra can detect retention of the probe in tumors after intravenous injection [59,60]. Hypoxia in tumors can also be detected with ^{19}F MRS of hexafluorobenzene, a reporter molecule with an oxygen-dependent T_1 relaxation rate [56]. A ^1H MRS-detectable analog of hexafluorobenzene, hexamethyldisiloxane, is also available for *in vivo* quantitative oxygen partial-pressure measurements [57].

In preclinical studies, optical imaging was used to detect hypoxia by using the hypoxia response element (HRE) to drive the expression of imaging reporters in tumors derived from stably transfected cells. Hypoxic regions were detected in human tumor xenografts stably expressing GFP under control of HRE [61,62]. These xenograft models have proven to be useful for understanding the effect of hypoxia on vascularization [61], choline metabolism [63] and the ECM [62].

Studies using combined MRI to determine vascular volume and permeability and optical imaging to determine hypoxia in such tumors revealed that low vascular volume regions, measured by MRI, were associated with high fluorescence and *vice versa* [61]. Regions with high permeability were coincident with low vascular volume and high fluorescence. These observations are consistent with the possibility that regions of low vascular volumes are hypoxic and, with hypoxia acting as a transcriptional activator of VEGF, a potent vascular permeability factor [61].

While metabolism is not strictly considered a TME parameter, cancer cells can influence the metabolism of stromal cells. In addition, the aberrant metabolism of cancer cells themselves can result in TME conditions that influence invasion and metastasis. Conversely, physiological environments, such as hypoxia, will influence metabolism. Increased total choline levels are typically observed in tumors and occur mostly owing to increased phosphocholine (PC) levels [64], which have been linked to oncogenic Ras signaling and oncogenic transformation [64]. Hypoxic regions were found to colocalize with regions of elevated total choline [63]. The presence of putative HREs was observed within the promoter region of choline kinase, the enzyme that converts free choline to PC [63]. Therefore, hypoxia may contribute to the heterogeneous distribution of the total choline signal frequently observed in preclinical and clinical cancers [63]. Conditioned medium from MDA-MB-231 breast cancer cells has also been observed to increase PC in human vascular endothelial cells, demonstrating the ability of paracrine factors secreted by cancer cells to alter the metabolism of stromal cells [65].

By combining SHG microscopy of tissue slices with optical detection of hypoxia, Kakkad *et al.* have reported on the relationship between hypoxia and Col1 fibers in MDA-MB-231 breast and PC3 prostate cancer xenografts [62]. As shown in Figure 6A–e, significant differences in Col1 fiber patterns were observed between hypoxic and normoxic regions [62]. Hypoxic tumor regions contained fewer and less dense Col1 fibers than normoxic

regions in both MDA-MB-231 breast tumor and PC3 prostate tumor xenografts [62]. Further studies are necessary to understand the impact of reduced collagen fibers in hypoxic tumor regions on metastasis and on drug delivery.

pH

Tumor pHe is usually acidic, whereas the pH_i is neutral–alkaline [66]. Even in the presence of oxygen, high glycolytic activity, termed the ‘Warburg effect’, together with poor blood flow and hypoxia contribute to this acidic pHe. The molecular mechanisms underlying acidic pHe may also be, in part, mediated through the stabilization of HIF-1 α , even under oxygenated conditions in cancer cells [67]. HIF-1 α expression mediates a switch in glucose metabolism, through the induction of lactate dehydrogenase, to convert pyruvate to lactate and the inactivation of pyruvate dehydrogenase, which converts pyruvate to acetyl-Coenzyme A. Tumor pH plays an important role in tumor progression, metastasis and treatment efficacy [67]. Neutralization of the acidic TME has been shown to reduce metastasis [68].

Tumor pH can be measured *in vivo* from the pH-dependent chemical shift of the phosphate inorganic signal in ^{31}P MR spectra. Since inorganic phosphate is mostly intracellular, it reports on pH_i [69], which has been shown to be mostly neutral to alkaline [66]. pHe can be measured from the pH-dependent chemical shift of extracellular ^{31}P MR detectable probes such as 3-aminopropylphosphonate [70]. In preclinical studies, ^1H MRS imaging has been used to obtain pHe maps of tumors by determining the chemical shift of extracellular compounds such as (imidazol-1-yl)3-ethoxycarbonylpropionic acid or 2-(imidazol-1-yl)succinic acid [71,72].

Another MR technique that can be applied to measure pHe *in vivo* consists of using hyperpolarized ^{13}C -labeled bicarbonate [73]. The hyperpolarization of spins allows an improvement in the sensitivity of detection of the MR signal of ^{13}C -labeled substrates by >10,000 and hyperpolarized ^{13}C -labeled substrates can be injected systemically, allowing real-time metabolic mapping [74–76]. Using this technique, pHe maps were obtained from a subcutaneous EL4 lymphoma tumor model. The spatial distribution of $^{13}\text{CO}_2$ and $\text{H}^{13}\text{CO}_3^-$ were imaged using a gradient echo pulse sequence and images of tumor pHe were generated from the ratio of the signal intensities of hyperpolarized $\text{H}^{13}\text{CO}_3^-$ and $^{13}\text{CO}_2$ [73]. pH imaging has been widely used in preclinical studies to better understand tumor physiology and to identify metastasis-permissive microenvironments. While these preclinical studies are valuable in cancer discovery, clinically translatable methods are currently not available and are required to develop pH imaging as a predictive biomarker for metastatic environments.

Multiparametric imaging

Since the physiological and metabolic environment can influence invasion and metastasis, one goal for multimodality molecular and functional imaging is to determine if there are combinations of these parameters, which represent the most ‘permissive’ regions in a solid tumor and identify the molecular makeup of these regions. The metabolic phenotype of cancer cells can contribute significantly to the physiological environment. High glycolytic activity and poor perfusion can result in increased lactate and extracellular acidosis. Significant differences in vascular, physiological and metabolic characteristics have been identified in metastatic and nonmetastatic human breast and prostate cancer models with MRI and MRS [6,71]. The malignant and metastatic phenotype is characterized by high total choline, high lactate, low pHe and high permeability. By using a multiparametric approach, we can begin to evaluate which combinations represent the highest ‘metastatic threat’. Such information can be used to design therapeutic strategies targeting multiple phenotypic

characteristics both in the primary tumor and in the premetastatic niche (discussed later) to prevent metastasis.

Metastases occur more readily when cancer cells are implanted in orthotopic sites rather than heterotopic sites. Multimodal imaging was performed in an experimental model of prostate cancer to compare vascular, metabolic and physiological characteristics between xenografts implanted orthotopically on the prostate or implanted subcutaneously in the flank. A multiparametric analysis of hypoxia, pHe, vascularization and metabolism identified environments that are characterized by higher vascular volume, permeability and total choline, and a more acidic pHe were more permissive for metastasis to occur (Figure 7) [6]. Moreover, metastases observed in the lymph nodes as well as cancer cells found in ascites fluid were hypoxic, which may explain, in part, the refractory nature of metastatic disease [6].

The potential of multiparametric breast MRI to predict lymph node metastases has been recently described in a study of 350 patients with invasive breast cancer [77]. Histological analyses were performed in parallel to define the nodal status of each patient. A close association was found between the patient nodal status and selected breast MRI descriptors. Among 17 descriptors selected in the study, seven were associated with the presence of lymph node metastases. Skin thickening and internal enhancement after GdDTPA injection were the most accurate descriptors [77].

Recent clinical studies have demonstrated the potential of using diffusion-weighted MRI, which reports on the degree of restriction of water in tissues, to identify primary tumors that are most likely to metastasize [77,78]. Diffusion-weighted images (DWIs) were acquired from 44 patients with localized prostate cancer on a 1.5T. The apparent diffusion coefficient values demonstrated differences between aggressive and indolent tumors [78].

Imaging the premetastatic niche

Recent studies have identified ‘premetastatic niches’ created by stromal cells to provide a microenvironment conducive for the formation of metastases [79]. Cancer cells establish micrometastases in these niches, ultimately evolving into macrometastases. These premetastatic niches result from tumor-secreted factors inducing tissue parenchymal remodeling at target sites [79]. In response to these tumor-secreted factors, tumor-associated cells such as hematopoietic progenitor cells and macrophages are recruited to the niches, where they secrete chemokines, growth factors, matrix degrading enzymes and adhesion molecules to facilitate the establishment of metastases [79]. Cells of hematopoietic origin have been shown to play a key role in the construction of premetastatic niches [80,81]. Premetastatic niches can also be formed in distant organs by invading bone marrow-derived cells. Lysyl oxidase plays a critical role in premetastatic niche formation in the lungs [81]. The enzyme is secreted by hypoxic breast cancer cells and accumulates in premetastatic niches where it cross-links collagen IV in the basement membrane, which is required to recruit for myeloid cells. These recruited cells adhere to cross-linked collagen IV and produce MMP-2, which cleaves collagen and enhances the invasion and recruitment of CD11b⁺ bone marrow-derived cells and metastasizing tumor cells [81]. Although to the best of our knowledge, *in vivo* imaging studies of the premetastatic niche have not been published, the application of molecular and functional imaging techniques could significantly advance the understanding and characterization of the premetastatic niche.

Primary & metastatic tumors

Since metastases typically become refractory to treatment, identifying imaging-based differences between primary and metastatic tumors can lead to the development of image-

guided therapeutic strategies to treat metastatic disease. VEGF expression, hypoxia, proliferation and vascular phenotype have been explored in a series of primary non-small-cell lung carcinomas (NSCLCs) and corresponding brain metastases [82]. The proportion of mature vessels was greater in brain metastases than in their matched primary NSCLCs. Differences were also observed in the vascular patterns, with a predominance of alveolar, basal and diffuse patterns in the primary tumors, and a predominance of diffuse and papillary patterns in the secondary tumors. Proliferation index, VEGF expression and microvessel density were different between primary and secondary cancers. However, the expression of carbonic anhydrase 9, a marker of hypoxia, was similar between matched primary NSCLCs and brain metastases, suggesting similar levels of hypoxia in matched cases. The proportion of mature vasculature was higher in brain metastases, suggesting that these lesions may be refractory to anti-VEGF therapy. These results highlight the importance of characterizing the vasculature of the primary tumors and metastases for clinical trials and in biomarker studies evaluating antiangiogenic agents in brain metastases treatment [82].

Imaging metastases & their environments

Since metastasis is one of the most lethal aspects of cancer, the ability to noninvasively track the journey of cancer cells and their environments during metastatic dissemination would advance our understanding of the metastatic cascade. In the clinical setting, the ability to detect meta-static dissemination is important for staging and to evaluate treatment efficacy.

The sensitivity of the detection of metastatic lesions depends upon the imaging modality being used. With optical imaging of fluorescent reporters or bioluminescence imaging, nodules containing a few hundred cells can be detected, but because of poor penetration depth, the use of optical imaging clinically is restricted to the intra-operative setting and it is mainly used in preclinical studies. PET, MRI and MRS are clinically translatable. PET imaging has exquisite sensitivity of detection in the nanometer range but the spatial resolution is of the order of approximately 1 mm. Intrinsic contrast, generated by the diffusion of water, is being used to detect metastases with spatial resolutions of approximately 100 μm using diffusion-weighted MRI. The elevated total choline signal that is typically observed in most cancer cells can also be used to detect metastases, but since the sensitivity of detection of ^1H MRS metabolites are approximately 0.1 mM, spatial resolutions is of the order of a few millimeters.

Preclinical studies

In preclinical models, noninvasive optical tracking of cancer cells following metastatic dissemination can be performed by inoculating cancer cells expressing optical reporters such as TdTomato-red fluorescent protein [83]. MRI can also be used to follow the occurrence of metastases in distant organs. As an example, following splenic injection of murine pancreatic tumor cells, the formation of metastases in the liver was followed over time by acquiring high-resolution T_1 -weighted images following injection of gadolinium ethoxybenzyl diethylenetriamine pentaacetic [84]. Since the agent is taken up by liver cells, the liver appears hyperintense and metastases are evident from nonenhanced hypointense regions in T_1 -weighted images [84]. Bioluminescence imaging can also be applied to follow metastases progression. PC3 prostate cancer cells transfected to express the *FLuc* gene were used to detect tumor growth and dissemination *in vivo*. Quantification of disease burden is possible since the optical signal is proportionate to the number of cells [85]. In another study, MDA-MB-435-HAL-Luc cells were used to track metastases to the lungs by bioluminescence imaging [86].

Several PET probes are being developed to detect primary tumors as well as metastases, but high specificity and sensitivity are necessary to detect small metastatic lesions. The chemokine, CXCL12, and its receptor, CXCR4, play an important role in metastasis. Inhibitors of the CXCL12–CXCR4 axis have been shown to reduce metastasis occurrence [87]. A PET imaging agent was developed using [^{64}Cu] AMD3100, a positron-emitting analog of the stem cell mobilizing agent plerixafor, to detect metastases in a preclinical model of breast cancer [88]. Lung metastases derived from human MDA-MB-231 breast cancer cells expressing CXCR4 were imaged by PET following injection of the probe [88].

Clinical studies

In the clinical setting, whole-body imaging techniques are most suitable to determine the extent of the disease and for detecting metastases in different organs. Bone is a common site of metastases in breast cancer patients [89,90]. In MRI studies investigating bone metastases in breast cancer patients, T_1 -weighted images were found to have the highest sensitivity to detect metastases in the spine and pelvis. The specificity of the MR analysis was improved by combining T_1 -weighted images with DWIs [91]. Detection of cancer-positive pelvic lymph nodes in patients with cervical and uterine cancers reached a sensitivity of 83% and a specificity of 99% by combining size analysis with apparent diffusion-coefficient values, [92]. The overall bone metastasis detection rate was reported to be 92.3% for DWI, compared with 56.5% and 23.1% for PET and bone scintigraphy, respectively [93].

MRI detection of lymph node metastases can be improved by intravenous injection of highly lymphotropic superparamagnetic particles that localize in the lymph nodes through interstitial–lymphatic fluid transport [94]. The presence of cancer cells results in an abnormal pattern of superparamagnetic particle accumulation that can be detected in T_2 -weighted images [94]. Using lymphotropic nanoparticles, occult lymph node metastases were identified in several prostate cancer patients who had undergone radical prostatectomy, although these positive lymph nodes were not enlarged [95]. Precontrast T_2^* -weighted images showed homogeneous high signal intensity of the lymph node, but in the postcontrast T_2^* -weighted image, signal heterogeneity indicative of metastatic involvement was observed. In the CT scan image, the 5-mm node did not demonstrate any morphologic features characteristics of malignancy [95].

^1H MRSI of total choline has also been used to determine the presence of cancer cells in enlarged lymph nodes in a patient with recurrent prostate cancer [96]. ^1H MRSI acquired at 3T, revealed high total choline in an enlarged lymph node that was confirmed to contain cancer cells in histopathologic analysis [96].

Combining imaging modalities can also be used to investigate the microenvironment of metastases as was recently shown by Jansen *et al.* in 13 patients with neck node metastases [97]. In these studies, DCE-MRI was combined with ^{18}F -fluoromisonodazole PET imaging and demonstrated that hypoxic nodes were poorly perfused compared with nonhypoxic nodes [97].

Conclusion

Some of the broad challenges for molecular and functional imaging are to reduce mortality from metastasis by understanding the metastatic cascade. With a molecular functional approach (Table 1), as demonstrated in Figure 8, noninvasive imaging can determine the effect of the TME on cancer cells and *vice versa*. These insights can provide new therapeutic strategies to prevent the occurrence of the metastatic cascade as well as to design effective image-guided treatments that would target metastases and minimize damage to normal tissue.

Future perspective

The direction of evolution of multimodal molecular and functional imaging over the next 5–10 years will be to identify primary tumors that are likely to metastasize. For this to be achieved, imaging biomarkers of specific microenvironments and molecular pathways that identify tumors with a predilection for metastasis will have to be identified. In pathology assessment of biopsy or excised tumor tissue, localized regions of the tumor or biopsied material are sampled. Imaging provides sampling of the entire tumor and, therefore, a comprehensive assessment of metastatic risk. This, together with the ability to obtain temporal and spatial information, would allow image-guided targeting of specific microenvironments that are most likely to permit metastatic dissemination. Another major challenge is to visualize changes in the premetastatic niche, and exploit this to identify and develop image-guided therapeutic strategies in inhibiting the establishment of the premetastatic niche as a road-block in the metastatic cascade.

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Executive summary

- Multimodal molecular and functional imaging can be used to explore several processes in the metastatic cascade and can identify potential physiological and metabolic biomarkers that can be applied to identify metastasis-permissive environments and metastatic lesions.
- Imaging can be used in cell culture systems to study the interaction of the cancer cells with other stromal cells or with the different extracellular matrix components. The physiological environment can also be modified to assess the impact of changes on cancer cell invasion, proliferation and metabolism.
- Imaging can also be applied *in vivo* to explore the interactions between cancer cells, stromal cells and the extracellular matrix. Proteolysis, angiogenesis, lymphangiogenesis, hypoxia and acidic extracellular pH are among the parameters that can be imaged and analyzed noninvasively either alone or in combination.
- In the future, molecular and functional imaging techniques should be applied to further characterize the premetastatic niche. Advances in the understanding and characterization of niches that provide a microenvironment conducive for the formation of metastases could lead to the discovery of new targets and new therapeutic approaches against the metastatic cascade.
- Differences between primary and metastatic tumors need to be characterized to improve treatment efficacy, since metastases typically become refractory to treatment. Identifying imaging-based differences could lead to the development of image-guided therapeutic strategies to treat metastatic disease.

Figure 1. Metastatic cascade
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Figure 2. Imaging cancer cell–stromal cell interaction

(A) Representative axial magnetic resonance images of two contiguous 0.5-mm thick slices containing human umbilical vein endothelial cell labeled with Feridex I.V.[®]. A lumen-like network formation of the cells is apparent in the T₂-weighted images 24 h postseeding.

(B) 3D reconstructed image of multislice data showing a lumen-like human umbilical vein endothelial cell network in the extracellular matrix gel (left). Orthogonal slices in the axial direction provide the ability to dynamically follow migration, invasion and network structure through the extracellular matrix gel (right).

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Figure 3. Imaging invasion and metabolism

(A) Photomicrograph of prostate cancer PC3 cells grown adherently on microcarriers (left) and schematic display of the sample structure (center). On the right are photomicrographs of the invasion chamber filled with ECM gel (top) and an example of the distribution of HUVEC on the ECM gel at the start of an experiment (bottom). (B–E) Representative 1D ¹H CSI spectra of 310-μm-thick slices (every third spectrum is shown) obtained along the sample at 47 h, acquired with or without water suppression, together with the corresponding ¹H magnetic resonance images for (B) oxygenated PC3 cells, (C) hypoxic PC3 cells, (D) PC3 cells in the presence of HUVEC under oxygenated conditions and (E) PC3 cells in the presence of HUVEC under hypoxia. †150–300 μm.

ECM: Extracellular matrix; HUVEC: Human umbilical vein endothelial cell.

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Figure 4. *In vivo* near-infrared fluorescence imaging of human fibrosarcoma HT1080 tumor-bearing animals

The top row shows raw image acquisition, with a representative untreated mouse shown on the left and a representative treated mouse shown on the right (150 mg/kg prinomastat, twice a day, for 2 days). The bottom row shows color-coded tumoral maps of matrix metalloproteinase-2 activity superimposed with white-light images of no treatment (left) and prinomastat treatment (right).

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Figure 5. Melanoma flavoprotein redox ratio images and corresponding histograms of C8161, A375P5, A375P10, A375M and A375P cell lines ranging from more invasive to less invasive *in vitro*, respectively

Differences were detected between the tumor cores and rims for all aggressive melanomas of (A) C8161, (B) A375P5, (C) A375P10, (D) A375M and (E) A375P cell lines, with the tumor cores more oxidized with higher Fp redox ratios than the rims. This is also evident in the bimodal distribution of the histograms showing the number of image pixels (y-axis) for specific Fp redox ratios (x-axis). (E) The least metastatic A375P melanoma is largely reduced with a single histogram peak of a relatively low redox ratio.

Fp: Flavoprotein.

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Figure 6. Hypoxic regions and collagen I fibers heterogeneous distributions in MDA-MB-231 breast tumor

(A) Bright-field, green fluorescence and overlay microscopic images (left to right) of a fresh 2-mm thick section from a MDA-MB-231–5HRE–enhanced green fluorescent protein tumor.

(B–C) Representative second harmonic generation collagen I fiber (red) and hypoxia (green) images from a normoxic (B) and a hypoxic (C) field of view, displayed as z-projection (left) and as 3D reconstructed image (right). (D) Mean distance distribution to the nearest fiber voxel (measure of fiber density) and (E) percentage of fiber voxels within the ROI (mean $\pm$ SD).

ROI: Region of interest.

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Figure 7. Double dendrogram illustrating the feasibility of clustering orthotopic and subcutaneous microenvironments on the basis of three imaging parameters measured *in vivo*: vascular volume, permeability-surface-area product and total choline concentration

The color bar represents the range of the variables color coded according to a log scale in order to accommodate the entire dynamic range of each variable. VV, PSP and total choline data clustered into two distinct groups, with the orthotopic group exhibiting elevated values for each parameter (red colors) compared with the subcutaneous group. The distance axis on the dendrogram represents the distance or dissimilarity between the two clusters. In addition, apparent from this 'double' dendrogram is that the PSP and total choline cluster together for this group of animals.

Ortho: Orthotopic; PSP: Permeability-surface-area product; Subcut: Subcutaneous; VV: Vascular volume.

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Figure 8. Overview of the multifaceted role of molecular and functional imaging in metastasis
DCE: Dynamic contrast-enhanced; DWI: Diffusion-weighted image; ECM: Extracellular matrix; MR: Magnetic resonance; MRS: Magnetic resonance spectroscopy; MRSI: MR spectroscopic imaging; pHe: Extracellular pH; pHi: Intracellular pH; SHG: Second harmonic generation.

Table 1

Imaging methods that are currently available for investigating specific aspects of the tumor microenvironment.

Tumor microenvironment characteristics	Methods	Ref.
Cancer cell–stromal cell interaction	Intact perfused cell MRI and MRS Optical imaging	[9,11,13,16]
Cancer cell invasion	Intact perfused cell MRI and MRS	[9,11,13]
Collagen fiber distribution	SHG microscopy	[18–20,62]
Fibroblast recruitment	MRI	[23]
Interstitial transport of macromolecules	Macromolecular contrast agent MRI	[24]
Immune cell imaging	MRI	[26–28]
Proteolytic enzyme activity detection	MRI Optical imaging	[29,30,32,34]
Lysosome imaging	Optical imaging	[36]
Tumor vasculature/angiogenesis	MRI PET SPECT	[40,41,44,45,48]
Lymphangiogenesis	PET	[49]
Hypoxia	MRS Optical imaging PET	[54–61]
Metabolism	MRS MRSI	[63]
pH	MRS MRSI	[70,71,73]
Metastases detection	MRI MRS Optical imaging PET	[83,85,86, 88,91,94–96]

MRS: Magnetic resonance spectroscopy; MRSI: Magnetic resonance spectroscopic imaging; SHG: Second harmonic generation; SPECT: Single photon emission computed tomography.