INTRAVITAL IMAGING OF CELL MOVEMENT IN TUMOURS

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Metastasis is the cause of death for patients with many types of cancer, but the process of tumour cell dissemination is poorly understood. As primary tumours are three-dimensional, departure of cells from primary tumours has been difficult to study. Multiphoton microscopy has been developed for *in vivo* imaging and, using this technique, we are beginning to understand how invasive tumour cells move.

LASER-SCANNING MICROSCOPY
Focuses laser light on each point in the field of view to generate the image by scanning the laser beam across the field of view.

MULTIPHOTON FLUORESCENCE EXCITATION
Uses a laser beam to focus a high density of photons of twice the excitation wavelength of a fluorophore on a single point. Simultaneous absorption of two photons of half the excitation energy results in excitation of the fluorophore. The efficiency of excitation depends on the square of photon density. Higher numbers of photons can also be used: for example, simultaneous absorption of three photons at one-third the excitation energy will excite the flourophore, with a cubic dependence on photon density.

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Treatments that prolong life and improve the quality of life of patients with cancer will probably depend on a combination of treatments that minimize the growth of existing tumours and limit their spread to new sites. Treatments for primary-tumour growth have clear, measurable end points in terms of tumour size and are in constant use in clinical trials. However, the development of anti-metastasis treatments is more difficult, and will depend on a detailed understanding of the basic steps of metastasis, beginning at the primary tumour and ending with the establishment of new tumours in the target organs.

Intravasation — the entry of tumour cells into the vasculature — is the result of migration of tumour cells towards blood vessels through intervening connective tissue and the basement membrane, and finally through the endothelium of the blood vessel. The importance of studying invasion and intravasation in the primary tumour is apparent when it is considered that intravasation of tumour cells from the primary, secondary and tertiary metastases are key steps in systemic spread of the cancer, and that this migration continues after removal of the primary tumour. Understanding the mechanisms behind invasion and intravasation will provide the insights that are needed to prevent formation of secondary and tertiary metastases.

Most studies of metastasis up until now have focused on the steps that occur after intravasation. These steps include arrest of tumour cells in the vessels of the target organ — either by adhesion to the vessel walls or by occlusion of small capillaries — exit from the vessels (extravasation) and growth in target organs[1,2]. Intravital-imaging studies have been especially useful for characterizing primary-tumour properties, growth rates and mechanisms of metastasis to target organs[1,3,4]. The initial escape of metastatic cells from tumours has been less well studied *in vivo* — in part because of the relative inaccessibility of this process to direct observation. Intravital multiphoton microscopy combines the advanced optical techniques of LASER-SCANNING MICROSCOPY with long-wavelength MULTIPHOTON FLUORESCENCE EXCITATION to capture high-resolution, three-dimensional (3D) images of living tissues that have been tagged with highly specific fluorophores. Advances in this technique, combined with animal models of cancer that use the stable expression of green fluorescent protein (GFP) from tissue-specific promoters, now make it possible to directly observe cell behaviour in primary tumours in live animals.

These advances in our ability to image cells in primary tumours have stimulated new insights and hypotheses about the mechanisms of cell migration during invasion and intravasation, and information about the microenvironment that is required for these key steps in metastasis. The interaction of cells with the extracellular matrix (ECM) and entry of tumour cells into the circulation in the primary tumour are believed to be important factors in tumour metastasis[5], and can now be directly observed using intravital multiphoton microscopy. Therefore, intravasation — which has always been assayed through indirect methods such as assaying for tumour-cell markers in the blood[6] — can be observed in real time. We can now address questions such as

Summary

- Carcinoma cells in the primary tumour can move at up to 10 times the velocity of similar cells *in vitro*.
- The highest velocities are observed for carcinoma cells in metastatic tumours that are moving along linear paths in association with extracellular-matrix (ECM) fibres.
- Carcinoma cell motility is characterized as solitary amoeboid movement and is unrestricted by networks of ECM in mammary tumours.
- Carcinoma cell motility is restricted at the basement membrane of blood vessels, where the cells must squeeze through small pores in the basement membrane/endothelium to gain access to the blood space.
- Carcinoma cells in non-metastatic tumours are fragmented during intravasation as they squeeze across the basement membrane/endothelium, whereas carcinoma cells in metastatic tumours cross this restriction as intact cells.
- Carcinoma cells in metastatic tumours are attracted to blood vessels, where they form a layer of cells that are morphologically polarized towards the vessel.
- Chemotaxis to epidermal growth factor is shown by carcinoma cells *in vitro* and *in vivo* in primary tumours, and might be responsible for the attraction of carcinoma cells to blood vessels.
- Cell polarity towards blood vessels is correlated with increased intravasation and metastasis.
- The ECM and its interaction with carcinoma cells can be observed directly using the second harmonic signal from multiphoton-illuminated tumours.
- Metastatic mammary tumours contain large numbers of rapidly moving macrophages and other leukocytes near blood vessels. These might be a source of chemotactic cytokines.
- Intravital imaging can be productively correlated with gene-expression profiling to generate new insights into the pathways that are responsible for invasive-cell behaviour.

how the *in vivo* motility of carcinoma cells differs from that of normal cells and what features of tumour cells determine their metastatic potential. These insights should provide important information that can be used in the diagnosis and treatment of metastases.

In vivo imaging

Before the introduction of GFP and its derivatives, intravital-imaging studies were limited to the study of tumour cells that were transiently labelled with vital dyes and a basic analysis of tumour vasculature[7–11]. Alternatively, other approaches involved the use of tissue preparations that were thin enough for bright-field microscopy, as in the pioneering work by Wood *et al.*, who used the rabbit ear chamber to show tumour cell motility during metastasis[12]. The combined use of intravital imaging with stable fluorescent labelling via GFP expression vectors now allows the direct imaging of intravasation at the single-cell level within the primary tumour in the context of metastatic potential.

CONFOCAL MICROSCOPY
A term that is mainly applied to specific light-microscopy techniques that are designed to minimize out-of-focus contributions from the vertical axis to an image. Typical single-photon microscopy makes use of a pinhole aperture to eliminate out-of-focus contributions, whereas multiphoton (also termed two-photon) microscopy makes use of the non-linear dependence of excitation on photon density to only excite molecules in a single plane of focus.

SECOND-HARMONIC GENERATION
The scattering of light by asymmetrically arranged electron orbitals in amino acids in α-helix-containing proteins such as collagen, elastin and laminin. The scattered light is polarized along the axis of the helix and is at half the wavelength of the incident multiphoton excitation.

Box 1 | Multiphoton microscopy in intravital imaging

Multiphoton microscopy in intravital imaging provides several technical advantages over conventional microscopy[24]:

- Bulk fluorophor bleaching and generation of toxic by-products is reduced because of the lack of fluorophore excitation in regions away from the focal plane.
- Multiphoton images are less prone to degradation by light scattering for two reasons. First, the longer wavelengths that are used for excitation suffer less scattering from microscopic refractive-index differences within the sample, allowing penetration further into tissues. Second, as all the resolution is defined by the geometry of the excitation beam, the fluorescence emission is unaffected by light scattering. Live tissues contain several refractive index interfaces, which cause significant light scattering.
- Multiphoton imaging is more efficient. As excitation in multiphoton imaging is confined only to the optical section being observed, a pinhole aperture is not required for confocality. So, for identical detector configurations, 100% of the collected light is measured at the external detector as compared with ~1% of the collected light in a conventional pinhole confocal microscope.
- True registration in the Z-axis is achieved. Multiphoton microscopy excites at one discreet diffraction-limited point and collects the emitted light without deconvolution to different focal planes. In standard multiprobe confocal microscopy, because wavelengths of different colours of light that originate from one point source focus at different focal planes, registration of many probes along the Z-axis is problematic.
- Second-harmonic generation allows the visualization of extracellular matrix that contains α-helical proteins such as collagen and laminin in a confocal plane without the need for fluorescent labelling.

These additive effects mean that images can be obtained from deeper within a sample than with any other type of light microscopy that is able to achieve single-cell resolution.

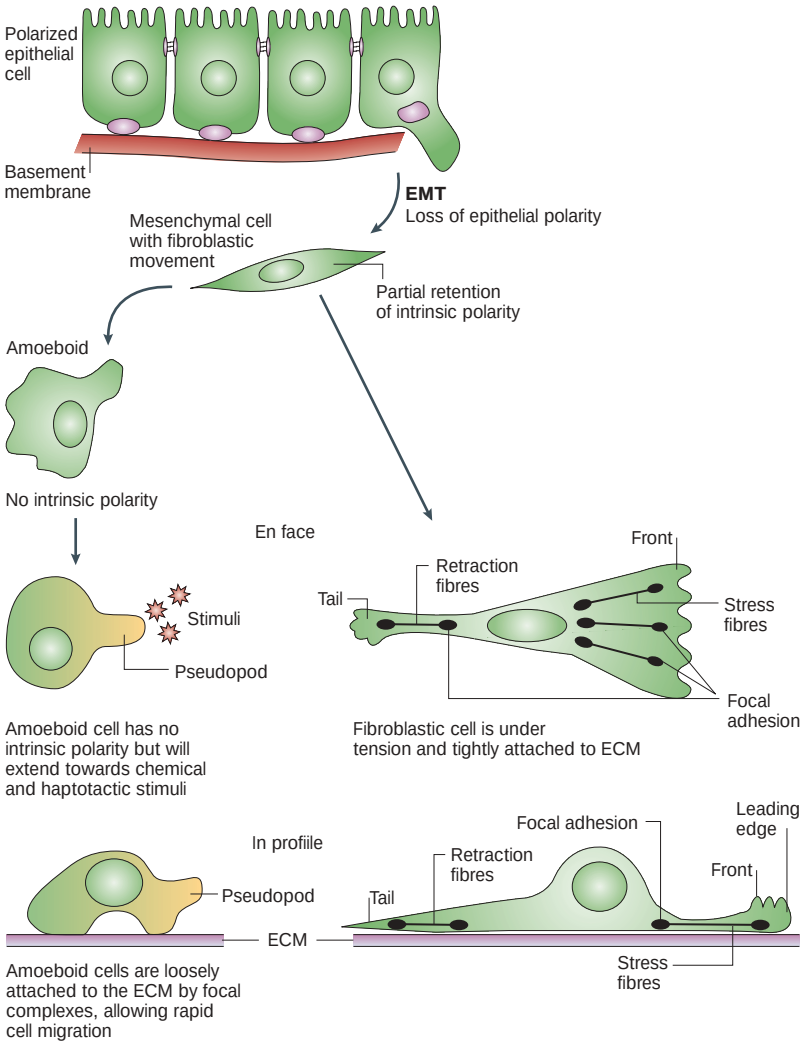

Figure 1 | **Definitions of types of cell movement.** During the epithelial–mesenchymal transition (EMT), tumour cells undergo a loss of intrinsic polarity and lose cell–cell junctions that are required for the integrity of the epithelium. These events require extensive reorganization of the cytoskeleton and initiation of actin-based cell motility[92]. Cells that partially retain their polarity still have a tail that contains retraction fibres and a front that contains stress fibres and can form focal adhesions. They can still attach to the extracellular matrix (ECM). Conversely, cells that lose all polarity are characterized as 'amoeboid'. These cells can chemotax towards stimuli and are only loosely attached to the ECM, allowing for rapid migration. The RHO family of small GTPases is known to be involved in EMT through its effects on junction stability[93], messenger RNA targeting to promote cell polarity[94] and actin dynamics that support cell motility[66]. We propose that as EMT progresses, the complete loss of intrinsic epithelial-cell polarity and the acquisition of random amoeboid locomotion is achieved, resulting in the extreme de-differentiated amoeboid phenotype of the invasive carcinoma cell.

Initial studies of tumour biology that used stable GFP expression focused on static images and examination of metastases[13–18]. The first use of stable GFP expression to characterize the motility, shape changes and migration of carcinoma cells at cellular resolution in live intact tumours *in vivo* was described by Farina *et al.*[19] using CONFOCAL MICROSCOPY. These studies were limited by the relatively poor optical depth of penetration of tissue due to the short excitation wavelengths, extensive photobleaching and phototoxic damage to cells in the tissue. The recent introduction of multiphoton microscopy[20,21] has allowed for much greater depth of penetration of tissue for imaging and has greatly decreased the amount of photobleaching and photodamage[3,22–24] (BOX 1). In addition, the ability to image ECM fibres using SECOND-HARMONIC GENERATION avoids the need to stain the ECM with dyes, making it possible to directly analyse cell–ECM interactions and proteolysis of the ECM in live tumours.

Studies involving the use of intravital multiphoton microscopy with animals that have received orthotopic injection of tumour cells (cell grafts, xenografts), or with transgenic mice that express GFP from tissue-specific promoters, has opened the primary tumour to direct evaluation at the cellular level of resolution[23–28]. For example, a vector that contains the mouse mammary tumour virus (*MMTV*) promoter can be used both to induce breast tumour formation, via expression of oncogenes such as *ERBB2* (also known as HER2/neu) and the polyoma middle T protein (*MMTV-PyMT*)[29], as well as to target expression of *GFP* to the mammary gland (*MMTV-GFP*). The promoter or oncogene can be changed to vary the characteristics of the tumour to be studied. In this review, we will discuss hypotheses that were inspired by our study of invasion and intravasation using these technologies. Detailed analyses of extravasating tumour cells and metastases are being performed[30–33] as well, but have been recently reviewed[1] and will not be covered here.

Concerns have been raised about the effects of chronic GFP expression on cell behaviour and about the viability of GFP-expressing cells in the tumour environment. GFP has been shown in several models to be a useful marker that does not affect the growth, invasion and behaviour of cells in both oncogene- and cell-graft-generated tumours[19,28,34,35]. Furthermore, the rapid development of tumours in *MMTV-PyMT* mice has allowed the comparison of GFP-expressing tumours with non-GFP-expressing *MMTV-PyMT* tumours. The defined histopathological stages of tumour progression, such as hyperplasia, adenoma, early- and late-stage carcinoma, are recapitulated with a high degree of reproducibility in *MMTV-PyMT* × *MMTV-GFP* mice[36,37]. In our studies, GFP expression did not affect the grades or stages of tumour progression, which indicates that it can be a neutral marker for following cell behaviour *in vivo*[28], but it is important to confirm this for each system studied[38]. In addition, the short latency, high penetrance and reproducible progression of the *MMTV-PyMT* tumour model has made it ideal for imaging of cell migration at defined points of tumour progression[39]. Its simplicity and ease of use is surpassed only by cell-graft models of mammary tumours.

Cancer cell movement *in vivo* and *in vitro*

Intravital imaging has identified several differences between the way in which carcinoma cells move *in vivo* (in tumours in live animals) compared with *in vitro* (culture models of invasion). These differences could provide important clues as to the molecular mechanisms of invasion in the primary tumour and reflect interactions between cells and the microenvironment of

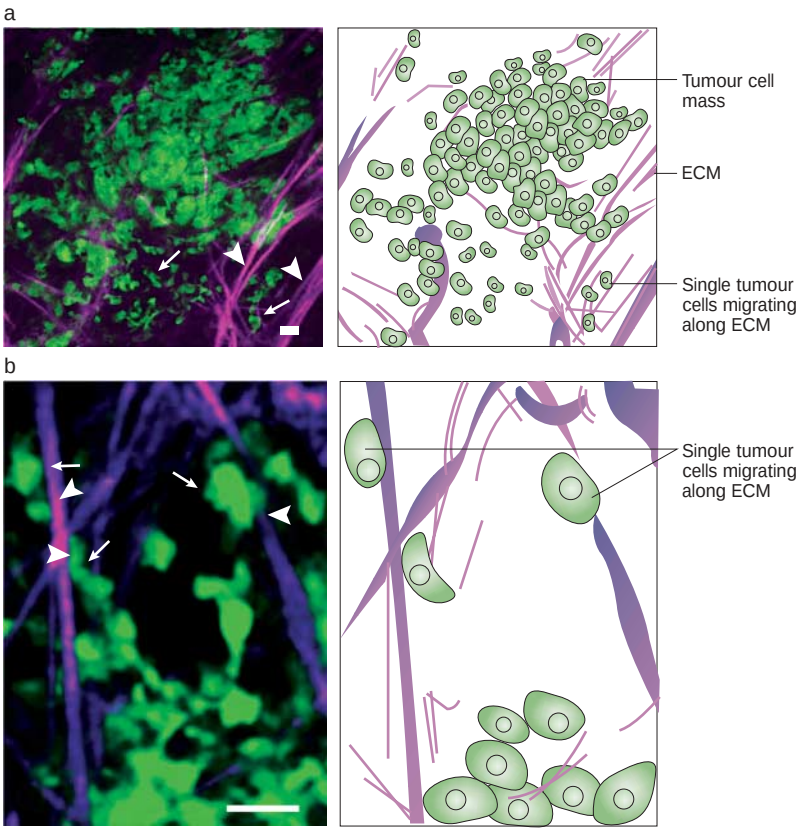

Figure 2 | **Carcinoma cells in primary mammary tumours move along ECM fibres.** Multiphoton microscopy shows that carcinoma cells (green) move on extracellular-matrix (ECM) fibres (purple) and do not seem to be constrained by the ECM networks. **a** | A low-magnification image that shows, in the lower third of the image, single carcinoma cells (arrows) invading away from the central tumour mass and accompanying ECM fibres (arrowheads). **b** | A higher-magnification image — visualized by second-harmonic generation — that shows carcinoma cells (arrows) in a tumour contacting collagen-containing fibres. Arrowheads point to cell–matrix interactions. Scale bar = 25 μm. From REF. 24.

the tumour that have not yet been duplicated *in vitro*. For example, in mammary tumours, carcinoma cells migrate as solitary amoeboid cells. They have not been observed to migrate as collections of attached cells as observed in several *in vitro* models of invasion (reviewed in REF. 40).

Another striking difference between migration of adenocarcinoma cells *in vivo* and *in vitro* is the difference in velocity. In tumours that result from both cell grafts and tissue-specific oncogene expression, migrating adenocarcinoma cells have been clocked at speeds greater than 3 μm/min — speeds tenfold higher than those seen for cells moving in two-dimensional (2D) culture[41], and as much as 30-fold faster than cells moving in 3D networks of ECM *in vitro*[40,42]. Another key difference between migration *in vivo* and *in vitro* is the high degree of persistent linear motion of carcinoma cells in tumours, compared with the random walking of equivalent cells in culture. For example, in 2D cultures, the metastatic cells we have studied are not POLARIZED in their locomotion and walk in random directions, compared with less metastatic cells and primary cultures of normal cells, both of which show polarized linear walking[43,44]. These properties could be crucial to allow metastatic cells to respond to chemotactic signals and to crawl along ECM fibres *in vivo*, as discussed below.

In mammary tumours, invasion and intravasation occur primarily during the carcinoma stage[19,36,45]. During this stage, extensive loss of ECM networks has already occurred and cell shape and movement are not constrained by dense networks of ECM (FIG. 1). ECM fibres that are composed of collagen and possibly other ECM proteins are identifiable and migration of cancer cells is most evident on these long ECM fibres[24,46] (FIG. 2). Cell migration of this type is characterized as 'amoeboid', meaning that the cells undergo marked shape changes while crawling and are not fibroblastic during migration. Fibroblastic migration is characterized by cells that are elongated and under tension, causing them to be rigid in shape except near their leading edges, where lamellipods are observed to extend (FIG. 1). *In vitro*, dense networks of ECM can induce amoeboid motility[40,42]. However, our *in vivo* imaging raises the possibility that such severe ECM constraints are not always necessary. If carcinoma cells are able to undergo amoeboid movement *in vivo* — in the absence of constraints of an ECM network that is postulated to cause the transition from fibroblastic to amoeboid motility[42] — other factors must be at work in the tumour to induce this transition (FIG. 1).

A likely candidate for such a factor is the tightness of contact between the tumour cell and the ECM fibre on which it migrates. The types of contacts that form between carcinoma cells and ECM fibres during rapid migration *in vivo* have not been characterized. However, *in vitro*, contacts between migrating cells and ECM are of two types — focal complexes and focal contacts/adhesions[47]. Both involve integrin-mediated interactions with fibronectin, laminin, vitronectin and collagen, but differ in stability. Focal complexes are transient structures that do not support significant tension and that assemble and disassemble rapidly near the leading edge of the cell, but can develop into focal adhesions and then persist to the middle of the cell. They are thought to mediate adhesion to ECM in rapidly moving cells. Focal adhesions are stable, support tension and are characteristically seen in slowly moving or static cells under tension, such as fibroblasts that are tightly attached to ECM. The molecular composition of both is similar, and focal adhesions are believed to mature from focal complexes.

The importance of the distinction between focal complexes and focal adhesions for tumour cell migration is that focal complexes can form transient contacts with the ECM. The rapid speed and shape of the tumour cells that are associated with ECM fibres that have been observed *in vivo* confirms this, as stable focal adhesions would slow movement considerably and cause the elongation of cells along ECM fibres because of the development of tension in cells[48,49]. This would produce a fibroblast-like mode of migration, which is not observed *in vivo*. So,

POLARIZATION
The orientation of the cell in a specific direction, often indicated by the alignment of the long axis of the cell with the direction of polarization.

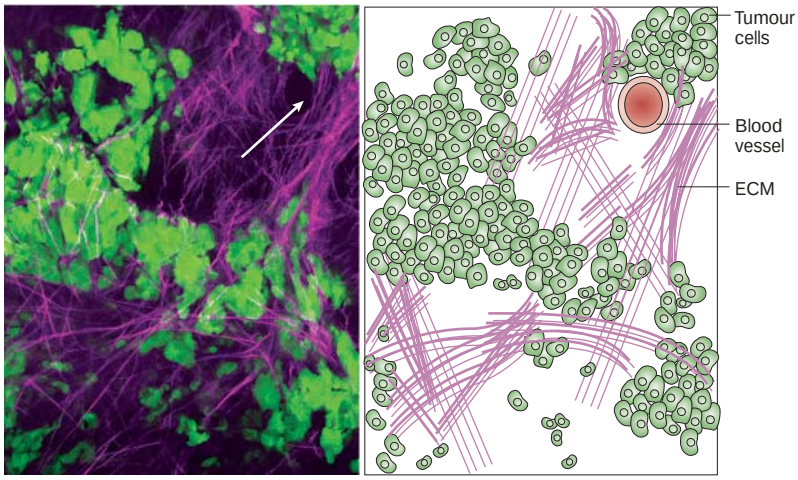

Figure 3 | **ECM fibres converge on blood vessels in mammary tumours.** Multiphoton microscopy shows that carcinoma cells (green) move on extracellular-matrix fibres (purple), some of which converge on blood vessels (arrow).

tumour cells probably move as amoeboid cells *in vivo* because of the formation of focal complexes, rather than because of the constraints of ECM networks. An analysis of the nature of the cell–ECM adhesions *in vivo* and *in vitro* in 3D matrices will be an important test of this hypothesis.

So, three factors — the absence of a dense network of ECM, amoeboid movement and linear ECM fibres — allow cells *in vivo* to undergo persistent linear excursions over long distances at very high speeds, while still maintaining contact with the ECM fibres. This contributes to the efficiency of invasion by allowing cells to traverse large distances within the tumour, and can enhance intravasation because some ECM fibres converge on blood vessels in mammary tumours (FIG. 3). These fibres therefore serve to deliver the highly motile invasive cells to vessels.

Multiphoton imaging has also shown that the phenotype of mammary carcinoma cell migration changes abruptly when carcinoma cells contact blood vessels. Second-harmonic imaging has shown that blood vessels in primary mammary tumours often have a dense coating of ECM (FIGS 3,4a). The dense ECM that is associated with the vessel surface causes slowing in the velocity of amoeboid movement as the cell body squeezes through the ECM network of the vessel wall to gain entry into the blood space (FIG. 4b). In this location, the migration of carcinoma cells in mammary tumours can resemble the non-proteolytic amoeboid phenotype that has been described for cell movement in 3D networks of ECM, in which both cell shape and locomotion are constrained by dense networks of ECM[42].

In vitro assays are being developed that mimic the *in vivo* environment more accurately, in terms of 3D structure, the presence of ECM networks and, possibly, the presence of other cell types[50]. Such assays will be extremely useful in allowing us to identify the elements that are necessary for motility of cancer cells *in vivo*.

Metastatic versus non-metastatic tumours

An important question is whether cell migration differs between metastatic and non-metastatic tumours. Multiphoton microscopy has allowed a direct comparison of cell motility behaviour in metastatic and non-metastatic tumours. These studies typically involve tumours that are derived from grafts of cells with differing metastatic potential. To identify important behavioural properties of metastatic cells within the primary tumour, we have compared the *in vivo* motility of cells of mammary tumours that are formed by the poorly metastatic MTC cell line with the highly metastatic MTLn3 carcinoma cell line. These are a well-characterized pair of cell lines that were originally derived from the same tumour, and retain their relative metastatic phenotypes even after prolonged culture[19,51]. These cells were used to generate primary tumours *in vivo* and the motility of the cells in these tumours was observed. The comparison of the properties of these primary tumours highlights some intriguing aspects of the metastasis process.

Migration of cancer cells on ECM

Carcinoma cells move at similar rates in both MTC tumours and MTLn3 tumours, but the character of the cell motility is quite different. As previously mentioned, rapidly migrating carcinoma cells *in vivo* are closely associated with collagen-containing fibres — this is true of MTLn3 but not MTC carcinoma cell migration. In MTC tumours, the cells move over each other, and the direction of motility is non-linear and does not seem to be guided by collagen fibres[24]. However, MTLn3 cells have no motility in areas where there are no vessels or collagen fibres, paradoxically making cell migration a rare event, even in the highly metastatic MTLn3 tumours.

High-resolution time-lapse images of cell movement within MTLn3 tumours shows the linear and fibre-associated locomotion of carcinoma cells *in vivo*. This result is consistent with the streaming-like, linear cell locomotion shown previously in MTLn3 tumours with conventional confocal microscopy[19]. In these earlier studies, the underlying fibres that directed the linearity of motion were not, however, identified. It was only the use of second-harmonic imaging of ECM[24] that revealed the fibrous substratum on which the cells were migrating.

Carcinoma cells can migrate towards epidermal growth factor (EGF)-like ligands that are formed during the proteolysis of ECM. This could be an important property of invasive tumours[5]. MTLn3 tumour cells upregulate expression of the γ2 subunit of laminin-5 (a component of the ECM fibres) and bone morphogenic protein-1 (BMP1)[24] . Laminin-5 contains EGF-like sequence repeats and BMP1 is a protease that cleaves the γ2 subunit of laminin-5 to release these EGF-like peptides[52], which could be chemotactic[53]. Therefore, the patterned proteolysis of ECM fibres, leading to the release of chemotactic peptides, could contribute to the rapid migration rates of carcinoma cells on ECM fibres[19,24].

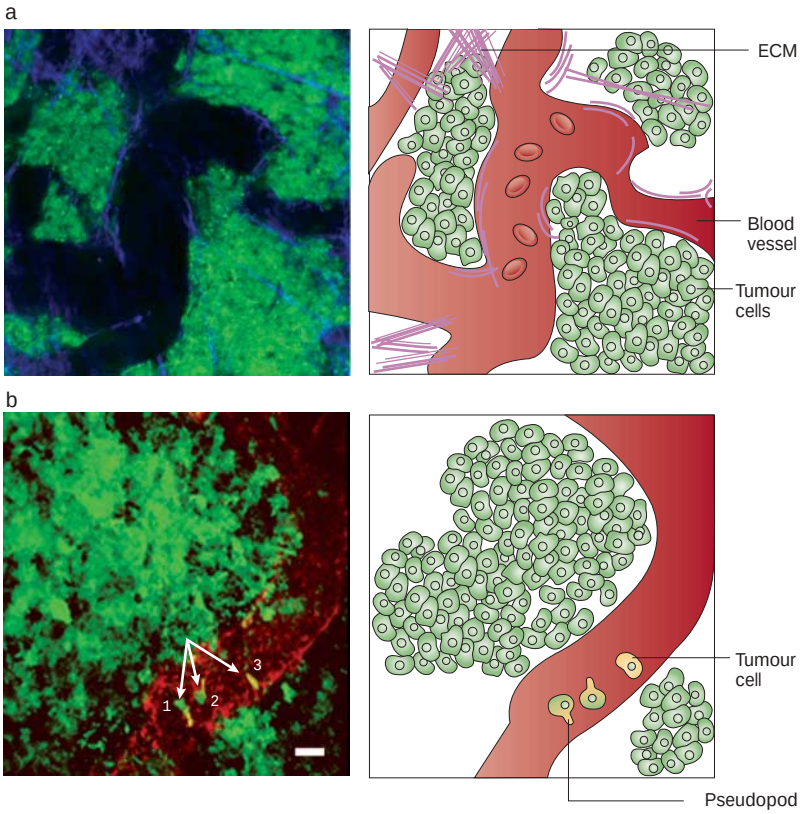

Figure 4 | **Intravasation in primary mammary tumours. a** | Blood vessels are seen as non-fluorescent regions. Extracellular-matrix (ECM) fibres (purple) line the border of the vessels and extend through the green fluorescent protein (GFP)-labelled tumour (green). The ECM of the vessels, as imaged here by second-harmonic generation, is believed to be associated with the basement membrane of the vessels. **b** | Intravasation of carcinoma cells in metastatic primary tumour as shown in a projected z-series that is ~180 μm thick. Rhodamine dextran was injected intravenously to image the blood vessel (red). GFP-labelled tumour cells are green, whereas portions of cells that have crossed into the blood space are yellow. Arrows indicate cells that are crossing or have crossed into the blood vessel. Cells 1 and 2 have yellow pseudopods but the cell body is green, indicating that these cells are still crossing the basement membrane/endothelium barrier. Cell 3 is uniformly yellow, indicating that it is entirely within the blood-vessel space. From REF. 24.

Chemotaxis towards blood vessels

Chemotaxis is the directed movement of cells in response to a soluble gradient of 'chemoattractant'. Chemotactic migration of carcinoma cells towards blood vessels could increase the efficiency of intravasation. Metastatic MTLn3 primary tumours contain carcinoma cells that form loose clusters of rounded, non-polarized cells — except near blood vessels, where they are highly polarized towards the vessel[45]. Non-metastatic MTC primary tumours contain carcinoma cells that show intrinsic cell polarity — they are elongated and form tight sheets, and their polarity is irrelevant to the position of blood vessels (FIG. 5). This has been confirmed by examination of intravital images and histopathological analysis of tumours[19,24,43], and could reflect the ability of metastatic cells to be more sensitive to chemotactic (or haptotactic) gradients[43,45]. Polarity of carcinoma cells towards blood vessels can be induced by chemoattractants that are secreted by vessel-associated cells, such as macrophages[36], or that diffuse into tissue from the blood. High rates of chemotaxis have been measured directly within metastatic primary mammary tumours of rats and mice, using MICRONEEDLE ASSAYS[54].

Consistent with this high rate of chemotaxis, expression-profile analysis[24,55] shows that MTLn3 cells in culture express higher levels of EGF receptor (EGFR) than MTC cells. *In vivo* MTLn3 tumours express even higher levels of EGF receptor, and cells from these tumours have been shown to respond to EGF *in vitro*[56,57]. On the other hand, MTC cells express much higher levels of the fibroblast growth-factor receptor and insulin-like growth-factor-binding protein than MTLn3 cells. As MTC cells do not polarize towards blood vessels, EGFR signalling might mediate tumour cell chemotaxis towards blood vessels.

In support of this concept, exogenous expression of the *EGFR* in MTC cells increases chemotactic responses to EGF *in vitro* and metastatic ability *in vivo*[58,56,59]. Expression of the *EGFR* and its homologues, such as *ERBB2*, has been correlated with poor prognosis in human breast tumours[60]. Chemotactic growth factors, including EGF, are present in serum, and are also expressed by macrophages, platelets and smooth-muscle cells near vessels[61–64].

Expression-profile analysis of MTLn3 cells has shown that other members of the EGFR signalling pathway are also upregulated[24,65]. Growth-factor signalling involves small GTPases of the RHO family[49,65,66], and EGFR transport involves small GTPases of the RAB family — both of which are upregulated in MTLn3 cells[24]. Lanzetti *et al.*[67] reported that the EPS8 (EGFR pathway substrate 8) coordinates EGFR signalling through RAC and transport through RAB5. MTLn3 cells express threefold higher levels of EPS8, compared with the non-metastatic MTC-derived tumours[24].

So, carcinoma cell chemotaxis towards blood vessels might be mediated by the EGFR signalling. This could provide an additional mechanism for enhancing metastatic capability, in addition to the well-characterized effects of EGFR signalling on MITOGENESIS. However, EGFR could be just one of many possible receptors that can induce polarized cell movement and promote metastasis. Given the wide variation in growth-factor expression that is seen in human tumours — even within a single category of cancer such as breast cancer[68] — the mechanistic contribution of each of these signalling pathways to metastasis will require careful study.

Cell fragmentation

A novel observation that resulted from intravital imaging of mammary tumours was the marked fragmentation of non-metastatic MTC-derived cancer cells when they come into contact with tumour blood vessels[45]. This was observed as the generation of subcellular fluorescent cell fragments that lie in contact with blood vessels. These fragments were swept away rapidly in the blood vessel, indicating that the fragmentation results from the shearing of pseudopods that are inserted into the blood flow.

MICRONEEDLE ASSAY
An *in vivo* invasion assay in which fine needles containing epidermal growth factor (EGF) in Matrigel are inserted directly into tumours of anesthetized animals. Cells that are attracted to the EGF are collected and counted.

MITOGENESIS
The initiation of the process of cell division, or mitosis.

Table 1 | Correlation of cell polarity to vessels and metastatic outcome

	Tumour size (cm^3)	Polarity (vessels)	Cells in blood (per 4 ml)	Cells in lung (per 40 HPF)	Lung metastasis/ section
Metastatic	31.0 ± 5.5	High	22.8 ± 13.6	35.7 ± 10.1	17.9 ± 13.6
Non-metastatic	44.5 ± 9	Low	0.25 ± 0.16	0.75 ± 0.75	0
p value	<0.4	—	<0.002	<0.003	<0.003

Results of analysis of metastatic MTLn3 and non-metastatic MTC mammary tumours. Tumour size is unrelated to metastasis, whereas carcinoma-cell polarity towards vessels is correlated with increased lung metastasis[41]. HPF, high-power field.

This 'fragility' of MTC cells, compared with MTLn3 cells, is consistent with changes in expression patterns of genes that are involved in regulating mechanical stability and survival[24]. MTLn3 cells, *in vivo* and in culture, express high levels of cytokeratins and apoptosis-suppressing genes, relative to non-metastatic MTC cells *in vitro* and *in vivo* — all of which might contribute to the ability of the MTLn3 cells to survive the intravasation process. The large relative overexpression of cytokeratins by MTLn3 tumours, relative to MTC tumours, might contribute to the mechanical stability that is necessary for cells to survive the shear forces that are encountered in the blood circulation. These keratins form the largest subfamily of intermediate-filament proteins and are important for the mechanical stability of epithelial cells that are subjected to shear forces[69]. Apoptosis, which results in cell lysis and fragmentation, might also be activated in these MTC cells undergoing intravasation. The ability of MTLn3 cells to withstand these factors might underlie their increased numbers in the circulation[45]. Similar properties could also contribute to their survival in target organs and extravasation efficiency[70,71].

Immune cells and metastasis

Another unexpected dividend of GFP-based intravital imaging has been the ability to image motile non-fluorescent host cells against a background of fluorescent tumour cells. This juxtaposition causes light scattering by the host cells, resulting in shadows that define their outlines. This can be used to observe the behaviour of these cells within the primary tumour and in association with carcinoma cells[45]. We observed increased numbers of non-fluorescent host cells in MTLn3 tumours, compared with MTC tumours, indicating that host immune cells might contribute to metastasis.

Complementing such observations, we have observed that highly motile macrophages are present in tumours of *MMTV-PyMT* transgenic mice that have GFP-expressing macrophages. These mice were created by the insertion of *GFP* into the lysozyme locus[72]. Macrophages and other leukocytes have been proposed to contribute to metastasis by producing chemotactic factors and by promoting degradation of the ECM barriers that form around blood vessels[73–77]. Macrophages have been also been found to promote tumour malignancy[78–80]. In mice that carry inactivating mutations in the colony-stimulating factor-1 (*CSF1*) gene — a cytokine that is important for macrophage development and migration — there is reduced metastasis from *MMTV-PyMT*-induced mammary tumours. In these tumours, macrophages might promote intravasation, which would explain the observations that CSF1 is required for cancer cell invasion and metastasis[36].

In summary, intravital-imaging studies in mouse models of cancer have shown several significant differences between cells from metastatic and non-metastatic tumours. These differences indicate specific mechanisms of metastasis that need to be tested in additional cell-graft and transgenic models. It will be important to evaluate changes in cell-migration characteristics during tumour development, as well as to compare tumours with different metastatic properties.

Cell polarity and metastatic potential

The intrinsic polarity of MTC cells, *in vitro* and *in vivo*, is connected with the stable polarization of actin polymerization at the cells' leading edge. Stable polarity can cause chemotactic inefficiency[43,44], and could

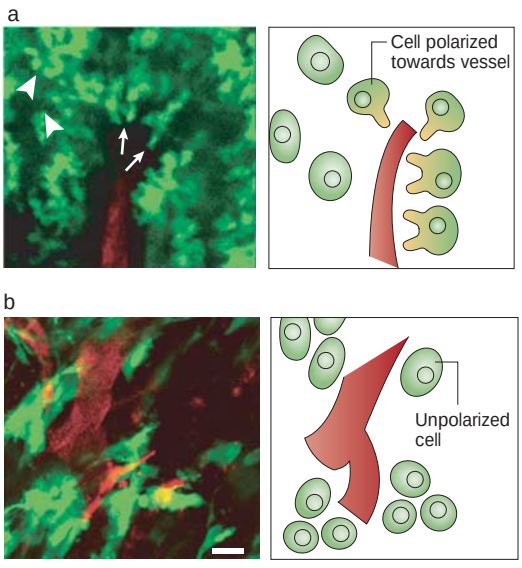

Figure 5 | **Metastatic cells orient towards blood vessels, whereas non-metastatic cells do not. a** | MTLn3-green fluorescent protein (GFP) carcinoma cells (green) near the vessel (red) are seen to orient towards the vessel by elongating pseudopods (arrows to single cells) compared with cells at a distance from the vessel which are rounded (arrowheads). **b** | MTC-GFP cells (green) do not orient towards the vessel (red), but remain elongated in random directions whether near or at a distance away from the vessel. Scale bar = 25 μm, which is close to the diameter of a single rounded cell of 20 μm. From REF. 45.

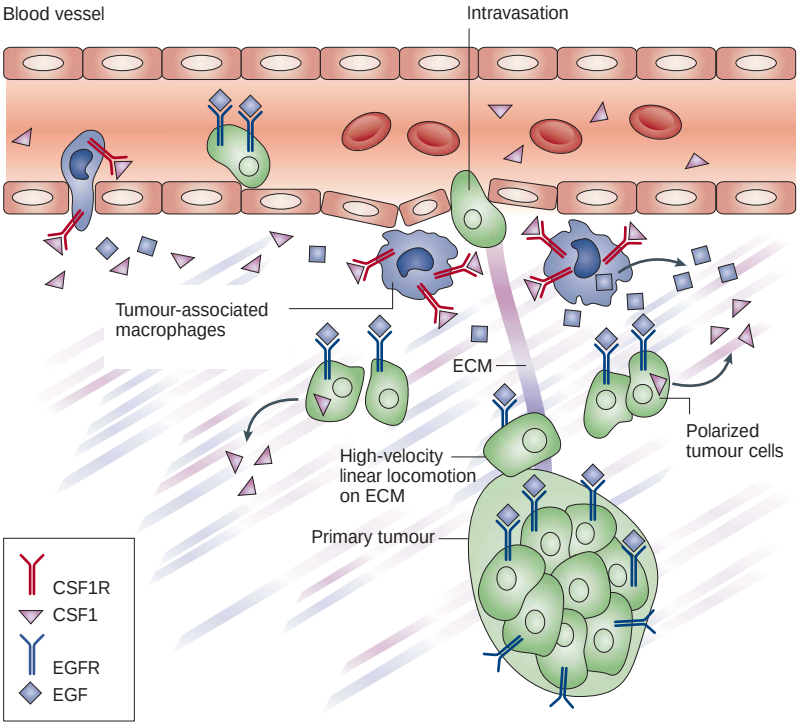

Figure 6 | **Model for intravasation of metastatic tumour cells.** An illustration that summarizes the interactions of carcinoma cells with blood vessels as seen by multiphoton-based intravital imaging. Carcinoma cells (green) of metastatic tumours collect near blood vessels as a polarized cell layer, which is a result of chemotaxis to the blood vessel in response to chemoattractants such as epidermal growth factor (EGF). Cancer cells express the EGF receptor (EGFR). Macrophages (purple) collect near the vessel in response to colony-stimulating factor-1 (CSF1), which is produced by tumour cells. Macrophages express the CSF1 receptor (CSF1R) and might be the source of EGF. The polarity of carcinoma cells is correlated with increased intravasation and metastasis.

reflect an incomplete transition to the amoeboid phenotype, resulting in reduced metastatic ability. By contrast, carcinoma cells in MTLn3 tumours are unpolarized — except when they are near blood vessels, where they become highly polarized (TABLE 1; FIG. 5). These results indicate that cells that have proceeded through the EPITHELIAL–MESENCHYMAL TRANSITION (EMT), such that all remnants of intrinsic cell polarity are lost, might be more sensitive to external chemotactic signals and more attracted to blood vessels in the primary tumour.

The inverse correlation between cell polarity and metastasis could be explained by the loss of the ability by metastatic cells (non-polarized, amoeboid cells) to localize the messenger RNA and proteins that are required to regulate cell polarity[43]. Highly metastatic cells have lost the ability to target mRNA for β-actin and related proteins, which are required for the assembly of dynamic actin filaments at the leading edge. Without a stable leading edge, the polarity of the metastatic cell can only be determined by external signals, such as chemotactic factors produced by blood vessels, resulting in intravasation. Gene-expression analysis of MTLn3 and MTC cells and tumours has shown that MTC cells and tumours express much higher levels of ZBP1 (REF. 24) — the zip-code protein that regulates mRNA targeting to the leading edge of cells[81] — than the metastatic MTLn3 cells.

Low levels of *ZBP1* expression in MTLn3 cells results in their inability to target β-actin mRNA to the leading edge[43]. Experimentally, this has been shown by disrupting mRNA targeting to the leading edge, using oligonucleotides that inhibit the interaction between ZBP1 and mRNA. This results in delocalization of mRNA and actin nucleation sites from the leading edge, and the disruption of intrinsic cell polarity[44]. ZBP1 is therefore a good candidate for a 'metastasis repressor' and, together with analysis of tumour cell polarity around blood vessels, might be used in prognosis.

Putting it all together

Intravital imaging has revealed the importance of chemotaxis for tumour cell invasion and metastasis. We propose that highly metastatic tumours show increased intravasation, via increased invasiveness and motility towards blood vessels, in response to chemoattractant gradients that are generated by blood-vessel-associated cells (FIG. 6). For epithelial tumours, amoeboid movement and intravasation can occur in association with blood vessels in localized regions and therefore amoeboid cells might not be easily detected in standard histopathological preparations. One of the challenges for future work is to determine at which point do cancer cells undergo the transition to amoeboid movement. Does it occur early in tumorigenesis or near the end of tumour progression?

The importance of intravasation in the primary tumour as a determinant of metastatic outcome is controversial, and the literature is inconsistent on this point[45,82–87]. In rat and mouse models of mammary tumour development, intravital imaging has shown that the cancer cell polarity towards blood vessels, intravasation and the number of tumour cells in the circulation are all correlated with metastatic outcome. This indicates that cell migration towards blood vessels and intravasation are key steps in metastasis[45] (TABLE 1). In addition, studies using cell-based assays indicate that tumour-cell burden in the blood can be correlated with poor prognosis[82,83]. However, PCR and antibody-based assays for tumour cells in the blood do not always show a strong correlation between the two[84–87].

This inconsistency might result from the methods that are used to score the blood burden of tumour cells. Indirect methods such as PCR and antibody reactivity can overestimate the number of viable carcinoma cells in the blood. Intravital imaging of intravasation has shown that cells become fragmented by shear forces that occur during intravasation, and possibly in the circulation. This fragmentation might cause the release of PCR- and antibody-detectable material, which is detected as 'cancer cells', but is obviously not able to produce metastases[45].

It will also be important to determine whether changes in cell polarity and chemotaxis also occur towards lymphatic vessels. Studies have shown that expression of the CXCR4 receptor enhances metastasis

EPITHELIAL–MESENCHYMAL TRANSITION
The transformation of cell morphology from the tightly coupled and polarized structure that is typical of a cell in an epithelium to the more irregularly shaped and isolated morphology that is typical of mesenchymal cells such as fibroblasts.

towards lymphatic vessels[88]. The motility of tumour cells towards and within lymphatics can be monitored using transgenic approaches to express fluorescent molecules in lymphatic cells. It will also be important to determine whether lymphatics are a route for cancer cells that leads to formation of metastatic tumours in other parts of the body, or whether cancer cells simply remain in the lymph nodes. Although lymph-node metastasis is a clear indicator for the ability of a tumour to invade, chains of lymph nodes with tumour cells that lead to the thoracic duct are rarely seen. So, can tumour cells intravasate from lymph nodes or just from the primary tumour?

Metastasis of some tumour types might not only be limited by intravasation. For example, large ascites tumours that have been shunted into the vena cava do not always colonize distant sites[89–91]. This could reflect heterogeneity among tumours, with some having different capabilities for different steps in the metastatic cascade, such as adhesion to blood vessel surfaces, extravasation or proliferation in the target organ.

Part of the challenge for the future is to identify the tumours for which 'anti-intravasation' therapy might be suitable. For inoperable primary tumours or for patients with a high likelihood of metastases present at the time of the removal of primary tumours, treatments that would reduce intravasation could reduce further spread of metastases and prolong the patient's life. Such treatments might enhance current cytotoxic strategies. Indeed, several agents that are being developed to target signalling pathways that are activated by growth-factor receptors also affect cell motility and chemotaxis. These treatments could be useful in preventing intravasation and metastasis, independently of their ability to reduce the size of the primary tumour.

Intravital-imaging studies have revealed new information about metastatic cells that indicate ways to improve patient diagnosis, prognosis and therapy. In determining the number of cancer cells in the blood, collection methods should be developed to exclude fragments and non-viable cells. Steps such as these should enhance the prognostic value of such assays. In addition, further evaluation of the factors that regulate cell polarity in metastatic versus non-metastatic cells should be performed. Molecules that promote polarity, such as ZBP1, might be developed as indicators of reduced metastatic ability. Conversely, reduced levels of ZBP1, combined with indicators of increased chemotactic ability (such as EGFR upregulation), might be used to predict increased invasiveness and poor prognosis.

The ability to directly observe the motility and chemotaxis of cells within the primary tumour could eventually lead to a well-characterized phenotype of the invasive population of carcinoma cells. This phenotype could then be referenced when manipulating the expression of candidate invasion genes, and would allow the correlation of cell motility with metastatic outcome at the molecular level. This approach, based on imaging technologies such as multiphoton microscopy and intravital microscopy, will allow direct experimental manipulation of cancer cell movement *in vivo*, and improve our understanding of the early steps of metastasis.

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Competing interests statement

The authors declare that they have no competing financial interests.

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