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The Different Routes to Metastasis via Hypoxia-Regulated Programs

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Abstract

Hypoxia is linked to metastasis; however, how it affects metastatic progression is not clear due to limited consensus in the literature. We posit that this lack of consensus is due to hypoxia being studied using different approaches, such as *in vitro*, primary tumor, or metastasis assays in an isolated manner. Here, we review the pros and cons of *in vitro* hypoxia assays, highlight *in vivo* studies that inform on physiological hypoxia, and review the evidence that primary tumor hypoxia might influence the fate of disseminated tumor cells (DTCs) in secondary organs. Our analysis suggests that consensus can be reached by using *in vivo* methods of study, which also allow better modeling of how hypoxia affects DTC fate and metastasis.

Hypoxia in Cell Fate and Cancer

Evolution and organism development have revealed how natural hypoxic environments influence cell survival and reprogramming. During evolution, organisms capable of ef ciently handling oxidative stress and using oxygen for energy production exhibited survival and evolutionary advantages [1]. Normal mammalian development occurs in a moderate-to-severe hypoxic environment that is responsible for aspects of developmental morphogenesis. Oxygen concentrations in the uterine environment range from 1 to 5%, and the placenta and embryonic cardiovascular system are formed under low cellular oxygen conditions [2]. During development, the expression and activity of the hypoxia inducible factor (HIF) transcription factor, is tightly controlled in space and time by oxygen availability in different developmental processes including placental development, trophoblast differentiation, and heart development, among others [3]. In low oxygen tensions the HIF protein is stabilized

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and not targeted for degradation by the Von Hippel Landau (VHL) tumor suppressor. Stabilized HIF then translocates to the nucleus where it participates in the expression of genes that drive adaptation to low oxygen tension [3].

The above literature shows how oxygen is an essential morphogen that regulates cell fate. This is clearly recapitulated, albeit in an aberrant manner, in cancer where hypoxia is a strong regulator of tumor cell fate. In cancer, hypoxia is associated with tumor progression, resistance to therapy, and metastasis. Sustained hypoxia in a growing tumor was described as being associated with a clinically aggressive phenotype, increased invasive capacity, perifocal tumor cell spreading, regional and distant dissemination, and resistance to different therapies [4,5]. However, hypoxia can also induce growth arrest, cause cell death, decrease motility speed while increasing invasiveness and directionality [6], and induce a dormancy-like program [7].

In this review we highlight the role of hypoxia during the steps of metastasis occurring in the primary tumor (invasion and intravasation) and in secondary organs (extravasation, dormancy, and reactivation) (for details on the metastatic cascade see [8]). We address several unresolved differences in the literature, which we attribute to limitations of the standard assays utilized to evaluate hypoxia in cancer and metastasis. As these standard *in vitro* assays have been thoroughly reviewed elsewhere in the literature [5,9–12], we instead focus on new technological developments, which enable studies of hypoxia in more physiologically relevant contexts, and highlight some of the surprising new revelations that these assays have provided. We further dissect apparently opposing functions of hypoxia and discuss how the context-dependent effects of hypoxia shape solitary DTC biology and metastasis development.

Hypoxia, Motility, and Directionality in the Primary Tumor

Hypoxia is one microenvironmental parameter historically implicated in both metastasis initiation [13] and therapy resistance [14]. Early on, two studies performed in mouse models showed that tail vein injection of tumor cells previously exposed to hypoxic conditions (<0.1% O_2), followed by reoxygenation, led to a dramatic increase in resulting metastases [13,15]. These experiments, along with the advent of a small polarographic needle sensor for measurement of tissue oxygen levels, enabled a series of clinical studies which showed that tumor oxygenation levels are prognostic for patient outcome in various cancers. For an excellent review of these studies, and the needle sensor that enabled them, see [16].

With the prognostic ability of tumor oxygen levels thus established, understanding how hypoxia influences the metastatic cascade is crucially important. Some researchers have taken a reductionist approach to analyzing hypoxia and relied on *in vitro* assays designed to break the metastatic process into its postulated steps and to test how hypoxia in uences these in isolation. However, as discussed below, traditional *in vitro* assays do not recapitulate *in vivo* biology from several aspects and are likely poor surrogates for migration and invasion during the metastatic cascade *in vivo*.

Traditional *In Vitro* Assays May Not Re ect *In Vivo* Biology

First, *in vitro* studies typically compare cells exposed to hypoxic conditions (typically <1%, but occasionally <0.1% O_2) with cells exposed to the rather nonphysiological 'normoxic' conditions (atmospheric oxygen levels of 21%). This is problematic since physiological (median) oxygen levels of normal tissues range from 3 to 7.4%. The de nition of normoxia as 21% does not match the lower levels of oxygen universally found in normal tissues, a condition that has been termed physoxia [17]. In addition, the choice of oxygen level considered to represent hypoxia may vary as many cell types respond differently to different levels of hypoxia, limiting comparative studies [18]. Second, *in vitro* systems cannot accurately re ect the fact that tumor cells do not exist in isolation from vascular and lymphatic systems, growth factors, cytokines, stromal, and immune cells, all of which have a signi cant impact upon tumor cell phenotype [19]. Third, the majority of published studies have used invasion through Matrigel, an arti cial matrix composed of laminin, nidogen, and collagen, which has been measured to dramatically alter tumor cell migration mode (collective vs. single cell), velocity, and distance of migration depending upon its mechanical properties [20]. Even in a study that avoided Matrigel – which contains several extracellular matrix (ECM) molecules including laminin as a major component – and instead utilized only collagen I [21], the situation was not improved as differences in ECM stiffness can dramatically impact migration and invasion. For example, focal adhesion formation and cell motility were enhanced on cultures utilizing a stiff versus a soft substratum, and this effect of substrate stiffness was dependent upon HIF activity, confounding any ability to determine the role HIF activity plays in motility phenotypes [22]. Finally, the extensive use of 2D *in vitro* assays limits the interpretation of the results as qualitative and quantitative differences in cell movement between 2D and 3D environments (such as 3D *in vitro* cultures and real tissues) exist [23,24] (see Box 1). Thus, while these *in vitro* studies have provided information on hypoxia-driven migration and invasion, they should be reconsidered as more sophisticated *in vivo*-like tests are developed.

Accordingly, recent studies have begun to shift away from these standard techniques and develop assays that attempt to recapitulate the *in vivo* complexity. These efforts are summarized in Figure 1 and include: more accurate 3D culture assays, microfluidic and controlled gas exchange platforms, and increased tumor–host mimicry by incorporation of additional host cell types. 3D culture assays (Figure 1A) address the geometrical constraints placed on cells that are plated on a glass or plastic surface by culturing cells either as a single-cell suspension, or as small clusters (known as spheroids [25]) within a deep but loose (low concentration) ECM. The ability of the cells to grow into large spheroids and to spread and invade into the surrounding matrix under different culture conditions are taken as indicators of the aggressiveness and invasiveness of the tumor cells. The increased physical dimensionality thus allows for a more nuanced analysis of cell migration. For example, one study looked at the impact of hypoxia on different modes of migration and found that hypoxia induces a switch from collective to single cell migration [26] a switch that was revealed thanks to the 3D nature of the assay.

Closely related to 3D culture assays are microfluidic and controlled gas exchange platforms (Figure 1B) that both remove the dimensional constraint of 2D assays and also provide

precise regulation and control of gas concentrations, either uniformly across the sample, or in gradients. These assays can also be combined with imaging systems to directly compare the motility of the cells under different oxygen conditions. These systems have revealed that tumor cells can respond to, and travel along, gradients of oxygen concentration leading to a net migration of cells towards higher oxygen levels [27].

Finally, since tumors comprise multiple cell types, coculturing them together is another way to increase sophistication and account for more complex cell–cell interactions (Figure 1C). Using Boyden chambers, one group determined that, in direct contrast to tumor cells, hypoxia dramatically inhibits monocyte and macrophage migration toward chemoattractants [28–30]. This led the group to speculate that macrophages and monocytes, which are observed to accumulate in hypoxic regions of tumors, do so by shutting down chemotaxis. This inhibition of migratory capacity *in vitro* was also seen in monocyte-derived dendritic cells [31]. Another group looked at the role of tumor-associated macrophages (TAMs) in gastric cancer using a 3D dynamic migration imaging system to directly visualize the motility of tumor cells under normal and hypoxic conditions, both alone and in coculture with macrophages. They found that tumor cells in 3D culture (i) exhibit reduced migration speeds when under hypoxia; (ii) show increased migration rates when cocultured with macrophages; and most surprisingly; (iii) show a variable response when cocultured with macrophages under hypoxia where the migration speed of some cell lines increase under hypoxic conditions, while others decrease [32]. These apparently conflicting data emphasize that *in vitro* models do not converge on a set of coherent conclusions, suggesting that the phenotypes observed *in vitro* may be more a property of the model than the functions these cells actually exhibit *in vivo* and ultimate validation of any conclusion obtained from these *in vitro* assays must come from the ability to observe cell phenotypes, *in vivo*.

Thus, while representing a step forward, these assays still show some limitations and have not significantly shifted understanding of tumor cell responses to hypoxia when compared to *in vivo* analyses. Since our goal is to highlight *in vivo* biology, we focus next on the assays that we propose will more accurately improve our understanding of hypoxia biology in metastasis.

Divergent Cell Motility Phenotypes in *In Vitro* versus *In Vivo* Hypoxia Assays

There is no doubt that advances in intravital imaging have revolutionized the analysis of biological systems [33,34]. Intravital imaging using multiphoton microscopy is a powerful technique capable of directly visualizing the phenotypes of individual cancer, immune, and other stromal cells, and their interactions in both intact solid tumors and in target organs. This technique demonstrated that tumor cells migrating in invasive carcinoma display two distinct motility patterns, slow and fast migratory phenotypes, which differ in migration speeds by a factor of ~10 [35,36]. The slow migratory phenotype *in vivo*, is characterized by cells that migrate with mean speeds of 8 mm/h, display increased numbers of invadopodia, increased matrix degradative ability, and increased intravasation over time [35]. Meanwhile, fast migratory cells (69 mm/h) comigrate in streams with macrophages along collagen fibers [37] (Figure 2); both of these phenotypes are required for metastatic dissemination [35,38].

Importantly, comparison of the speeds reported in the few *in vitro* assays that measured this parameter [22,26,27,32,39,40] reveals that *in vitro* assays only recapitulate the slow migratory phenotype (Table 1). While treatment of the cells in culture to hypoxic conditions in some studies does result in an increase in migration rates, these elevated speeds still fall well within the slow migratory phenotype [35]. Thus, because these *in vitro* assays lack the appropriate microenvironment found *in vivo*, they lock tumor cells into the slow locomotion, degradative phenotype and completely suppress the fast migration phenotype.

The fast migratory phenotype *in vitro* can be recapitulated by reconstituting the chemotactic gradients and ECM support found in living tumors. One study built upon the known CSF1–EGF paracrine loop discovered to exist *in vivo* between fast comigrating macrophages and tumor cells [38] and added to the culture 2-mm diameter micropatterned fibers recreating the physiologically relevant ECM utilized by these streaming cells *in vitro* and forcing cell–cell interactions to occur only along this 1D surface [41] (Figure 2). Furthermore, another group [40] expanded upon this 1D coculture model by adding endothelial cells, thereby modeling the fast migration phenotype associated with blood vessel trophic streaming on collagen fibers *in vivo* ending on microanatomical sites within tumor high masses (composed of a Mena tumor cell, an endothelial cell, and a macrophage) [42]. These sites, called tumor microenvironments of metastasis (TMEM), act as the doorways for hematogenous dissemination [42]. Thus, this study determined that endothelial cells supply an additional HGF/c-Met axis (HGF is a powerful promigratory morphogen also known as scatter factor) to the TMEM that breaks the migration symmetry and allows sustained and persistent fast streaming migration toward blood vessels and intravasation [40,42,43].

Finally, the 3D assays in Figure 1 and Table 1 assume that invasion into the stromal matrix at tumor edges is key when assessing tumor cell metastatic capability. While it has long been speculated that the invasive front of tumors is the location of metastatic dissemination [44], this connection has been based upon associations and correlations between features observed either in *in vitro* models [45] or in fixed tissue histology and eventual metastatic outcome [46–49]. The intravasation of tumor cells at the invasive front of tumors has not been observed in live tissues nor functionally linked to metastasis. In contrast, studies quantifying intravasation sites across entire primary mouse mammary tumors (possessing histology like that seen in breast cancer patients) and human tumors themselves, show the location of intravasation sites to be throughout the entire tumor mass and not localized to the tumor edge [42,50,51].

Furthermore, numerous intravital imaging studies have directly observed, with single-cell resolution and in real time, the intravasation process within the core of the tumor [42,52–56]. The mechanisms underlying these intravasation events have been well elucidated [42,54,57–59] and can be directly inhibited *in vitro* and *in vivo* by blocking their underlying pathways [42,58–60].

Recent evidence using large-volume, high-resolution imaging in tumor models in chick and mouse tissues also supports the conclusion that intravasation occurs through the tumor mass [61], and a study of early-stage cancer showed that cancer cells can also disseminate very efficiently from sites associated with atypical ducts in the core of the mammary gland [56].

A recent extension of this work revealed that $CD206^{high}/Tie2^{high}$ macrophages in TMEM assist early dissemination of tumor cells through vascular endothelial growth factor A signaling that causes a local loss of vascular junctions and induces transient vascular permeability and tumor cell intravasation [42], further supporting the importance of additional immune cell types in understanding and modeling dissemination [62]. Finally, the above studies correlate with clinical trials showing that TMEM sites for intravasation are found in the primary tumor interior and not the tumor stromal interface of breast cancer patients, and that TMEM numbers in the core are prognostic for distant metastatic recurrence [50,51,63,64].

In addition to advances in *in vitro* assays, a number of recently reviewed measurement techniques [65,66] have been developed to identify and measure hypoxic cells *in vivo*. We have summarized these techniques in Table 2 for easy comparison. The first of these techniques are those that label hypoxic areas of tissues *in vivo* and then analyze them *ex vivo* (i.e., in explanted tissues). This includes directly marking those tissue areas exposed to blood ow by utilizing an intravascular injection of a membrane permeable dye [67]. However, with this assay only areas perfused at the time of injection are marked. Tumor neovasculature, which is often only intermittently perfused, is therefore missed [68]. Techniques that can be applied directly to patients in the clinic include conjugated magnetic resonance imaging (MRI) [69] and positron emission tomography (PET) [70] probes and polarographic electrodes [71] or optical fibers coated with oxygen sensitive dyes [72,73]. However, these methods lack spatial resolution, do not provide information on cell type or viability, and cannot reveal oxygenation levels down to the single cell level.

Other techniques are designed around staining of excised tissues either for proteins within the hypoxia response pathway (e.g., HIF [74,75], glucose transporter (GLUT)1 [76], carbonic anhydrase (CA)IX [77]), or with molecules from the nitroimidazole class [78] (e.g., pimonidazole) that are reductively metabolized to form covalent adducts in hypoxic cells. These techniques provide the requisite spatial resolution to analyze hypoxia; however, as they are methods used on fixed tissue sections they cannot inform on cellular motility and invasion. In addition, these stains often do not correlate with each other owing to their dramatically different onset and decay lifetimes after hypoxic exposure. Comparison of HIF-1a, CAIX, and pimonidazole staining [79] showed that the onset of HIF-1a and pimonidazole only takes 1–2 h, while CAIX takes upwards of 16 h. Meanwhile, pimonidazole and CAIX last for days after exposure while HIF-1a rapidly decays (1–2 min).

Techniques that enable *in vivo* measurements of single-cell motility and invasion include the engineering of cells to express fluorescent or bioluminescent proteins that are expressed in response to hypoxic conditions. These hypoxia biosensors typically use multiple copies of the hypoxia response element (HRE) gene promoter to drive expression of the biosensor protein. Inclusion of the oxygen dependent degradation (ODD) domain of the HIF-1a protein further reduces background signal and increases hypoxia-speci city of the biosensor. Several reporter proteins were developed including GFP [80–82], YFP [83], and firefly luciferase [84,85]; albeit with some limitations. For example, GFP did not demonstrate expression at levels suf cient for unequivocal visualization in tumor sections or live tissues [86]. Additionally, the choice of GFP, YFP, and firefly luciferase complicated analyses as the

formation of these chromophores depends on the presence of molecular oxygen [87,88]. Thus, the uorescence intensity is not necessarily proportional to the amount of synthesized reporter protein [89]. Recognizing these chromophore limitations, one group has developed a fluorescence resonance energy transfer (FRET)-based biosensor called FluBO using a combination of YFP and a avin-binding fluorescent protein [89]. Additionally, another study used mCherry, which is not dependent on oxygen for folding and fluorescence [6]. Although these reporters allow the tracking of the motility and invasive phenotypes of hypoxic cells with single cell resolution, only two of the studies utilizing these *in vivo* probes [6,85] analyzed migration and invasion of tumor cells.

Another study utilized a novel luciferase-based reporter [85] that could be induced to permanently mark tumor cells and their progeny at a speci c time; cells were traced by sacri cing mice at different time points post induction and compared to other staining markers such as HIF-1a and pimonidazole. While innovative, the interpretation of much of the data was dependent upon the assumption that HIF-1a and pimonidazole areas remain static in the tumor. However, in the rapidly growing tumor, this assumption does not hold.

The discrepancies raised above were resolved using intravital imaging [6] as this study was the first to directly visualize the motility of hypoxic tumor cells, with single-cell resolution, in living tissue. The authors found that hypoxic cells show dramatically reduced migration speeds (~24 mm/h) compared to normoxic cells (~66 mm/h). This places hypoxic cells into the slow migratory phenotype associated with invasion and dissemination as observed previously [35]. Concordant with this, hypoxic cells were also observed to have dramatically increased invadopodia with degradative activity and be more chemotactic toward flowing blood vessels with which hypoxic cells were found to associate *in vivo* [6] (Figure 1).

Using the *in vivo* invasion assay [6], 83% of collected tumor cells were observed to be hypoxic. In contrast, only ~33% of the tumor cells in the primary tumor were observed to be hypoxic. Thus, although hypoxic tumor cells show dramatically reduced migratory speeds, their increased degradative ability and chemotaxis toward blood vessel associated growth factors resulted in greater intravasation and dissemination.

The above studies highlight the importance of *in vivo* imaging of tumor cell phenotypes associated with invasion and intravasation. Importantly, the phenotype of hypoxic cells can be long lived, and the slow but efficient movement towards vessels that they exhibit is more persistent than anticipated from *in vitro* studies. These findings then raise questions as to how this long-lived migratory phenotype of hypoxic tumor cells is carried over to distant sites by disseminating cells. The next section will explore how the hypoxic or normoxic phenotypes affect DTC behavior and its ability to initiate a metastatic colony.

Routes by Which Hypoxia Affects DTC Fate

A large body of literature that has been expertly reviewed recently links *in vitro* hypoxia to the epithelial-to-mesenchymal transition or EMT, which allows epithelial cells to reduce their interaction with other epithelial cells and become motile [5,90,91]. Given that these mechanisms and papers were reviewed in the past we focus here primarily on *in vivo*

studies. Also, because we attempt to understand how naturally occurring hypoxia in target organs affects DTC behavior, we do not focus on how therapies, either chemotherapy and/or antiangiogenic therapies affect hypoxia in tumors [5,92–96]. As mentioned earlier, most of the measurements and conclusions of the biology of hypoxia have been derived from primary tumor biology and *in vitro* studies. However, the impact of hypoxia on tumor phenotypes does not end in the primary site. How hypoxia affects intravasating cells also needs to be taken into consideration at the secondary organs where physiology and oxygen levels may vary. Additionally, acute, cycling, and chronic hypoxia can coexist in tumors and often lead to different biological consequences (See Box 2 and [5]), with both associated with poor clinical outcome. However, in clinical studies, the challenge is to determine where hypoxia speci cally in uences the endpoints of clinical outcome. We attempt to provide an analysis of this question, focusing mostly on *in vivo* experiments and mention the oxygen tension and exposure duration of the hypoxic conditions for context and comparisons.

Role of Hypoxia in Affecting Metastatic Capacity through Motility and Growth Programs

Limited oxygen supply has been historically proposed as a rate-limiting factor for primary tumor growth, where it is suggested that the tumor could not surpass 1 mm^3 if hypoxic cells do not mount a neovascularization response [97]. However, in a mouse model of melanoma [98], inactivation of HIF1a or HIF-2a did not affect primary tumor formation but instead selectively abrogated metastasis. This result suggests that in some cancers tumor growth is actually independent of HIF signaling but HIF function is critical for metastasis development. This unexpected result supports the need to understand how hypoxia shapes niches in the metastatic setting. Along these lines another study described that the ECM protein, lysyl oxidase (LOX), is regulated by hypoxia and HIF [99]; LOX upregulation correlates with poor distant metastasis-free and overall survival in human breast and head and neck tumors. When LOX is secreted, it is responsible for the invasive properties of hypoxic cancer cells (1–2% O_2 for 18 or 24 h) through focal adhesion kinase activity and cell-to-matrix adhesion [99,100]. Moreover, LOX blockade strikingly decreased metastasis, suggesting this enzyme to be a metastatic niche therapeutic target [99,100]. LOX secreted by hypoxic primary tumors was proposed to be critical for premetastatic niche formation as it results in crosslinked collagen IV and the recruitment of $CD11b^+$ myeloid cells; all factors that are prometastatic. These data suggest that hypoxia in the primary site can affect the secondary organ [19]. It will be important to understand how these mechanisms of hypoxia-regulated premetastatic niche organization are sustained after primary tumor surgery, as patients may not develop metastasis for years after the source of these signals is removed.

Hypoxia and HIF1α also in uence the splicing of a specific variant of the scaffold protein A-kinase anchor protein 12 (AKAP12). AKAP12v2 is a direct HIF target and regulates protein kinase A (PKA) activity under hypoxia (2% O_2 for 16 h), favoring tumor cell invasion and migration *in vitro*, but most importantly, metastasis in an *in vivo* orthotopic model of melanoma [101]. Additionally, metastatic samples have higher levels of AKAP12v2 than the primary tumor has [101–105]. It is possible that hypoxia-induced splicing of AKAP12v2 at the primary site provides an advantage for dissemination and/or colonization at the metastatic sites. The latter possibility would suggest that AKAP12v2 splicing is part of a posthypoxic program that is self-sustained in secondary organs.

Leukemia inhibitory factor (LIF), which is produced by osteoblast-lineage and bone marrow (BM) stromal cells, can induce disseminated breast cancer cell dormancy [106], a reversible growth arrest state. Accordingly LIF receptor (LIFR) functions as a metastasis inhibitor in different cancers [106,107] and, in breast cancer patients with bone metastases, low levels of LIFR are correlated with poor patient outcome. Intriguingly, hypoxia (0.5% O_2 for 24 h) and HIF signaling reduce the LIFR–STAT3–SOCS3 signaling pathway in breast cancer cells *in vitro* leading to downregulation of dormancy related genes. These data suggest that hypoxia may awaken dormant cells by inhibition of LIFR–STAT3-mediated dormancy [106]. *In vivo* validation of these findings may support some of the speculation derived from the *in vitro* ndings [106] that are countered by the findings that dormant DTCs can persist in the BM for years despite this being a proposed hypoxic niche [108]. It would also be interesting to know if primary tumor hypoxia already renders DTCs insensitive to LIF signaling in secondary sites.

The above studies comprise a handful of recent papers with *in vivo* data that support the notion that hypoxia can activate traits that render cancer cells more migratory, invasive, and apt to escape dormancy. However, given the heterogeneous nature of the tumor cell population, their response to hypoxia may be also as heterogeneous.

Hypoxia as a Regulator of Dormancy and Stress-Tolerance Programs in DTCs

Numerous studies suggest that hypoxia can induce as an adaptive survival response growth arrest and stem-cell-related programs [4,109,110]. This allows cells to pause proliferation while adapting to hypoxia and then resume growth. Thus, this transient growth arrest in growing tumors contributes to the viability of cells that can resume growth. Hypoxia activates an unfolded protein response (UPR) that results in the attenuation of translation initiation and the induction of cell cycle arrest genes [4,109,110]. Intriguingly, the UPR was found in dormant cancer cells in experimental models [111] and transcripts for speci c UPR genes were also found to be upregulated in dormant DTCs isolated from BM of prostate cancer patients [112]. These genes could also be turned on in DTCs in the target organ by the fact that they carry a signature epigenetically imprinted in the primary tumors (i.e., the DTCs carry a remodeled chromatin in response to hypoxia in the primary site) [7,113]. Accordingly, hypoxic micro-environments in primary lesions contain a fraction of cells that concomitantly turn on hypoxia (GLUT1 and HIF1a) and long-term dormancy genes such as NR2F1, DEC2, and p27. Presumably, these would be the slow, but persistent migratory cells that ef ciently reach blood vessels for dissemination (see first section on motility) [6]. Additionally, both NR2F1 and HIF1a are required for p27 induction in dormant DTCs originating from hypoxic primary tumor microenvironments, but these DTCs do not display $GLUT1^{high}$ expression, supporting the concept that in secondary sites, a dormancy program is sustained even after the hypoxic response is resolved (Figure 2). The dormancy-prone hypoxic cells also display accumulation of transcriptionally repressive histone marks (H3K27me3), suggesting that an epigenetic change is taking place in the dormancy-inducing primary tumor hypoxic microenvironments. Interestingly, this histone H3 repressive mark is found in dormant cells [114], suggesting that it could be a regulator of a persistent dormancy program retained in DTCs originating from hypoxic primary tumor niches. Importantly, hypoxia-induced dormant-like DTCs survive chemotherapy [7]. These findings lead to the

notion that these once hypoxic and now dormant DTCs may be the source of disease relapse and poor prognosis associated with hypoxia. Hypoxia-associated poor prognosis is derived from measurements in primary tumors, but survival is dictated by metastases. Thus, it is possible, that the dormancy-inducing capacity of hypoxia allows a subpopulation of DTCs to robustly survive treatment and then ultimately determine the fate of patients as they reactivate.

In an HPV^+ head and neck squamous cell carcinoma model, tumors express the viral E6/E7 oncogenes, which inhibit p53 expression/function resulting in induced senescence or cell death [115]. What was found is that under hypoxic conditions where E6/E7 are repressed, but p53 is not induced; instead, these cells experience growth arrest upon reoxygenation in a reversible manner controlled via the nutrient sensing mTOR/REDD1/TSC2 signaling axis [115]. This suggests that HPV^+ tumor cells low may enter a therapy resistant, $p53^{low}$, dormant phenotype in response to hypoxia, serving as a source of recurrence [115]. Overall, these studies provide additional evidence that some cancer cell subpopulations respond to hypoxia by activating a growth arrest program that, like dormancy, can lead to increased therapeutic resistance.

Analysis of receptor tyrosine kinase (RTK) signaling in lung cancer cells also revealed that hypoxia induced dormancy of mutant epidermal growth factor receptor (EGFR) (ErbB1) primary lung cancer cells through upregulation of MIG6; MIG6 results in the disassembly of the ErbB receptor heterodimers, blocking their signaling for growth. Critically this phenotype renders mutant EGFR lung cancer cells resistant to specific EGFR inhibitors, while MIG6 downregulation restores EGFR signaling and sensitivity to tyrosine kinase inhibitors and radiation. Lung cancer patients carrying EGFR mutations and MIG6 up-regulation showed shorter disease free periods. Thus, coexistence of dormant hypoxic tumor cells and proliferative cells might allow lung tumors to adapt to therapeutic stress and eventually progress unrestricted [116].

It has been recently described that successful metastasizing melanomas underwent reversible metabolic changes that increased their oxidative stress resistance through NADPH-generating enzymes in the folate pathway [117]. Using cells with high primary tumor forming capacity, but low metastatic efficiency, the authors showed that tumor cells in the blood and visceral organs experienced oxidative stress not observed in the bulk primary tumors. Antioxidants promoted distant metastasis without significantly affecting primary tumor growth [117]. While the authors did not explore dormancy mechanisms, we propose that perhaps hypoxia induced oxidative stress may prime melanoma cells to enter dormancy, and as a consequence limit distant metastasis *in vivo*.

Concluding Remarks and Future Perspectives

Our analysis of recent literature reveals that understanding how hypoxia affects metastasis will require high resolution intravital imaging at single cell resolution and/or other methods that complement intravital imaging *in vivo*. These are key to help de ne the actual phenotypes of cells in primary tumors and secondary organs both in hypoxic and normoxic microenvironments. It is expected that *in vivo* studies will then inform more physiologic *in*

vitro assays. Finally, the notion that hypoxia in primary sites can have long term effects on secondary organ DTC biology needs to be explored in more detail (see Outstanding Questions). The integration of efficient hypoxia biosensors with fate mapping tools that allow for longitudinal tracking of DTCs may be necessary to link primary tumor biology to secondary organ DTC fate. Overall, understanding the fate of DTCs *in vivo* will ultimately guide us to understand how to target these hypoxia-influenced events to prevent and treat metastasis more ef ciently.

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Highlights

Hypoxia is associated with resistance to therapy and metastasis onset but mechanistically this link is still unclear.

In vitro assays to study hypoxia in metastasis have limitations that lead to limited consensus on how it drives metastasis.

New intravital imaging technologies enable studies of hypoxia and metastasis in physiological contexts, revealing unanticipated roles of hypoxia not observed *in vitro*.

In vivo studies revealed that hypoxia could decrease motility speed while increasing invasiveness and priming tumor cells for dormancy after dissemination.

In vivo analysis of hypoxia and DTC fate may reveal its link to metastasis and poor prognosis and how to prevent recurrence.

Box 1.

Variability in the *In Vitro* Measurements of Tumor Cell Responses to Hypoxia

While it is generally accepted that hypoxia increases migration and invasion, *in vitro* studies show a variable response to hypoxia across culture conditions [118] and cell lines [119]:

- Hypoxia reduces tumor cell cycling, but tumor cell invasiveness is not affected [120]
- Hypoxia increases ability of tumor cells to invade [121]
- Overexpressing HIF-1a increases invasion [122]
- Silencing HIF-1a with siRNA inhibits migration and invasion [123]
- Acute hypoxia (≤6 h) enhances cell migration, invasion, and clonogenic survival [124]
- Chronic (≥24 h) hypoxia results decreases cell proliferation and increases cell death [124]
- Intermittent hypoxia inhibits cell proliferation and increases cell migration and motility compared to both chronic hypoxia and normoxia [118]

The crucial question is which of these phenotypes is observed *in vivo* and related to tumor progression in animals and in patients.

Box 2.

Acute versus Chronic Hypoxia

Acute or perfusion hypoxia (0.05–2.3% O_2) [5]:

- is an abrupt and brief (of the order of minutes) exposure to hypoxia due to structural and functional abnormalities in the tumor microvessels [125];
- Ieads to cell survival by activation of autophagic, apoptotic, and metabolic adaptation pathways and a decrease of oxidative metabolism [126,127].

Chronic or diffusion limited hypoxia (≤0.05% O_2) [5]:

- occurs due to hyperproliferation of cancer cells that surpasses the capabilities of the vascular supply leading to tumor cells being exposed to continuous hypoxia for long periods [125];
- activates cell survival and progression programs through increased ROS production [128];
- has been linked to a more aggressive tumor phenotype, usually measured by *in vitro* parameters of motility and invasion, but also through measurements of lung colonization and metastasis development [21,119,129–139].

Outstanding Questions

How is hypoxia linked to the fate of DTCs?

How does hypoxia affect the manifestation of metastasis years after primary tumor surgery and what happens to premetastatic niches in uenced by hypoxia regulated mechanisms?

How could hypoxia lead to increased therapeutic resistance of DTCs, which may serve as reservoirs for tumor recurrence after reoxygenation?

What is the role of epigenetic mechanisms in driving hypoxia in uenced DTC fate and how do organ cues required to maintain organ homeostasis affect the fate of DTCs derived from hypoxic and normoxic microenvironments?

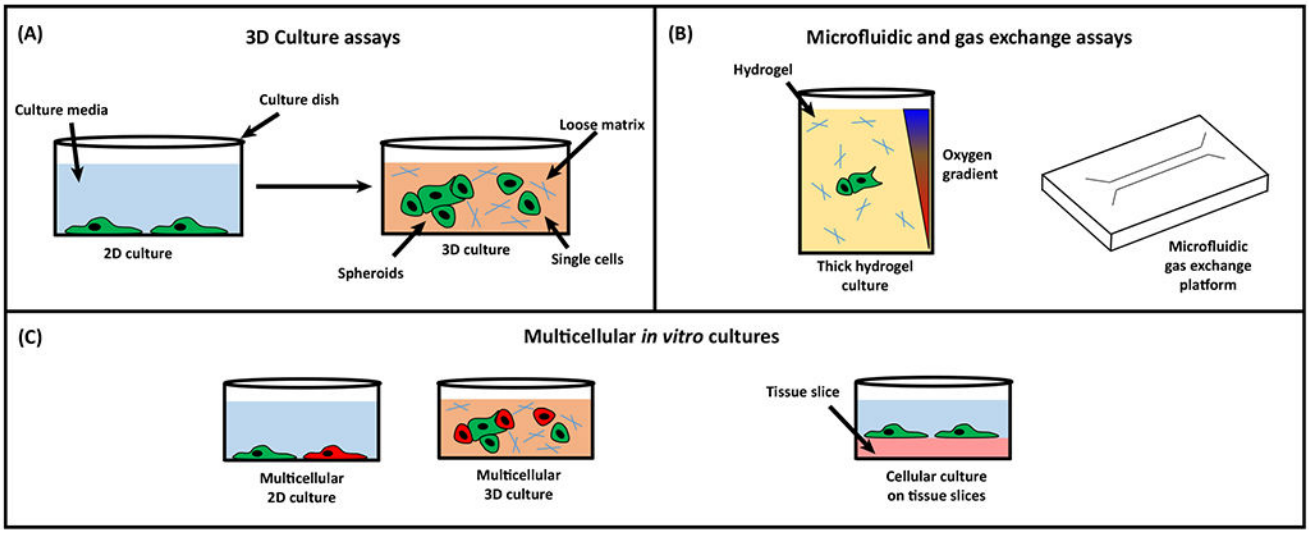

Figure 1. Moving away from 2D and toward More Sophisticated Assays.

(A) Traditional 2D cultures consist of tumor cells plated on a glass or plastic surface. These geometrical constraints are alleviated by moving to 3D cultures where single tumor cells or tumor cell spheroids are grown in suspension in a deep but loose (low concentration) matrix. (B) Micro uidic and gas exchange assays provide precise control of oxygen levels for cell cultures allowing the utilization of physoixia, oxygen levels which are physiological for normal tissues and tumors. These assays have the added bene t of being able to create gradients of oxygen that can then be used to evaluate migration phenotypes. (C) Since tumors comprise multiple cell types, coculturing them together is another way to increase sophistication. This can be done either in standard 2D assays, 3D cultures, or even on top of tissue slices which can provide the most physiologically relevant extracellular matrix.

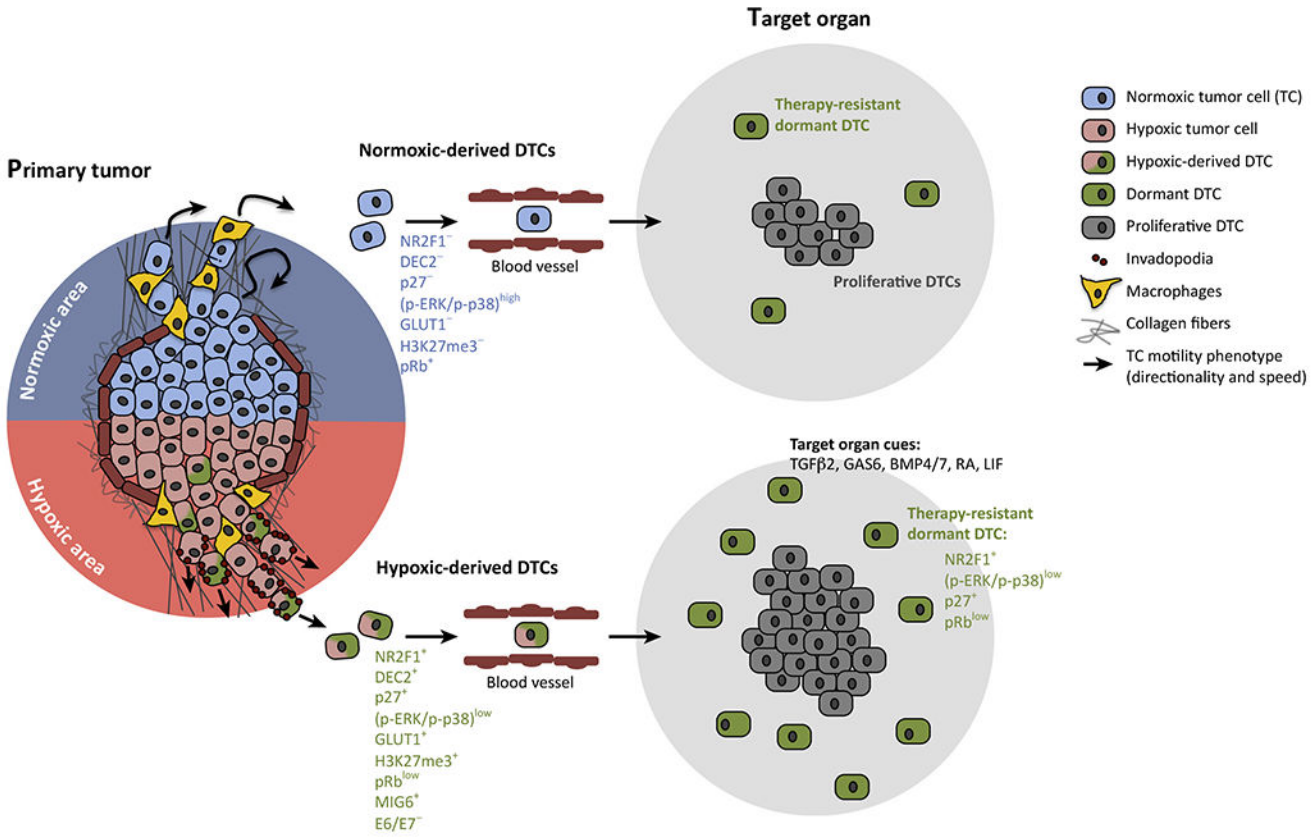

Figure 2. Hypoxia-Regulated Migration and Metastasis Programs.

Tumor cells in solid tumors display two distinct motility patterns that are linked to hypoxic microenvironments: (Top Left) the fast migratory phenotype observed in normoxic cells, in which cells migrate rapidly, and in streams with macrophages (yellow cells), along collagen fibers; and (Bottom Left) the slow migratory phenotype observed in response to hypoxia, characterized by slower but more directional migration, where cells display increased numbers of invadopodia (red dots), increased matrix dissolving ability, and increased levels of intravasation over time. Tumor cells in hypoxic areas (red) are usually dormant or slow cycling, which is also true in secondary organs (green cells). DTCs originating from primary hypoxic microenvironments carry a hypoxia and dormancy signature (pink and green cells – $DEC2^{+}$ $p27^{+}$ $(p\text{-}ERK/p\text{-}p38)^{low}$ $GLUT1^{+}$ $H3K27me3^{+}$ pRb^{low} $MIG6^{+}$ $E6/E7^{-}$), while cancer cells originating from normoxic regions (blue cells) do not carry a dormancy signature. Once in secondary organs, unlike normoxia-exposed cancer cells, cells emanating from hypoxic microenvironments can display a long-lived dormancy program (green cells – $NR2F1^{+}$ $(p\text{-}ERK/p\text{-}p38)^{low}$ $p27^{+}$ pRb^{low}) reinforced by secondary organ signals (target organ cues). These hypoxic microenvironment-derived cells may have high ROS levels, silence RTK signaling or alter oncogene signaling evading chemo, targeted and radiation therapies. Adapted from [10].

Abbreviations: DTC, disseminated tumor cells; ROS, reactive oxygen species; RTK, receptor tyrosine kinase.

Table 1.

Recapitulation of the *In Vivo* Slow and Fast Migratory Phenotypes with *In Vitro* Assays

***In vitro* assay**	**Speed normoxia (μm/h)**	**Speed hypoxia (μm/h)**	**Refs**
3D collagen I microfluidic platform	10	14	[39]
3D collagen I matrix	6	6	[26]
3D collagen I matrix	24	24–35	[22]
3D Matrigel matrix	4–9	4–14	[32]
3D hydrogel platform	0–8	4–18	[27]
1D fibronectin tracks	60–90	-	[41]
1D fibronectin tracks	60–90	-	[40]

Table 2.

Comparison of Different Technologies Used to Study Hypoxia *In Vitro* and *In Vivo*

Assay	Pros	Cons	Refs
In vitro assays			
Scratch assay	Easy, economical, establishes cell-cell contact before start of assay	2D, nonphysiological, no chemical/oxygen gradients	[140,141]
Boyden chamber	Easy, economical, utilizes a chemical gradient	2D, nonphysiological, removes cell-cell contacts prior to assay, no oxygen gradient	[142]
Invasion assay (Boyden chamber with matrix)	Easy, economical, utilizes a chemical gradient	2D, nonphysiological, removes cell-cell contacts prior to assay, sensitive to matrix composition and density, no oxygen gradient	[143]
Culture dish hypoxia chambers	Easy, economical, creates gradients of oxygen	2D, nonphysiological	[144]
3D culture	Moderately easy, mimics 3D structure of tumors	Nonphysiological, sensitive to matrix composition and density	[145,146]
Invasion into tissue sections	Mimics 3D structure of tissue, realistic matrix	Lacks connection to vasculature	[123]
Thick hydrogel	Moderately easy, mimics 3D structure of tumors, creates gradient of oxygen	Complex, nonphysiological, limited to hydrogel	[27]
Microfluidic controlled gas exchange platforms	Mimics 3D structure of tumors, creates controllable oxygen gradients	Complex, nonphysiological	[39]
Ex vivo assays			
Staining for endogenous hypoxia markers in sectioned tissue	Reflects true physiology at time of excision	Requires sectioning of tissue, different makers do not colocalize and reflect different timing post exposure to hypoxia	[76,147–149]
Nitroimidazole adducts for sectioned tissue	Reflects true physiology at time of excision, can be used in clinic, most sensitive at low oxygen levels	Requires sectioning of the tissue	[150–152]
Dye injections	Only stains perfused cells	Indirect marker only showing perfusion, requires sectioning of tissue	[67]
Soluble phosphorescent probes	Directly measures oxygen levels	Requires sectioning of tissue, does not indicate history of cells	[153,154]
In vivo assays			
Polarographic electrodes	Gold standard, approved for use in clinic	Susceptible to oxygen consumption by probe, damaging to tissue, averages over many cells, limited to only a few locations	[155,156]
MRI, PET or CT probes	Noninvasive	Lacks single cell resolution	[69,157]
Fiber optic phosphorescent probes	Does notconsume oxygen, higher sensitivity at physiological oxygenation	Damaging to tissue, averages over many cells, variable results	[73,158]
Fluorescent protein biosensors	Genetically encoded, allows realtime, single-cell visualization	Cannot be used in clinic, may be delayed with respect to exposure to hypoxia	[6,80,86,159]