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From good to bad: Intravital imaging of the hijack of physiological processes by cancer cells

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ABSTRACT

Homeostasis of tissues is tightly regulated at the cellular, tissue and organismal level. Interestingly, tumor cells have found ways to hijack many of these physiological processes at all the different levels. Here we review how intravital microscopy techniques have provided new insights into our understanding of tissue homeostasis and cancer progression. In addition, we highlight the different strategies that tumor cells have adopted to use these physiological processes for their own benefit. We describe how visualization of these dynamic processes in living mice has broadened to our view on cancer initiation and progression.

1. Introduction

Both prevention of cancer and development of new therapies depend on the complete understanding of the mechanisms underlying initiation and progression of tumors. Over the years, it became increasingly apparent that some cells within a tumor can hijack processes that would normally drive development, homeostasis and regeneration of tissues (Fig. 1 and reviewed in e.g. Condeelis et al. (2005), Ellenbroek and van Rheenen, (2014) and Scheele et al. (2016)). For example, from the billions of cells within a primary tumor a small number of cells can adopt a migratory phenotype that is normally used by epithelial cells during wound repair. However, detection of these rare cells, and especially their dynamic behavior, is lost in population measurements using standard biochemical techniques or static histological images. Therefore, to visualize the behavior of individual cells in living mice in real time at (sub)cellular resolution, various high resolution intravital microscopy (IVM) techniques have been developed. Here, we briefly describe IVM techniques, and review the unique insights IVM provided to the understanding of physiological processes and how cancer cells use their hijack for their own benefit.

1.1. High-resolution fluorescent intravital imaging at a glance

Most high-resolution IVM techniques are based on fluorescent light microscopy, where light of specific wavelengths can excite fluorescent objects, such as GFP-labeled tumor cells, resulting in emission of photons and detection of these objects. Unfortunately, light within the visible range has the tendency to get distorted, scattered and absorbed when it travels through tissues. This severely compromises the quality of fluorescent images acquired deep into tissues, since it leads to phototoxicity, signal loss, and imaging distortions. By contrast, multi-photon microscopy is based on the excitation of fluorophores with low-energy infrared light that has fewer phototoxic effects and suffers less from absorption and scattering. Therefore, depending on the tissue of interest and experimental set-up, multi-photon-based IVM enables good imaging contrast up to 1.6 mm deep (in brain tissue (Kobat et al., 2011)) and is therefore often the preferred high-resolution IVM technique. Moreover, infrared light can generate label-free signals such as second harmonic generation (SHG) that allows detection of non-centrosymmetric molecular structures, such as collagen type I fibers, and third harmonic generation (THG) for visualization of the interface between structures with a different refractive index, such as lipid bodies in an aqueous environment. These signals can be detected at half (SHG) and one third (THG) of the excitation wavelength (Campagnola et al., 2002; Weigelin et al., 2014). For a good overview of all the available optical techniques for in vivo cancer imaging, we refer to (Condeelis and Weissleder, 2010).

The use of multi-photon excitation increases imaging depth, but many tissues (except the skin) are still not optically accessible. Surgical exposure of sites of interest has been extensively used for imaging sessions up to 40 h (Ewald et al., 2011a, 2011b, 2011c). In addition, various imaging windows have been developed that provide repeated

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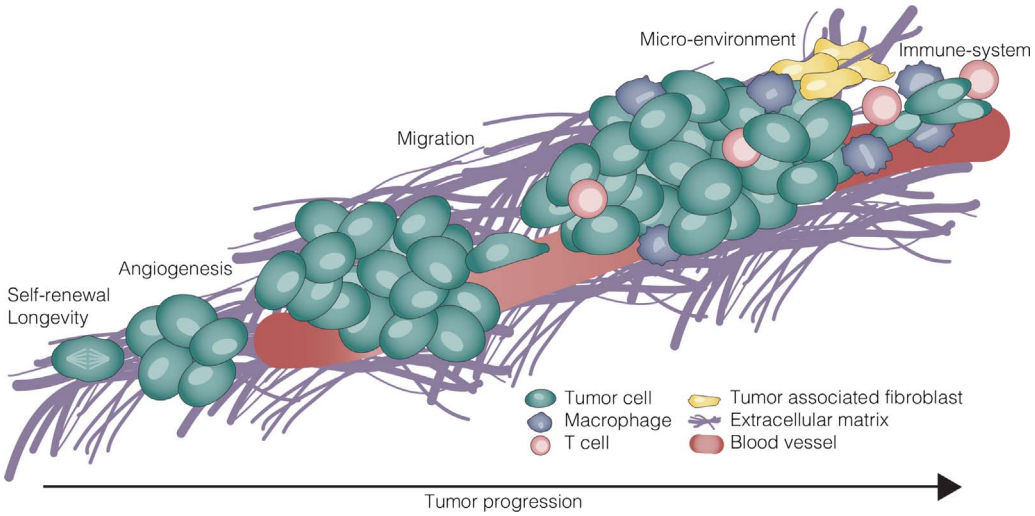

Fig. 1. Schematic overview of different stages of tumor progression. The various processes that tumor cells can hijack and use to promote their growth have been indicated.

optical access (up to weeks or even years) to a variety of tissues, including the brain, breast, and abdominal organs (for a review, see (Alieva et al., 2014)). During the last decade, the above-described high-resolution microscopy techniques have been widely used to study the plastic behavior of cells under physiological conditions. In the next sections, we will first describe these findings followed by a description of how cancer cells have turned this physiological behavior to their own advantage.

2. Tumor initiation

Tumor induction and progression is mediated by accumulation of genetic alterations. However, as a result of the fast turnover of tissues during homeostasis, most cells live too short to accumulate the genetic alterations required to initiate a tumor. By contrast, adult stem cells are long-lived due to their capacity to self-renew and therefore have the opportunity to accumulate genetic oncogenic alterations (Blokzijl et al., 2016) and act as the cells of origin of tumors. In addition to being potential cancer-initiating cells, stem cells have properties to proliferate infinitely, which is speculated to be a trait that cancer cells need to hijack in order to fuel tumor growth (Reviewed in Hanahan and Weinberg (2011), (2000)). Furthermore, it has been shown that, at least for some tumor types, the vast majority of tumor cells are short-lived and quickly replaced by the progeny of a small population of long-lived cancer stem cells (Vries et al., 2010).

2.1. Stem cells

The location and activity of stem cells within different tissues were previously determined with the help of lineage tracing and ex vivo staining (Barker et al., 2007; Clayton et al., 2007; Van Keymeulen et al., 2012). However, in these static images, the behavior and fate of individual stem cells could not be identified. Real-time visualization of stem cells in their natural environment has led to new unexpected insights in many different tissues, and has shown that also differentiated cells can adopt a stem cell state in a plastic fashion. For example, it was previously thought that only single isolated spermatogonia provide stem cell function in the testis. However, with the use of IVM it was shown that all GDNF family receptor alpha-1-expressing (GFRα1+) cells, including those with a syncytial state, could acquire stem cell capacity by becoming single cells. The different cell states (syncytial and single) were found to be reversible, and through conversion to a single cell state the whole pool of GFRα1+), has the potential to become a functional stem cell (Hara et al., 2014; Nakagawa et al., 2010). Furthermore, muscle stem cells, which are quiescent and immobile during homeostasis, can be activated to migrate and divide upon injury. IVM showed that regeneration is mediated by migration that is bidirectional along the axis of remnants of injured muscle fibers (Webster et al., 2016).

The hair follicle continuously cycles between phases of growth and regression and has therefore emerged as a valuable tool to study stem cell behavior using IVM. The growth phase is dependent on a mesenchymal niche and is driven by a combination of spatially organized divisions and coordinated cell movements (Rompolas et al., 2012). Regression of the hair follicle occurs by elimination of epithelial cells through apoptosis of basal cells and differentiation of suprabasal cells. This regression phase is essential to maintain normal homeostasis and counter balance growth (Mesa et al., 2015). Static analysis of the mouse skin has illustrated the importance balancing growth, because activation of an oncogenic pathway (e.g. expression of mutant H-Ras) in hair follicle cells produces epithelial tumors with highly malignant characteristics (Brown et al., 1998).

Moreover, besides location and activity, real-time visualization with IVM also allows the study of stem cell fate. It has been shown that the position of stem cells in their niche is a good predictor of precursor or differentiation fate (Ritsma et al., 2014; Rompolas et al., 2013; Scheele et al., 2017). Repeated intravital imaging of intestinal stem cells showed that, although all ISCs have the potential of long-term maintenance in the crypt, those that are positioned within the center of the niche have a higher chance of remaining in the niche than those positioned in the border region (Ritsma et al., 2014). Static lineage tracing of APC, p53 or K-Ras mutant ISCs has shown that these cells can generate a biased drift towards clonality. Although, this biased drift is not deterministic, it is a potential method by which tumor cells hijack homeostatic stem cell competition (Snippert et al., 2014; Vermeulen et al., 2013).

Recent analysis of the developing mammary gland during puberty has shown that morphogenesis of this organ also relies upon a functional and transcriptional heterogeneous population of mammary stem cells localized at the terminal end buds (TEBs) (Scheele et al., 2017). Only the MaSCs localized at the border of TEBs can temporally contribute to ductal expansion. However, IVM showed that bifurcation of TEBs causes rearrangements of cell positions and therefore of positional biases, resulting in long-term equipotent pools of MaSCs. Due to the plastic nature of the behavior, fate and even identity, stem cells cannot always be labeled by specific markers but should be defined functionally (Scheele et al., 2017). The observed heterogeneity of MaSCs is not limited to healthy cells in this tissue, as the stem cell state of mammary cancer stem cells was also found to be plastic and dynamic of nature (Zomer et al., 2012) (Fig. 2A). This is a mechanism

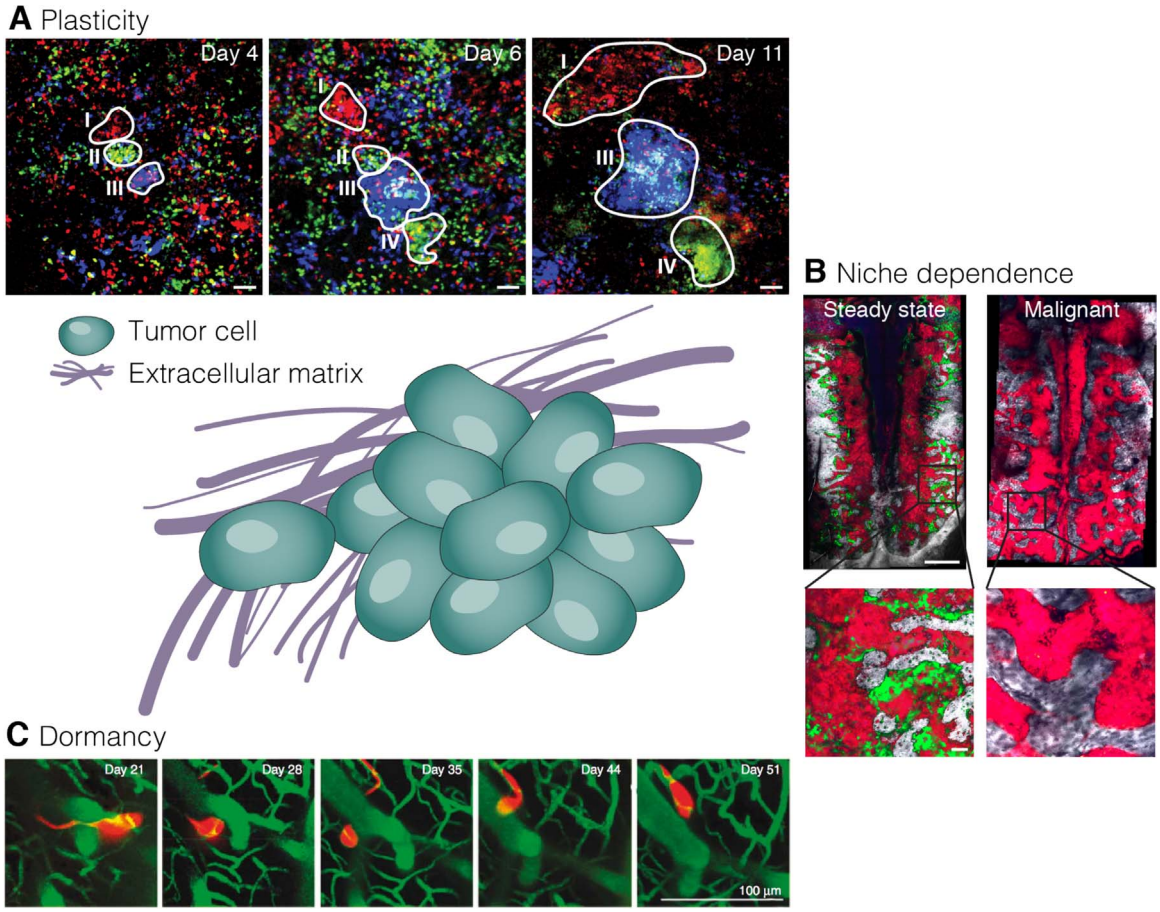

Fig. 2. Tumor initiation through the co-option of normal processes by tumor cells. (A) Plasticity: Intravital images of a growing lineage traced mammary carcinoma. The growth of independent clones (outlined with white lines) was followed over time. The diversity in behavior (continuous growth and alternating growth and regression) displays plasticity of cancer cells. Scale bars represent 50 µm (adapted from Zomer et al. (2012)and reprinted with permission). (B) Niche dependence: T-ALL cells (red) cause remodeling of the bone marrow niche (osteoblasts, green) in the mouse calvarium 22 days after transplantation (right images). The niche is unaffected in steady state conditions (left images). Bone (grey) and vasculature (blue) are shown, Scale bars represent 500 µm (top) and 50 µm (bottom) (adapted from Hawkins et al. (2016)and reprinted with permission). (C) Dormancy: Extravasated melanoma cell (red) is highly motile in close proximity of the vasculature (green) in the mouse brain and remains dormant over a long period of time (adapted from Kienast et al. (2010)and reprinted with permission).

by which these cancer cells could quickly adapt to changing environments, such as those induced by therapy, and thereby promote their own survival.

The environment in which stem cells reside can have a major impact on their behavior and activity. A good example is the bone marrow niche that harbors hematopoiesis driving hematopoietic stem cells (HSCs). Intravital tracking of transplanted HSCs in the mouse calvarium has shown that the state of this niche, which consists of blood vessels, osteoblasts and endosteal surface, determines HSC behavior (Khorshed et al., 2015; Lo Celso et al., 2009). Furthermore, subsets of HSCs localize to different sites within their niche depending on their differentiation state. Healthy HSCs are stable and immotile under homeostatic conditions, but upon stress (such as infection) they become more migratory and interact with a larger niche (Rashidi et al., 2014). This is in contrast to T-cell acute lymphoblastic leukemia (T-ALL) cells, which are instead highly motile and independent on interactions with the stroma. Interestingly, T-ALL cells can induce remodeling of bone marrow stroma by inducing apoptosis of osteoblastic cells and hereby hijack this system (Fig. 2B). Moreover, as a consequence of stroma-independence, loss of the supporting stem cell niche does not have a negative impact on leukemic cells. However, it leads to rapid loss of healthy HSCs and thereby benefits tumor cell expansion (Hawkins et al., 2016).

Together these studies illustrate how imaging in living mice revealed the dynamic behavior of various populations of stem cells. Importantly, only through visualization of stem cells in their natural environment the influence of interactions with other cells and niche components on their behavior during homeostasis and cancer initiation was discovered.

2.2. Dormancy

Not all adult stem cells are continuously cycling; many populations remain dormant under homeostatic conditions. For example, dormant HSCs will only divide about five times per lifetime, but can be reactivated upon external insults to the hematopoietic system, such as serial transplantation, bleeding, infection, and chemotherapeutic agents (Foudi et al., 2009; van der Wath et al., 2009; Wilson et al., 2008). Cancer cells have been described to co-opt dormancy in order to decrease chemo-sensitivity and promote relapse. For example, acute lymphoblastic leukemia (ALL) cells can secrete osteopontin, which functions as an extracellular matrix (ECM) molecule in the endosteal niche that promotes anchoring of leukemic cells and supports dormancy (Boyerinas et al., 2013). The endosteal niche can also control dormancy of myeloma cells. By live tracking of single cells in the mouse tibia it was found that a quiescent niche repressed proliferation of myeloma cells and induces their dormancy. This state is reversible and small numbers of cells can cause relapse of cancer upon re-activation on actively resorbing bone surface (Lawson et al., 2015). Dormancy is not limited to leukemic cells, and IVM of metastasized melanoma and lung carcinoma cells showed that reduced angiogenic growth after inhibition of VEGF-A can induce dormancy of these cells in the brain (Kienast et al., 2010) (Fig. 2C).

Hence, the hijack of dormancy is a dangerous strategy by which tumor cells can avoid response to therapy. Therefore future investigation of the mechanisms involved in dormancy is an important step to prevent cancer initiation and relapse of cancer.

3. Tumor progression

Over time, the accumulation of genetic alterations and a changing microenvironment enables tumors to progress to a metastatic stage. At this time cancer cells detach from the primary tumor and spread to distant sites. IVM of tumor cells showed how tumor cells dynamically hijack physiological processes at various phases of tumor progression.

3.1. Communication

Cells continuous communicate to coordinate growth and signaling during homeostasis. Many of these cell-cell interactions are short-ranged and involve direct contacts. However, some types of communication strategies used by cells can span longer distances. For example, the formation of canals between cells is a way for cells to transport signaling molecules, proteins or even organelles. The first illustration of such structures was found in the developing *Drosophila* wing disc. Small tubes, called cytonemes are formed towards a morphogen gradient and grow as long as the size of the wing disc (Ramírez-Weber and Kornberg, 1999). Recently, it was observed with IVM that glioma cells form ultra-long protrusions that are used as tracks for transport. Cancer cells can form a network through these gap-junction based tumor microtubes that are very similar to axons on neurons. Communication via this network through intracellular calcium waves is essential for tumor progression and chemo-resistance (Osswald et al., 2015).

Another way of long-distance communication is by means of extracellular vesicles (reviewed in McGough and Vincent (2016) and Zomer and van Rheenen (2016)). These are used in many different processes amongst a variety of species, including fly wing disc patterning, left-right determination in the chick embryo and development of the vertebrate limb bud (Callejo et al., 2011; Tanaka et al., 2005; Zeng et al., 2001). Live imaging of thymoma cells has shown release of extracellular vesicles containing membrane-bound and soluble proteins in vivo (Lai et al., 2015). Furthermore, using a Cre-based recombination system, exchange of extracellular vesicles between tumor cells was visualized in living mice. This method allows the fluorescent distinction of cells based on their uptake of locally and systemically transferred extracellular vesicles (Fig. 3A). Analysis of the differential behavior of these two cell populations showed that aggressive behavior can be phenocopied by less malignant tumor cells through extracellular vesicle transfer (Zomer et al., 2015). Thus, tumor cells can use physiological long-distance communication methods to propagate malignant behavior to less aggressive cells and this contributes to their metastatic potential.

3.2. Angiogenesis

Proper vascularization is essential for the supply of oxygen and nutrients to and removal of waste products from tissues during homeostasis. Most blood vessels are formed during development. However, in some cases, for example during trauma, angiogenesis is required to re-establish normal tissue function. Over the years IVM has been a valuable tool to study the dynamics of blood vessel formation and many tools have been established to analyze angiogenesis. Vessels can be imaged in vivo at single cell resolution using the genetic Tie2-reporter model (Motoike et al., 2000) and injectable dyes or quantum dots ((Xiang et al., 2015) and reviewed in Fang et al. (2012)). Furthermore, functional characterization of levels (Lecoq et al., 2011) and pressure of oxygen, pH (Helmlinger et al., 1997) and multiple parameters of blood flow (Kamoun et al., 2010) have given information on the state and behavior of vessels. Together, these tools have been used to study early formation of vessels and their maturation in vivo. Visualization of transplanted Islets of Langerhans using imaging windows showed rapid signs of angiogenesis, which was completed to a micro vascular network within ten days (Gurp et al., 2016; Menger et al., 1989). Strikingly, co-option of this process by human tumor cells in immune-deficient mice holds very similar kinetics, as was shown using dorsal skin fold IVM (Leunig et al., 1992). Furthermore, tumor cells infiltrate better in well-vascularized areas of the tumor compared to areas devoid of vessels (Kedrin et al., 2008). In an orthotopic transplantation model for brain metastasis of human epidermal growth factor receptor-2 (HER2)–amplified breast cancer it was found that decreased angiogenesis, induced by a combination therapy, results in slower growth of brain metastases (Kodack et al., 2012). Thus, these studies all point towards a stimulating role of angiogenesis in tumor growth.

In homeostatic skin, blood vessels are quite impermeable and can retain molecules, such as dextran, as small as 70 kDa for a few hours. However, upon challenges like an allergic reaction, quick leakage of molecules with sizes up to 2000 kDa is observed (Egawa et al., 2013). Interestingly, tumor-associated vessels generally have a distorted irregular morphology, which can coincide with leakiness (reviewed in Jain et al. (2002)) and (Fig. 3B)). This can lead to increased interstitial fluid pressure in tumors, which may result in decreased delivery of therapeutics (reviewed in Fukumura et al. (2010)). However, other studies have shown an opposite effect of vascular permeability on tumor growth and response to therapy. For example, increased vascular leakage caused by deletion of matrix metalloproteinase-9 benefits to the response of mammary carcinoma's to chemotherapy (Nakasone et al., 2012). Similarly, ultrasonography showed increased delivery of the chemotherapeutic drug Gemcitabine after a vascular promotion therapy in models for lung and pancreatic cancer. This resulted in reduced growth of primary tumors and metastasis (Wong et al., 2015). So, manipulation of the vasculature by tumors can cause a plethora of effects on their growth and response to therapy. More IVM studies are required to better predict the efficacy of therapeutics. For example, the use of IVM showed a heterogeneous response to the anti-angiogenic drug Sunitinib. The overall tumor vascular density was reduced upon treatment, but at the tumor margin it had no significant effect (Manning et al., 2013). Moreover, IVM can be used to characterize the state of the tumor vasculature in a particular tumor as described above and this can be combined by the analysis of penetration, efficacy, engagement and turnover of drugs in tissues (Dubach et al., 2017). This has revealed a heterogeneous effect of treatment with the PARP-inhibitor Olaparib by analysis of a DNA damage reporter in living tissues (Yang et al., 2015). Furthermore, IVM of genetic reporters that map activity of specific signaling pathways can be used to assay the efficacy of small molecule inhibitors designed to target the respective pathways. This strategy was successfully adapted to determine the efficacy of treatment with the small molecule inhibitors Dasatinib or NSC23766 on Src and Rac pathway activation (Johnsson et al., 2014; Nobis et al., 2013).

3.3. Migration

Cell migration plays an essential role and is a driving force during development, starting as early as gastrulation and continues throughout most stages of embryogenesis. During adult life cell migration remains to be crucial. For example, when cells of the immune system move throughout the body to detect and protect against insults or when epithelial cells travel to repair a wound. Cells have developed various ways by which they can move depending on cell intrinsic and external factors (for an overview and classification see Friedl et al. (2012)). Mesenchymal migration of single cells is dependent on interactions with the ECM and the capacity of migrating cells to induce degradation of this matrix (reviewed in Boekhorst et al. (2016)). In contrast, amoeboid movement, which is used by for example leukocytes, requires

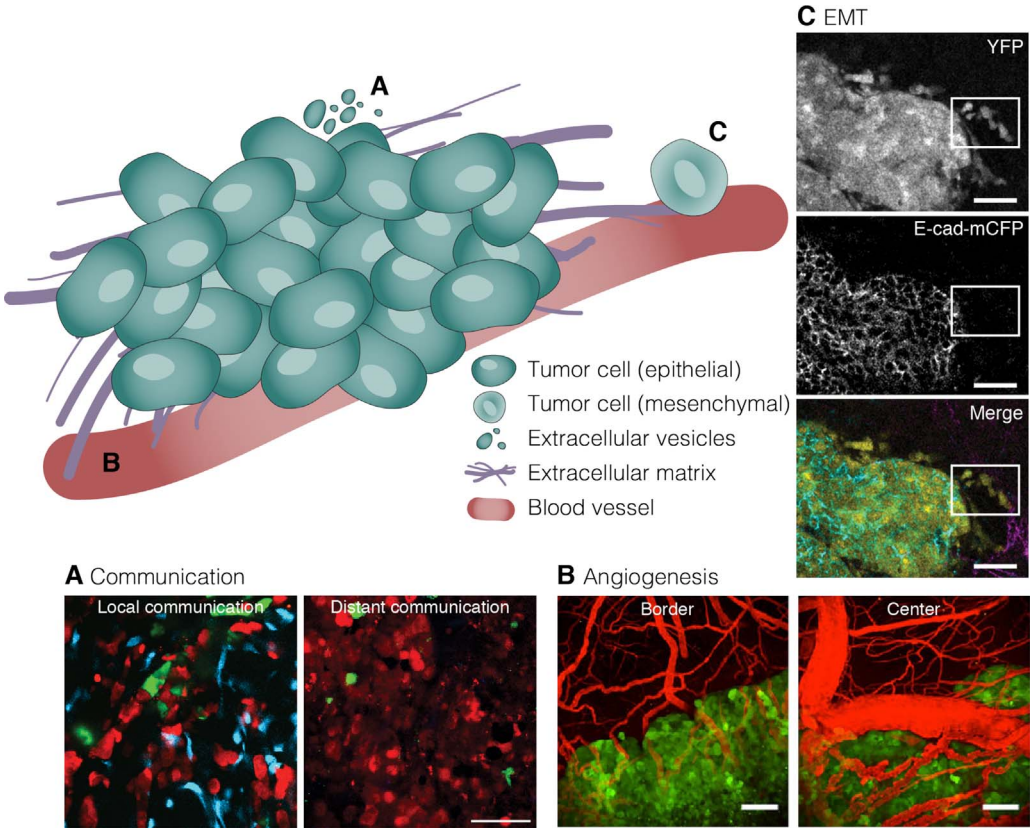

Fig. 3. Physiological processes that can be hijacked by tumor cells in order to drive tumor progression. (A) Communication: Local (left) and systemic (right) functional transfer of extracellular vesicles by mammary cancer cells was detected by GFP expression (green) in reporter cells (red) upon uptake of Cre+ vesicles released by donor cells (blue). Scale bar represent 50 μm (adapted from Zomer et al. (2015)and reprinted with permission). (B) Angiogenesis: Abnormal vasculature (red) in the center of a mammary brain metastasis (green, right) compared to vasculature at the border of the metastasis (left). Scale bars represent 100 μm (adapted from Kodack et al. (2012)and reprinted with permission). (C) Epithelial-to-mesenchymal transition: Migratory mammary cancer cells (YFP) that underwent EMT (square outline) that indicated by the loss of membrane-bound E-cadherin (blue). Scale bars represent 50 μm (adapted from Beerling et al. (2016b)and reprinted with permission).

contraction of cortical actomyosin and formation of bleb-like protrusions and is independent on the matrix degradation (Renkawitz et al., 2009). In certain instances, including migration of neural crest cells derived from the ectoderm during embryogenesis and of neural precursors in the mouse brain, multiple single cells move together as a multicellular stream (Kulesa and Fraser, 1998; Lois et al., 1996). Guided by cues like chemokines, these cells move independently but directed occasionally with weak cell-cell interactions. In addition, a cluster of cells can move as a connected group. This collective movement, which is also used by border cells in the *Drosophila* ovary and primordial cells during development of the zebrafish lateral line organ (Gompel et al., 2001; Montell et al., 1992), is characterized by maintenance of cell-cell junctions throughout the migration process (reviewed in Friedl and Gilmour (2009)).

Tumor cells have been demonstrated to hijack these different modes of migration and use it at the various steps of metastasis. Overexpression of the actin-binding protein Mena (an Ena/VASP protein) is found in many types of cancer. In particular, the alternative splice variant Mena^{INV} associates with highly motile and invasive behavior in vitro (Philippar et al., 2008). In vivo Mena^{INV} increases motility, promotes intravasation and metastasis of mammary cancer cells (Philippar et al., 2008; Roussos et al., 2011). Signaling between Notch1 and Mena^{INV} contributes to this invasive behavior by promoting formation of protrusive structures, called invadopodia, via macrophages in vitro (Pignatelli et al., 2016). Importantly, invadopodia formation is essential for extravasation and intravasation of tumor cells and promotes lung metastasis in mice (Gligorijevic et al., 2014; Leong et al., 2014; Pignatelli et al., 2016). Cancer cells can also hijack the amoeboid migration strategy in order to invade surrounding tissue and escape from the primary tumor (reviewed in Pinner and Sahai (2008)). Interestingly, IVM also showed that migration is not always the limiting step of metastasis formation since cells from both benign and metastatic tumors can acquire migratory properties. However, only the former cell type has a higher chance of intravasation and thereby of forming metastases (Wyckoff et al., 2000). Besides migration of single cells, coordinated streaming of mammary carcinoma cells correlates with intravasation and numbers of circulating tumor cells in the blood (Beerling et al., 2016a; Patsialou et al., 2014). TGFβ signaling induces the switch from collective to single cell migration, thereby promoting intravasation and formation of metastases by cells that have disseminated through the blood circulation (Giampieri et al., 2009).

One of the first steps cells have to take to gain migratory behavior is detachment from their neighboring cells. Through a developmental program called epithelial-mesenchymal transition (EMT), polarized epithelial cells undergo morphological, biochemical and functional changes towards a mesenchymal phenotype, which is characterized by loss of adherence proteins from the cell surface. EMT results in increased migration and invasiveness and decreased sensitivity to induction of apoptosis (reviewed in (Kalluri and Weinberg (2009)). During development certain cells are plastic and reverse between different epithelial and mesenchymal state. For example, trophectoderm cells in the blastocyst use EMT to migrate and anchor in the placenta. Furthermore, neural crest cells, derived from the developing neuroectoderm, migrate to diverse regions in the embryo after undergoing EMT (Newgreen and Gibbins, 1982). In addition, EMT is associated with organ fibrosis upon inflammation in adults (Kim et al., 2006; Zeisberg et al., 2007a, 2007b). Static end-point analysis has shown that tumor cells can hijack the EMT process to acquire a

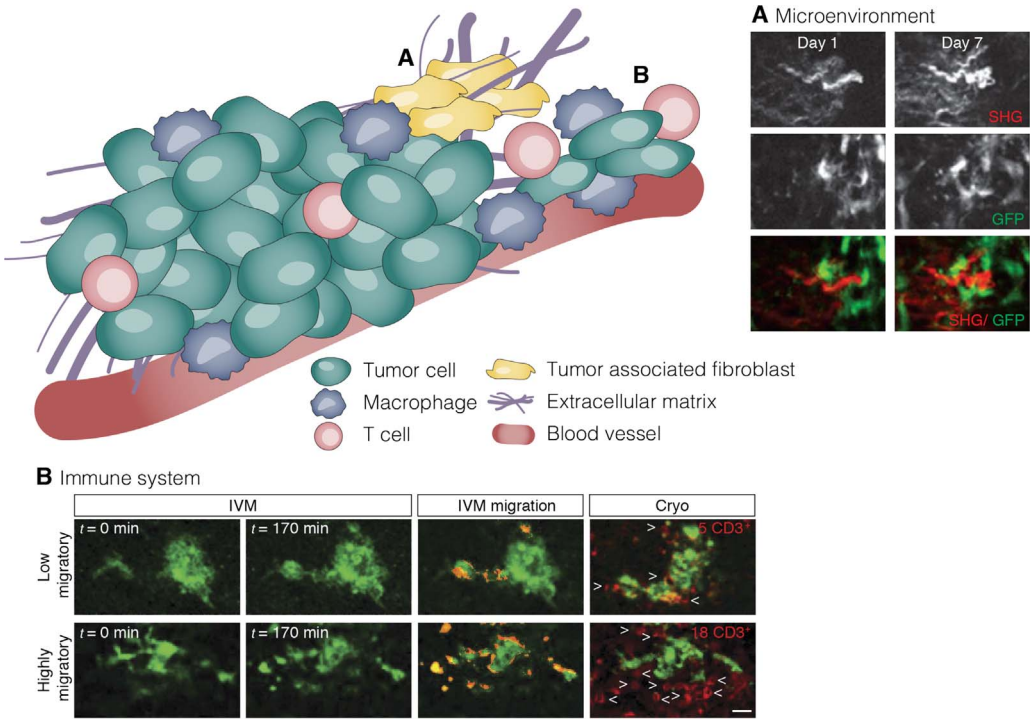

Fig. 4. Manipulation of homeostatic processes by cancer cells that promote tumor growth. A) Microenvironment: Remodeling of the extracellular matrix (red) by tumor-associated fibroblasts (green) in a tumor treated with the hormone relaxin (adapted from Perentes et al. (2009)and reprinted with permission). (B) Immune system: The presence of T cells (red) increases the migratory behavior of mammary tumor cells (green). T cells were detected by correlative immuno-staining of tumor regions that contained a low (upper panels) or high (lower panels) number of migratory tumor cells (indicated by orange pixels). Scale bar represent 15 μm (adapted from Ritsma et al. (2013)and reprinted with permission).

more invasive mesenchymal state in vivo (reviewed in Kalluri and Weinberg (2009)). However, recent studies indicate that suppression of EMT does not affect metastasis formation and tumor cells within metastases mainly have an epithelial state (Beerling et al., 2016b; Fischer et al., 2015; Zheng et al., 2015). These observations can be explained by plasticity of tumor cells. With the use of IVM it was observed that, without experimental induction, a small pool of breast tumor cells (~1%) can undergo EMT and become motile (Fig. 3C). Although this is not the only mechanism used by tumor cells to form metastases, the newly gained migratory capacity allows cells to disseminate at a distant site. Importantly, this study also showed that the (disseminated) mesenchymal tumor cells have the ability to revert back to an epithelial state. This plasticity appeared to render the reported differences in EMT-induced stemness irrelevant for metastasis, because mesenchymal cells, when reverted to an epithelial state, will obtain the same stem cell potential as epithelial counterparts (Beerling et al., 2016b). Interestingly, the reversal of tumor cells to an epithelial state in the metastatic niche is triggered by fibroblasts (del Pozo Martin et al., 2015). In addition, IVM showed that Wnt-dependent partial EMT can be used by mammary cancer cells for dissemination from early lesions. These partially reverted cells can subsequently spread to target organs and form metastasis after a period of dormancy (Harper et al., 2016). In addition to supporting metastasis, EMT also plays a role in resistance to chemotherapeutics. EMT thereby contributes to recurrence after therapy and causes decreased overall survival of tumor-bearing mice (Fischer et al., 2015; Zheng et al., 2015). However, these studies are based on static end-point analysis and could not address the effects of plasticity in EMT. Therefore future investigation is essential to determine in which state, mesenchymal or reversed epithelial, cells are when they are most resistant to chemotherapeutics.

4. Tumor promotion

Although many tumor cells may carry genetic alternations that give them the intrinsic capacity to metastasize, IVM showed that only a small population of these tumor cells would be able to migrate, disseminate and grow at the metastatic site. Extrinsic stimuli from the local microenvironment can promote and dictate cellular behavior and fate including migration, stemness and chemoresistance. In the next section, IVM studies will be discussed in which these factors and their contribution to tumor growth have been investigated.

4.1. Microenvironment

The extracellular matrix (ECM) is an essential regulator of growth and differentiation through direct interactions with cells. The ECM can, for example, guide morphogenesis of the intestine and organ branching through remodeling of matrix structure and composition (reviewed in Bonnans et al. (2014)). IVM analysis of different collagen scaffolds using SHG showed that, in tissues, these networks are very heterogeneous in pore size and density (Wolf et al., 2009). Other IVM studies have shown that the heterogeneity in the matrix environment dictates the migration strategy of cancer cells. By a combination of computer modeling and IVM, it was shown that blebbing-driven amoeboid migration is the most rapid form of migration in matrix environments with heterogeneous structure (Tozluoğlu et al., 2013). At high matrix densities cells can adopt a mesenchymal migration strategy. This type of migration is slower and dependent on the formation of protrusions and invadopodia that disintegrate ECM (Gligorijevic et al., 2014). In addition to tumor cells, tumor-associated fibroblasts (TAFs) can also degrade ECM and thereby influence tumor cell behavior. Treatment with the matrix-modifying hormone relaxin causes an increased interaction of TAFs with collagen through increased β1-integrin activity. With IVM it was shown that this promotes collagen fiber remodeling by matrix metalloproteinases that are produced by TAFs (Perentes et al., 2009) (Fig. 4A). Together these studies illustrate how tumor cells can hijack matrix-remodeling pathways and use this to promote their invasive capacity.

4.2. Wound healing and inflammation

An inflammatory wound healing response is essential for recovery of trauma and protection against infections. IVM has been widely used to study this response and with the aid of transgenic mouse models, such as fluorescent labeling of Csf-R1-expressing cells of the mononuclear phagocyte system with a Gal4-system or the MacGreen reporter (Sasmono williams, 2012; Ovchinnikov et al., 2007) and Ly6G-expressing neutrophils in the Catchup model (Hasenberg et al., 2015) many different cell populations has been followed live. For example, IVM showed that monocytes act as steady-state surveillance in lung, complementary to resident macrophages and dendritic cells without differentiating into macrophages. They patrol within large vessels of lung or at the interface between lung capillaries and alveoli (Rodero et al., 2015). In addition, an original and valuable labeling approach was taken by photo-switching areas of skin and draining lymph nodes in order to label and track migration and turnover of endogenous dendritic cells derived from skin and lymph (Tomura et al., 2014). With a combination of transgenic models, IVM has shown that upon scalp injury monocytes infiltrate the wound bed through small hemorrhages within hours, which is as early as classical neutrophils infiltrate (Rodero et al., 2014). Furthermore, neutrophil recruitment was visualized at the site of sterile inflammation in the liver of living mice. This recruitment is caused by ATP release from necrotic cells and allows adherence of neutrophils to liver sinusoids. In addition, release of chemokines and formyl-peptides directs neutrophils towards the site of damage and aid migration through non-perfused areas (McDonald et al., 2010). Recruitment of neutrophils is enhanced by lipid leukotriene B4, which acutely amplifies local cell death signals to enhance the radius of highly directed interstitial neutrophil recruitment (Lämmermann et al., 2014). Interestingly, the inflammatory response is aided by rapid recruitment of monocytes coming from the body cavity, which is also triggered by released ATP and results in dismantling of the damaged nuclei to cover the site of injury (Wang and Kubes, 2016). Together IVM showed that these signals inflict a response that protects the organ from further damage.

Cancer has often been referred to as a wound that never heals (Dvorak, 1986) and the responses that are invoked by tumors can have either an tumor-supporting or an anti-tumor effect. For example, for the latter effect, IVM showed that adoptively transferred GFP-labeled natural killer cells can form dynamic cytotoxic contacts with subcutaneous tumor cells (Deguine et al., 2010). Moreover, IVM of introduced activated CTLs showed that these cells are highly migratory at the tumor periphery, but upon getting in close contact to tumor cells expressing their cognate antigen they arrest. Only upon tumor cell death these CTLs resume migration and enter in deeper tissue if the antigen is expressed (Boissonnas et al., 2007). The contacts between CTLs and tumor cells are stable and depend on entry of extracellular Ca^{2+}(Deguine et al., 2010). A systemic response can be evoked with the aid of follicular dendritic cells and macrophages. In vivo imaging of these cells visualized capture of subcellular particles of tumor derived antigen in draining lymph nodes, which is dynamically scanned by circulating B cells and thereby driving a humoral response to melanomas (Moalli et al., 2015). Prolonged inhibition of such antigen presenting cells, by neutralizing CSF-R1 antibodies, delays primary mammary tumor growth (Lohela et al., 2014). In addition, a synergistic response between adoptively transferred and endogenous CCD8+ T cells can be induced against melanoma cells by co-stimulation via CD137 (Weigelin et al., 2015). These mechanisms all contribute to an anti-tumor response that provides the organism with a method to fight cancer.

Inappropriate immune responses can be prevented through the induction of dominant tolerance, which ensures that T cells become unresponsive to certain antigens. One of the mechanisms to evoke this tolerance was analyzed by IVM of cytotoxic T cells in lymph nodes. Cytotoxicity caused by these cells can be reversibly suppressed by the presence of activated regulatory T cells through TGFβ (Mempel et al., 2006). Furthermore, adoptively transferred CTLs that were activated in vitro engage a direct interaction with each target cell, which results in a slow response and intrinsically limits the anti-tumor T cell response (Breart et al., 2008). The arousal of endogenous immune-suppressive mechanisms could potentially be a way by which tumor cells could avoid detection of the immune system and prevent a cytotoxic response. Indeed, IVM of transplanted melanomas showed that regulatory T cells form an immunosuppressive ring around tumors and depletion of these cells by cyclophosphamide enhances the efficacy of immunotherapy of adoptive cell therapy (Qi et al., 2016). Also, a subset of myeloid cells with similarities to dendritic cells and macrophages continuously ingest tumor-derived proteins and present those antigens to activate T cells. These infiltrating tumor-specific T cells engage long-lived interactions at mammary tumor borders that are non-productive and do not cause cytotoxicity (Engelhardt et al., 2012). Furthermore, tumor-derived dendritic cells can limit the anti-tumor response of T cells. Live visualization of these tumor-derived dendritic cells showed that they form a dynamic meshwork that can trap T cells through formation of long-lasting antigen-specific interactions (Boissonnas et al., 2013). Thus cancer cells can develop strategies to avoid detection of the immune response.

In addition to anti-tumor effects, tumor cells have been found to hijack parts of the immune response and use this to drive tumor progression. For example, in vivo visualization of tumor cells and either T cells or macrophages showed that these immune cells promote migration of mammary carcinoma cells (Ritsma et al., 2013; Wyckoff et al., 2004) (Fig. 4B). The macrophage-tumor cell interaction is driven by a synergistic CSF1-EGF paracrine loop. In this loop, tumor cells attract macrophages by releasing CSF1 and macrophages can in their turn promote migration of tumor cell through release of EGF (Wyckoff et al., 2004). Furthermore, IVM showed that macrophages support the intravasation of tumor cells into the bloodstream (Wyckoff et al., 2007). This is strengthened by an induction of local loss of vascular junctions and increased permeability through VEGFA signaling of macrophages to the tumor microenvironment of metastasis (Harney et al., 2015). Furthermore IVM has shown that the innate immune system can be hijacked by mammary tumor cells through induced expression of the transcription factor ELF5. Activity of this transcription factor promotes recruitment of myeloid-suppressor cells that can promote angiogenesis and metastasis (Gallego-Ortega et al., 2015). Together, these studies highlight the many ways by which cancer cells have hijacked wound healing related processes and turned the immune response to their assistance.

5. Concluding remarks

Over the years, real time visualization of cancer cells and their microenvironment has led to many new discoveries. Here we have given a glance of these exciting techniques and how they are utilized to better understand cancer. As discussed in our review, IVM has the unique ability to visualize the dynamic behavior of (small) populations of cells and has identified many physiological processes that are hijacked by cancer cells and turned to their own assistance. With the vast development of optics and new fluorescent mouse models, we expect that intravital microscopy will become increasingly important to study the synergy between developmental processes and cancer biology.

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