

Hypoxia and Tumor Dormancy: Can the Two Tango?

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Abstract

Majority of cancer patients die of metastatic disease that can develop decades after primary tumor removal. During this period the disseminated tumor cells (DTCs) may stop proliferating and survive in a dormant state, a phenomenon also referred to as minimal residual disease (MRD). MRD is a well-known clinical phenomenon and studying the mechanisms behind this stage of tumor progression specifically the contribution of the microenvironments are areas of active investigation. Hypoxic microenvironments, which result from a decrease in oxygen levels, are common during tumor progression, but its role in the induction and maintenance of the dormant state remains unclear. This chapter focuses on some of the experimental as well as theoretical evidence supporting how tumor hypoxia both in the primary tumor as well as at target organ sites can influence disseminated tumor cells (DTCs) to enter dormancy. Furthermore, the interplay between hypoxic and the unfolded protein response (UPR) signaling in promoting the survival of dormant tumor cells, which is critical for both long term survival as well as therapy resistance is also reviewed. Lastly, the chapter emphasizes on the parallels between the concept of tumor dormancy and cancer stem cells (CSCs) and the overlapping roles of hypoxia mediated signals in the maintenance of quiescence of CSCs as well as and

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dormant tumor cells. This information is essential to design urgently needed therapeutic strategies aimed at either maintaining these DTCs in a dormant state or eradicating them before they progress to overt metastasis.

Introduction

Metastatic disease rather than the primary tumor itself is one of the major causes of morbidity and mortality among cancer patients. Traditionally metastasis was thought to happen during later stages of cancer progression. However, accumulating evidence suggests that tumor cells disseminate from the primary lesion at early stages of tumor development even before the acquisition of full malignant properties (Aguirre-Ghiso 2007). Although disseminated tumor cells (DTCs) are highly prevalent among cancer patients (up to ~70% in prostate cancer patients), only a fraction of patients with DTCs will carry overt metastasis at the time of or shortly after primary tumor resection (Aguirre-Ghiso 2007). Others may remain free of clinical evidence of the disease for years or even decades after primary tumor removal. The appearance of tumor relapse after such a prolonged disease free period implies that these DTCs remain in the body in a dormant state.

Classically, tumor dormancy is described as the time it takes for the cancer to relapse after clinical remission. It refers to a protracted stage in which DTCs can remain undetected and asymptomatic for prolonged periods of time by entering either (1) growth arrested state (Cellular dormancy) or (2) exist as micrometastases (small masses of tumor cells), which results from a balance between proliferation and cell death due to the lack of angiogenesis (angiogenic dormancy). The definitions and mechanisms regarding cellular and angiogenic dormancy are discussed in detail in other excellent reviews ((Aguirre-Ghiso 2007) and references therein). Although we have made progress in our understanding of the underlying mechanisms responsible for tumor dormancy, there still remains some unanswered questions. The first question is in regards to

whether the cells are already dormant at the time of dissemination. Another question is whether cues from the primary tumor microenvironment pre-program DTCs and micrometastases to enter dormancy. Alternatively, the stress of dissemination process, or the stress of seeding in a non-permissive target organ microenvironment itself can be growth suppressive favoring the induction of dormancy either at the solitary cell level or after some initial proliferation (Sosa et al. 2013). A third question is whether dormant tumor cells are equivalent/similar or distinct from cancer stem cells. This review will focus on role of the hypoxic niche and the activation of stress signals such as the unfolded protein response in determining the dormant phenotype. The role of hypoxia in dormant tumor cell survival following therapy will also be addressed. Discussions regarding the striking parallels between dormant tumor cells and cancer stem cells and their relevance to clinical tumor dormancy will be highlighted as well.

Influence of Tumor Hypoxia on Angiogenic Dormancy

In most normal mammalian tissues the oxygen tension ranges from 2 to 9% O₂. Tumor tissue hypoxia arises due to an imbalance in the supply and demand of oxygen. Both chronic or diffusion-limited hypoxia ($\leq 0.05\%$ O₂), which arises due to hyper-proliferation of cancer cells that outstrips its vascular supply and acute or perfusion limited hypoxia (0.05–2.3% O₂), which is due to structural and functional abnormalities in the tumor microvessels co-exist in tumors. Unlike normal healthy cells, tumor cells can adapt to such oxygen fluctuations by decreasing energy consumption and increase anaerobic metabolism. Hypoxia presents a “Janus face” in tumor biology. On one hand it promotes adaptive processes that leads to tumor aggressiveness, metastatic potential and enhanced resistance to conventional cancer therapies that adversely affect overall patient survival (Hockel and Vaupel 2001). But on the other hand, it is also associated with restrained proliferation and apoptosis. It has been shown to alter gene

expression that impairs neoplastic growth through molecular mechanisms that result in cellular quiescence, differentiation, apoptosis and necrosis (Hockel and Vaupel 2001).

Hypoxia via activation of hypoxia-inducible factor-1 α (HIF-1 α) stimulates the transcription of angiogenic mediators such as vascular endothelial growth factor (VEGF), growth factors like transforming growth factor- β , platelet derived growth factor, enzymes such as urokinase-type plasminogen activator, which are all involved in the invasion and expression of several factors shown to enhance resistance to therapy (Hockel and Vaupel 2001). In contrast, hypoxia can also induces apoptotic cell death by a number of HIF-1 α dependent and independent pathways. These include the HIF-1 α mediated increase in expression of pro-apoptotic proteins NIX and BNIP3 (Sowter et al. 2001), which mediate cell death through activation of both apoptotic and necrotic pathways. HIF-1 α also promotes apoptosis via increased p53 levels involving Apaf-1 and caspase-9 as downstream effectors (Hockel and Vaupel 2001). These findings indicate that although most of the hypoxia- induced pathways promote tumor growth, apoptosis is also induced by hypoxia. Therefore the balance between these pathways is critical for hypoxia to effect tumor outcome- proliferation, death or dormancy.

Microscopic tumors (<2 mm diameter) often found during early stages of primary tumor development or as micrometastatic lesions, can remain dormant by their inability to expand beyond a microscopic size. Such microscopic tumors have been shown to be avascular and enter dormancy known as tumor mass or angiogenic dormancy due to the inability of the nascent tumor mass to recruit blood vessels. Characterized by a balance in proliferation and apoptosis, these microscopic tumors maintain a small but constant metabolic demand (Aguirre-Ghiso 2007). In comparing a small cohort of microscopic melanoma metastases and macroscopic melanoma metastases obtained from sentinel lymph nodes of patients, Barnhill et al. demonstrated that while the micrometastases demonstrated balanced rates of proliferation and apoptosis i.e. angiogenic dormancy, the macrometastases

showed significantly higher rates of proliferation than apoptosis consistent with progressive growth (Barnhill et al. 1998).

But these pre-angiogenic micrometastases can emerge from dormancy if they acquire the ability to become vascularized either by down-regulating angiogenesis inhibitors or by upregulating proangiogenic factors (Aguirre-Ghiso 2007). While there is overwhelming evidence that hypoxia is one of the primary physiological regulators of this angiogenic switch (Bergers and Benjamin 2003), it remains unclear whether tumor hypoxia has a role in the angiogenic dormancy process. For instance, it is unknown whether these microscopic tumors albeit being avascular bear regions of hypoxia. Studies have shown that microscopic tumors that are <1 mm diameter were largely hypoxic (hypoxic fraction >90%) with little or no blood perfusion while those of a greater size (~1–4 mm diameter) were relatively well vascularized and well perfused and showed little to no hypoxia. Such microscopic tumors bearing a single or multiple hypoxic foci remained either avascular (Li and O'Donoghue 2008) or showed a vascular network characterized by low blood flow velocity, high vessel tortuosity (Simonsen et al. 2010). Li et al. observed that while the non-hypoxic rim of these microscopic tumors exhibited proliferation, the hypoxic core of these tumors were not, suggesting that dormant tumors may be hypoxic and that hypoxia may be causally related to low proliferation rates and thus tumor dormancy (Li and O'Donoghue 2008). Also is it possible that the hypoxic/anoxic core of these microtumors undergo apoptosis that balances the proliferation of the vascularized rim of these tumors to promote a balance in proliferation and apoptosis and thus angiogenic dormancy. For instance microtumors that overexpress BNIP3 instead of VEGF might undergo slower growth because the cells would undergo high levels of apoptosis and a small amount of proliferation (Sowter et al. 2001). Further studies are needed to analyze the expression pattern of hypoxic response genes in these microtumors to determine if the balance in the levels of apoptotic vs. proliferative signals would favor dormancy or metastatic outgrowth.

Hypoxia Regulation of Cellular Dormancy

Cellular dormancy occurs when DTCs enter a state of quiescence through a G_0/G_1 arrest. Experimentally, these dormant DTCs are characterized not only by the absence of proliferation but also by their ability to survive the interlude between entry to and exit from the dormant state. Studies using various tumor model systems highlight the important role of microenvironment/ niche in governing cellular dormancy. These studies have unraveled niche signals such as loss of integrin and/or extra cellular matrix (ECM) function, reduced urokinase receptor expression as well as activation of stress signals as mediators of cellular dormancy. These signals not only promote cell-cycle arrest but also activate stress adaptive responses that promote survival of dormant tumor cells (Aguirre-Ghiso 2007).

It is interesting to note that one of the early responses to oxygen deficits in tumor tissue is to reduce the rate of oxygen consumption. It does so by decreasing proliferation of hypoxic cells, hence tumor cells in hypoxic areas are usually dormant, dividing far more slowly but remaining viable for prolonged periods (Gatenby et al. 1985). In fact the dividing fraction in tumors is low (5–20%) suggesting that large fractions are either dying or quiescent. Studies have shown that cells exposed to hypoxia have disproportionately long G1 phase or arrest in G0/G1 phase of cell cycle. Using immunocytochemical techniques, investigators have demonstrated that hypoxic tumor cells are in a non or slow proliferating state as a vast majority of these cells are negative for proliferation markers (e.g. PCNA, BrdU). The absence of proliferation in hypoxic cells has been attributed in part to the upregulation of cell cycle inhibitors such as p21 (WAF1) and KIP1 (p27) (Carmeliet et al. 1998). Similar studies from our laboratory have also demonstrated the lack of proliferation in hypoxic regions of tumors (Kumar et al. unpublished results).

Recently, using dormancy expression profiles from a variety of cancer types Kim et al. identified a dormancy gene signature. They found that

this signature was significantly overrepresented in estrogen receptor positive breast cancer patients (HDS-high dormancy score) vs. estrogen receptor negative breast cancer patients that had a low dormancy score. Moreover they found that the estrogen receptor positive patients whose tumor had high dormancy score experienced longer metastasis-free periods. In contrast, when this score was underrepresented patients recurred with metastasis more frequently (Kim et al. 2012). This suggests that DTCs from Estrogen receptor positive tumors with a high dormancy score were more likely to enter a dormancy state. Several of the genes in the dormancy signature are regulators of quiescence and angiogenesis. Furthermore, several of the genes in the dormancy signature are regulated during hypoxia. Taken together, these data highlight the possibility that if the DTCs originated from within the hypoxic niche of the primary tumor then they may already be pre-programmed to be growth-arrested and enter a dormant state.

Another possibility is that the microenvironment in the target organs where the DTCs arrive can induce a dormant state. There is plenty of evidence for the role of hypoxic signaling in establishing the premetastatic niche, at distant sites to allow for engraftment of DTCs. However, whether hypoxia signaling also prepares the “metastatic soil” in distant organs such that the disseminated tumor cells that arrive can remain present in a prolonged state of dormancy is unclear. For instance, hypoxia sensitive metastasis suppressor genes such as MMK4, GPR56, KISS1 and CD82 (KAI1) suppress growth and expansion of DTCs at the target organ without affecting primary tumor growth suggesting a possible mechanisms for hypoxia in creating a dormancy favorable niche in the target organ (Aguirre-Ghiso 2007). Similarly, in several different types of cancers the bone marrow (BM) is a common homing organ for DTC. In most instances DTCs are detected in the BM at a much higher rate (15–70%) than the frequency at which metastasis (10–30%) occurs in the BM. Despite the hostile environmental conditions such as hypoxia, hypoglycemia etc. these DTCs can persist in the BM over many years with the potential

to recirculate into other organs. Pantel and his colleagues showed that a vast majority of these DTCs were in a quiescent state. Recently, they showed that these DTCs in the BM activate markers of the unfolded protein response (UPR) or the endoplasmic reticulum (ER) stress response as cellular adaptation strategy to survive the hypoxic microenvironment in the BM from where they originate (Bartkowiak et al. 2010). These findings suggest that the hypoxic milieu in the BM may not only prime the DTCs to enter a quiescent state but could also activate adaptive mechanisms such as the UPR to survive.

UPR Signaling Regulates the Growth Arrest and Survival Programs During Cellular Dormancy

Previous studies from the Aguirre-Ghiso laboratory discovered that HEP3 head and neck squamous carcinoma cells (HNSCC) that display a high ERK1/2 to p38 α / β signaling ratio showed enhanced proliferation *in vivo*. They further demonstrated that a reversion of this ratio resulting in a low ERK1/2 to p38 α / β signaling ratio results in a spontaneous reprogramming of these cells into dormancy (D-HEP3 cells) *in vivo*. D-HEP3 cells inoculated *in vivo* enter a G₀-G₁ arrest characterized by the induction of p21, p27, p18 and p15 (Adam et al. 2009). Additionally activation of at least three transcription factors p53, BHLHB3/Sharp-1 and NR2F1 is required for the dormancy of these cells *in vivo* (Adam et al. 2009).

While exploring the mechanisms that drive the reprogramming of HEP3 cells into dormancy, the same group showed that these D-HEP3 cells develop an UPR that is required for the dormancy of these cells *in vivo*. ER stress has previously been shown to induce G₀/G₁ arrest and cell survival, the two components of dormancy. While all the three sensors of UPR—PERK, ATF6 α and IRE1 α are activated in these dormant cells; only PERK activation contributes towards the growth-arrest of D-HEP3 cells. This occurs by attenuating translation of G1-S transition regulators cyclin-D1, -D3 and CDK4 (Ranganathan et al.

2008). Moreover inducible activation of PERK signaling in tumorigenic HEP3 cells using a dimerizable Fv2E-PERK was sufficient to fully abrogate tumorigenicity and induce a “dormancy-like” state that results in a transient growth arrest and delayed tumor growth *in vivo* (Ranganathan et al. 2008).

In addition to activating these three arms of UPR, these dormant cells also induce the expression of chaperone BiP/Grp78, whose upregulation is linked to survival against several stresses that affect the ER homeostasis including chemotherapeutic drugs (Ranganathan et al. 2006). While the upregulation of BiP expression and PERK activation was not advantageous to basal survival of these dormant cells *in vivo*, they protected these cells from therapy induce cell death both *in vitro* as well as *in vivo* (Ranganathan et al. 2006) (and Ranganathan et al. unpublished results). Mechanism analysis revealed that this occurred via a BiP dependent inhibition of Bax activation (Ranganathan et al. 2006). On the other hand activation of both IRE-1 α and ATF6 α were required for the basal adaptation and survival *in vivo*. This occurred in part via the ATF6 α mediated Rheb induction and a strong inhibition of mTOR signaling (Schewe and Aguirre-Ghiso 2008).

Another pathway that has emerged as an important regulator of tumor dormancy and is activated by microenvironmental stressors such as the UPR is autophagy, a process of “self-eating” of intracellular materials, to temporarily sustain energy production during starvation or stress conditions. Recent studies show that autophagy may be the survival mechanism of the dormant cells. Using models of ovarian cancer, investigators have shown that autophagy is critical for the survival of dormant tumor cells (Lu et al. 2008). Studies have also demonstrated that impaired integrin signaling, which can promote tumor dormancy also induces autophagy that protects cells from anoikis (detachment induced apoptosis) (Fung et al. 2008). Since such impairment in integrin signaling is possible in DTCs, not engaging efficiently in a foreign ECM, it has been speculated that this could stimulate autophagy and promote survival and maintenance of a dormant state.

In summary the studies presented above highlight an overlooked aspect of dormancy, the ability of these cells either from early primary lesions or from advanced tumors to survive in the long interlude between dissemination and metastatic relapse. Because chemotherapy targets mostly dividing cells, it is commonly assumed that the lack of proliferation of dormant cells could lead to chemotherapy treatment failure (Aguirre-Ghiso 2007). However, the above results suggest that dormant cells have survival mechanisms in place that are uncoupled from proliferation programs that selectively protect them from a hostile microenvironment or from stress imposed by cancer therapies.

One of the well-known inducers of ER stress is tumor hypoxia. Elegant studies from Koumenis' laboratory have demonstrated that PERK-eIF2 α pathway is activated in response to hypoxic stress independent of HIFs (Koumenis et al. 2002). They showed that increased PERK signaling enhanced the tolerance to chronic hypoxic stress (<0.02% O₂) and promoted tumor growth *in vivo* that was dependent on eIF2 α and ATF4 expression (Bi et al. 2005). In addition, studies from Wouters laboratory have shown that during tumor hypoxia PERK activation is also critical for the induction of the essential autophagy genes MAP1LC3B and ATG5. They further demonstrated that this induction was critical for the enhanced resistance of tumor cells to treatment such as radiation (Rouschop et al. 2010). In addition to PERK, BiP is also linked to resistance to various stresses that affect the ER homeostasis, serves as a survival factor and is upregulated in the hypoxic regions of several primary tumors (Kaufman 2002). A role for hypoxic signaling in promoting UPR mediated survival of dormant tumor cells was recently demonstrated in BM DTCs. Using cell lines derived from breast cancer BM DTCs as well as fresh BM samples from breast cancer patients it was demonstrated that similar to the dormant D-Hep3 cells, these DTCs upregulate UPR genes such as grp78, grp94, PDI but not their cytoplasmic homologues. The levels of these UPR markers were further induced upon exposure to hypoxia suggesting that these changes were an adaptive response to the hypoxic

microenvironment of the BM (Bartkowiak et al. 2010). Moreover, these investigators also found that these UPR- positive DTCs also shared a CD44^{high}/cd24^{low} cancer stem cell (CSC) phenotype, which was also upregulated upon exposure to hypoxia. These findings raise three important questions: (1) Do metastasis founding DTCs that survive long-term in the BM arise from quiescent CSCs, (2) how does hypoxia regulate CSC behavior and (3) can non-stem cells be converted towards a cancer stem cell phenotype? These questions are fundamentally important to our understanding of tumor dormancy. In fact there is striking parallels between dormant tumor cells and CSCs, which can also undergo periods of dormancy and preferentially resist therapy and survive in hostile microenvironments.

Influence of Hypoxia on Cancer Stem Cell Maintenance and Plasticity

Just as pluripotent stem cells are critical for the maintenance of regenerative tissues in the body, increasing evidence has accumulated to suggest that cancer stem cells (CSCs) also known as tumor initiating cells (TICs) also exist within a heterogeneous tumors and are important for initiation and maintenance of tumor progression. The term CSC refers to the functional trait of the cancer cell rather than its cellular origin and biological properties within normal tissues. The existence of these CSCs was first demonstrated in human acute myeloid leukemia (AML) by pioneering work from John Dick and his colleagues. Since then using specific surface markers such CSC sub-populations have been identified in a number of solid tumor types including those of the breast, bladder, central nervous system, colon, head and neck, ovaries, pancreas and the skin (Lobo et al. 2007).

Classically cellular differentiation of normal stem cells has been perceived to follow a unidirectional, hierarchical and irreversible progression. It was therefore hypothesized that tumor growth follows a hierarchical cancer "stem" cell (CSC) model. This suggests that only a specific

subpopulation of cancer cells have “unlimited” tumor initiating/CSC properties and hence the ability to sustain cancer growth while the rest of the tumor cells have limited or no growth potential (Lobo et al. 2007). Since tumor cells have greater plasticity it is possible that they can change their phenotypes depending on microenvironmental cues. Reports have suggested a more dynamic regulation of CSC phenotype and function allowing for a bidirectional interconversion between the CSCs and non-CSCs in response to signals from the microenvironment (Chaffer et al. 2011). This implies that non-stem cell subpopulations could dedifferentiate into stem cells—leading to reversibility.

How then are these different behaviors of CSCs regulated/controlled? Similar to normal stem cells CSC behavior is critically dependent on contextual and configurational cues from the surrounding niche environment. Several reports have shown that the presence of low oxygen tension (hypoxia) in stem cell niches offers a selective advantage to maintain a more stem-like and undifferentiated phenotype. While the reasons why a hypoxic niche favors the maintenance of stem cells is largely unknown it is postulated that a low oxygen environment reduces the extent of ROS induced DNA damage during radiation or chemotherapy (Mohyeldin et al. 2010).

Recent reports have demonstrated that HIFs through signaling pathways such as Notch and Oct4 are crucial regulators of the stem cell phenotypes (Mohyeldin et al. 2010). Consistent with these findings hematopoietic stem cells have been shown to reside in hypoxic niches and rely on HIFs to maintain an undifferentiated phenotype. Similarly, presence of hypoxic areas within a growing tumor suggests possible niches for CSCs. Recently, excellent studies from different laboratories have shown that the fraction of cells positive for CSC markers or the side population in established cancer cell lines or cultures from human tumors increases following exposure to low oxygen conditions (Li and Rich 2010). While hypoxia clearly promoted the undifferentiated state in CSC population, the molecular mechanisms underlying these observations remained obscure until recently.

Studies have shown that prolonged exposure of several cancer cell lines as well as glioma non-stem cells to hypoxia resulted in the induction of genes essential for stem cell function such as Oct4, Nanog and c-myc and augmented the tumorigenic potential of non-stem cells (Li and Rich 2010). Recently, it was identified that both HIF-1 α and HIF-2 α are critical for CSC maintenance. However, HIF-2 α is selectively induced only in CSC and not normal stem cell populations and is critical not only for the maintenance of CSCs in an undifferentiated state but also for their plasticity and correlated with poor patient outcome (Li and Rich 2010). These findings establish a clear link between hypoxia, HIFs and molecules that are crucial for the regulation of CSC behaviors.

Additional evidence for the role of hypoxia in the dynamic behavior of CSCs was provided by findings from Roesch et al. who identified that histone demethylases JARID1B is essential for the maintenance of a small subpopulation of slow cycling melanoma cells and is essential to both initiate and sustain melanoma growth. They further demonstrated that JARID1B⁻ cells could however switch to JARID1B⁺ cells and support tumor growth. Also these studies further emphasized the significance of low oxygen environment for the dynamic regulation of CSC phenotype as JARID1B expression in JARID1B⁻ melanoma cells was rapidly enhanced at low oxygen and steadily reverted to normal expression intensity (Roesch et al. 2010). These landmark studies suggest that hypoxia could potentially serve as the trigger that allows the reprogramming of non-CSC to CSC.

Recently, using experimental HEP3 model of HNSCC, Bragado and colleagues also demonstrated the existence of such marker enriched CSC subpopulation of G0-G1 arrested slow cycling, ALDHA1^{high}/ α 6^{high} integrin^{high} expressing tumor cells (Bragado et al. 2012). They found that this sorted ALDHA1^{high}/ α 6^{high} subpopulation had immediate and enhanced tumorigenic potential *in vivo*. In agreement with the above findings they also demonstrated that with time even the non-CSC subpopulation regained tumorigenic capacity *in vivo*, which was linked to the

restoration of ALDHA1^{high}/α6^{high} cells. In addition, they also found that the tumorigenic potential was functionally dependent on α6 expression.

They further demonstrated that α6^{high} CSCs were associated with low levels of the repressive histone modification H3K27me3 and H3K9me3. In contrast, the α6^{low} cells were associated with high levels of these repressive marks. This suggests that the CSCs maybe held in a transcriptionally ready state, while the non-CSCs are primarily in a transcriptionally repressed state demonstrating a strong correlation between epigenetic mechanisms and the dynamic states that dictate tumorigenic capacity.

Although in these studies the ALDHA1^{low}/α6^{low} were able to form tumors they had a longer latency period *in vivo* (15–20 days) before they regenerated ALDHA1^{high}/α6^{high} expressing tumors. While these studies did not examine the role of hypoxia in this dynamic regulation, it is possible that during this latency period *in vivo*, these cells could have experienced periods of hypoxia that may have resulted in the induction of markers such as α6 essential for its “stem-like” potential. To this end we examined whether tumor hypoxia can serve as a micro-environmental “trigger” that, allows for the reprogramming of α6^{low} cells to α6^{high} cells and recover tumorigenic capacity. Our studies found that *in vitro* exposure of α6^{low} non-CSCs from these HEP3 tumors to low oxygen environment (0.1–1% O₂) resulted in a significant increase in the α6 mRNA and protein levels, which was comparable to that seen in the α6^{high} cells and is sustained as long as the α6^{low} cells are maintained in hypoxia. In correlation with these findings, we found that a vast majority of α6^{high} cells resided within or near the vicinity of hypoxic regions (Kumar et al. unpublished observations). These results imply that tumor hypoxia may serve as a haven for CSCs. This hypoxia induced shift in α6^{low} subpopulation to a α6^{high} expressing cells could represent a true phenotypic switch to an enhanced tumorigenic program rather than a mere stress adaptation mechanism.

Alternatively it is plausible that a reversion in the chromatin states of the non-CSCs to a more permissive state for transcription may require the erasing and/or writing of new histone 3 post-translational

modifications, which could explain why longer periods are required for non-CSCs to fully restore their tumorigenic potential. Such epigenetic mechanisms can also be regulated by a hypoxic niche as recent studies have found that a majority of histone demethylases including JMJD2B/KDM4B and JMJD2C, which are H3K9me3 demethylases are upregulated in response to hypoxia, which can have profound implications in cancer biology (Yang et al. 2009).

All of the data discussed above show that as previously postulated cancer stem cells may not strictly adhere to the traditional hierarchical model, where the stem cell competence is only restricted to a discrete subset of tumor cells as characterized by the expression of certain markers. But rather they suggest that the different subpopulations within a tumor are in a highly dynamic state with respect to CSC phenotype and functions and can undergo bidirectional interconversion between CSC and non-CSC states while still maintaining a hierarchy with the CSC at the top of the hierarchy and the non-CSC are the “differentiated” progeny. While the transition between the two state is still under investigation the data discussed above point to a crucial role of tumor hypoxia in regulating this phenotypic heterogeneity within tumors.

Hypoxic Signaling in the Regulation of CSC Quiescence and Tumor Dormancy

Alongside self-renewal and multipotency, stem cell potential is frequently associated with dormancy/quiescence, which can also be applied to CSCs. Since CSCs by definition are known for their ability to initiate robust tumor growth, and are competent to differentiate into various non-self renewing tumor bulk populations, it is quite paradoxical to associate them with dormancy. However, there are striking parallels between CSC quiescence and tumor dormancy. For instance, dormant tumor cells and CSC alike have the ability to survive non-permissive microenvironments that are incapable of nurturing tumor growth and survive cancer therapy (Aguirre-Ghiso 2007;

Schillert et al. 2013). Recent evidence indicates that CSC population itself comprises of heterogeneous subpopulations including a dormant, slow-cycling fraction. Using fluorescent markers to specifically label quiescent cells (label retaining cells, LRCs), elegant studies from different groups provide strong evidence for the existence of such slow cycling, dormant cells as a subpopulation of CSCs in tumors of the breast, colon, pancreas, ovary, head and neck and skin that contributed towards CSC mediated tumor progression ((Schillert et al. 2013) and references there in). Additionally this slow cycling/quiescent population were more resistant to chemotherapy and retained the capacity to proliferate after withdrawal of chemotherapy (Schillert et al. 2013). This is analogous to the reversible quiescence observed in dormant tumor populations following therapy. Unlike dormant tumor cells that can remain quiescent for prolonged periods, it is unclear if CSCs undergo long phases of quiescence. While in the hematopoietic stem cells (HSCs) such long-term quiescent stem cells are present, such populations have not been described yet in solid tumor CSCs (Mohyeldin et al. 2010). Pantel and colleagues have demonstrated that DTCs from BM of cancer patients are quiescent and can persist in the BM for several years with the potential to give rise to late recurrences. This coupled with the findings that these DTCs express putative CSC markers: $CD44^{high}/CD24^{low}$, suggest that DTCs may arise from CSCs at least in breast cancer patients that similar to HSCs can remain quiescent for prolonged periods (Bartkowiak et al. 2010). Taken together these findings suggest that these slow-cycling CSC compartment may contribute towards the latent disease and delayed recurrences caused by the dormant tumor cells.

It is clear from the preceding discussion that a hypoxic microenvironment clearly promotes the induction and maintenance of tumor dormancy and that it is also critical for maintaining the CSCs self-renewal capacity and plasticity. But whether hypoxia also contributes towards the quiescence of CSC subpopulations and if these same mechanisms also operate to maintain the slow cycling or quiescent state of dormant tumor cells needs to be investigated.

Several investigators have demonstrated that slow-cycling HSCs are more likely to localize in the low oxygen areas of the BM away from the blood vessels whereas the fast cycling hematopoietic progenitors with limited self renewal capacity reside in areas much closer to the vasculature (Mohyeldin et al. 2010). In contrast CSCs have been proposed to reside in the perivascular niche, in an intimate relationship with the tumor vasculature (Mohyeldin et al. 2010). But in recent studies by Li et al. CSCs have also been proposed to exist in a secondary niche within cancers that is far away from the vasculature and as a consequence more hypoxic (Li et al. 2009). CSCs found in these hypoxic niches were maintained in an undifferentiated, quiescent state in several solid tumors including neuroblastoma, breast and cervical cancers (Axelson et al. 2005). These studies suggest that hypoxic/angiostatic niche environments could be conducive for the maintenance of CSC dormancy. For instance hypoxic induction of p21, which leads to cell cycle arrest in dormant tumor cells is also activated in quiescent CSCs (Carmeliet et al. 1998). In mouse models of cancer Felsner and his colleagues demonstrated that MYC inactivation induced tumor regression that required the enhanced expression of antiangiogenic protein Tsp-1. In addition, MYC inactivation also uncovered stem-like properties that induced differentiation but some cells remained in a dormant state and turned cancerous upon reactivation of the oncogene (Bellovin et al. 2013). Furthermore, independent studies have shown that hypoxic stabilization of HIF-1 can cause cell-cycle arrest via inhibition of myc expression and can also induce Tsp-1 expression (Keith et al. 2012). These findings demonstrate that there is a direct link between tumor dormancy and CSC quiescence and suggest a possible role for the angiostatic/hypoxic niche in regulating this process. Similarly, NDRG1 (N-myc downstream regulated gene-1) a metastasis suppressor gene, and a target of hypoxia, has also been implicated in the quiescence of CSCs in prostate cancer as well as tumor dormancy (Kobayashi et al. 2011). An additional link between hypoxic niche environments and CSC

and dormant tumor subsets, is their use of mTOR signaling to promote survival and growth inhibition. Activation of mTOR signaling is essential for the survival of dormant tumor cells as well as for CSC quiescence (Gaur et al. 2011; Schewe and Aguirre-Ghiso 2008). Taken together these findings highlight the critical importance of a hypoxic microenvironment in regulating the quiescence of CSC and dormant tumor populations.

Conclusion

Our knowledge on the mechanisms of tumor dormancy, which makes cancer cells refractory to conventional therapies, is limited. Even less understood is the role of the microenvironment in regulating the dormant phenotype. This chapter gives an overview of the role of tumor hypoxia in regulating the dormancy program during the initial stages of tumorigenesis as well as in micro-metastatic disease and in DTCs. The genomic state of the tumor cells, microenvironmental epigenetic factors and the degree of hypoxia are all factors that determine whether the net phenotypic result of hypoxia induced changes in gene expression would result in tumor dormancy. One can hypothesize that in the early stages of primary tumor progression when the tumor cells have not acquired genetic alterations that lead to immortalization such as loss of tumor suppressors such as TP53, RB1 (retinoblastoma 1) etc., hypoxia induced growth arrest programs may favor tumor dormancy. Since dissemination occurs early in tumor progression microenvironment restrictions such as hypoxia or stress signals resulting from hypoxia could influence the genetic progression of DTCs and slow down the metastatic progression by keeping the cells in a growth arrested state. Alternatively, even in genetically progressed DTCs that carry oncogene activation, signals from the hypoxic niche of the primary tumor could induce dormancy gene signature and activate survival mechanisms in DTCs that can overcome the pro-growth oncogenic signals and allow them to survive in a dormant state. Traditionally, tumor hypoxia has been considered a therapeutic hindrance as it renders solid tumors more resistant

to therapy. The findings that dormant tumor cells as well as DTCs can tap into hypoxia induced UPR survival signals to survive and persist for prolonged periods holds potential for developing therapies that target specific components of the UPR machinery to eradicate dormant DTCs.

There are interesting parallels between tumor dormancy and the role of CSCs in tumor propagation such as the ability to engage several survival-promoting mechanisms to resist therapy. Furthermore the commonalities in the molecular pathways that are integral to CSC biology and maintenance of tumor dormancy, point to a partial overlap between these two populations and that the hypoxic niche could serve as a direct link between these two populations. The studies discussed in this chapter highlight the important role of hypoxia and HIFs in the maintenance, plasticity and quiescence of CSCs. Therefore, disruption of the hypoxic niche while being detrimental to the maintenance of CSC properties, could serve as a critical therapeutic target to kill tumor cells. These studies also underscore the fact that the standard culture conditions using ambient air may fail to maintain the heterogeneity present within the tumors and may not represent *in vivo* situation. Hence understanding the importance of hypoxia and the role of factors such as HIF2 α in the maintenance of CSCs and facilitating ways to destroy the niche are important from both a basic science standpoint as well as for drug development. For instance, improving oxygenation through “vessel normalization” via repair of tumor vessel abnormalities might be able to disrupt CSC phenotype by normalizing the CSC microenvironment. Furthermore disrupting endothelial cell homeostasis in the CSC niche could also reduce tumorigenesis by depleting the CSC pool as these niche endothelial cells release trophic ‘angiocrine factors’ that can activate dormant CSCs (De Bock et al. 2011).

The existence of dormant/slow-cycling cell sub-population in CSCs suggest that these cells may survive treatment but still retain the ability to proliferate in response to a yet to be identified stimuli and cause disease recurrences. Accordingly it has been postulated that low dose metronomic chemotherapy can in fact induce tumor dormancy in cancer cells. In agreement with this

Martin-Padura et al. demonstrated that following low dose antiangiogenic metronomic therapy of hepatocellular carcinoma, the residual population identified in the liver parenchyma were CSCs that remained dormant after treatment and is responsible for the tumor regrowth (Martin-Padura et al. 2012). Since such anti-angiogenic strategies could induce tumor hypoxia, one can speculate that the emergence of a dormant CSC subpopulation could be a result of hypoxia-induced shift in the tumor cell phenotype. The existence of such plastic states could pose both obstacles and opportunities. For instance, activating dormant tumor cells prior to treatment could sensitize them to conventional therapy and favor the eradication of minimal residual disease. An alternative strategy would be to keep these cells long-term in a dormant state, which could prevent relapses and potentially convert cancer into an asymptomatic-chronic condition.

Therefore, the successful eradication of cancer requires a better understanding of the mechanisms that regulate the onset of dormancy and dormant cell drug-resistance. Given the significant overlap between CSCs and dormant tumor fractions and the crucial role of hypoxic niche in governing the CSC fate and dormancy maintenance, unraveling the molecular complexities that dictate dormant state will lead to a better understanding of tumor progression in cancer patients with important implications for improved diagnosis and treatment.

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