Published in final edited form as:

Curr Osteoporos Rep. 2017 August ; 15(4): 231–238. doi:10.1007/s11914-017-0378-8.

Hypoxia and Bone Metastatic Disease

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Abstract

Purpose of review—This review highlights our current knowledge of oxygen tensions in the bone marrow, and how low oxygen tensions (hypoxia) regulate tumor metastasis to and colonization of the bone marrow.

Recent findings—The bone marrow is a relatively hypoxic microenvironment, but oxygen tensions fluctuate throughout the marrow cavity and across the endosteal and periosteal surfaces. Recent advances in imaging have made it possible to better characterize these fluctuations in bone oxygenation, but technical challenges remain. We have compiled evidence from multiple groups that suggests that hypoxia or HIF signaling may induce spontaneous metastasis to the bone and promote tumor colonization of bone, in particular in the case of breast cancer dissemination to the bone marrow.

Summary—We are beginning to understand oxygenation patterns within the bone compartment and the role for hypoxia and HIF signaling in tumor cell dissemination to the bone marrow, but further studies are warranted.

Graphical abstract

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Conflict of Interest

Miranda Sowder, Rachelle Johnson, and Amato Giaccia declare no conflicts of interests.

Human and Animal Rights

All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

Keywords

Hypoxia; bone; breast cancer; colonization; metastasis

I. Introduction

Hypoxia (low oxygen tensions) is a common feature in solid tumors. The increased proliferation rate of cancer cells results in high tumor cellularity and increased oxygen consumption, but since blood flow is frequently compromised in solid tumors, this results in intratumoral regions in which oxygen tensions are quite low. These fluctuations in oxygen arise from the leaky and abnormal vasculature that is commonly found in large solid tumors [1], including breast. Instability in the oxygen gradient complicates both the signaling and biology of large solid tumors, as well as drug delivery aimed at tumor eradication. Indeed, induction of tumor hypoxia or hypoxia inducible factor (HIF) signaling is clinically associated with a poor prognosis [2–6] and metastasis [7–11]. Breast tumor cells have a high predilection for homing to the bone marrow, where they encounter another hypoxic, but physiologically and mechanically different microenvironment: the skeleton [12].

When oxygen tensions are low, the hypoxia inducible signaling pathway is induced, beginning with inhibition of the prolyl hydroxylases (PHDs). Under normal oxygen (normoxic) conditions, PHDs hydroxylate hypoxia inducible factor 1 α (HIF1 α) and HIF2 α [13–15], which are the major downstream signaling mediators of hypoxia. Hydroxylation of HIF1 α and HIF2 α leads to polyubiquitination of the HIFs by von Hippel-Lindau (VHL) and subsequent degradation in the cytosol [16–20]. In hypoxic conditions, PHDs are inhibited, stabilizing HIF proteins and allowing their translocation into the nucleus, where they complex with the co-factor ARNT, bind to hypoxia responsive elements (HREs) and activate gene transcription [21–23]. HIF signaling affects a number of cellular processes, including glycolysis, angiogenesis, drug resistance, and many steps within the metastatic cascade [24].

When tumors metastasize to the bone marrow, they disrupt physiological bone remodeling and coupling between osteoblasts and osteoclasts through the release of cytokines (e.g. PTHrP, etc.) that stimulate RANKL production by osteoblasts. This enhances osteoblast^{RANKL}-osteoclast precursor^{RANK} signaling and results in increased osteoclastogenesis and resorption of the bone matrix and localized release of cytokines and growth factors (e.g. TGF- β). These factors stimulate tumor proliferation and further increase

production of osteolytic factors, and PTHrP in particular [25] (Figure 1A). Osteocytes (terminally differentiated osteoblasts) may also promote tumor-induced bone disease through RANKL production [26], although this contribution has not been specifically tested, and serve as mechanosensors of intraosseous pressure in the bone microenvironment to promote tumor growth [27]. Tumor cells may also enter a dormant, or latent phase through interactions with the bone microenvironment and emerge years to decades later as a clinically detectable bone metastasis [28].

II. Hypoxia in the bone marrow

Despite being a highly vascularized tissue, the bone is a particularly hypoxic environment. Oxygen tension in most normal tissues falls between 2% and 9% (14 – 65 mm Hg) [29], however, in the bone it is widely accepted that oxygen levels range from < 1% – 6% (~7 mm Hg – 43 mm Hg) [30–32]. Oxygen tension in the bone is likely determined by the level of cellularity and oxygen consumption rate in particular regions of the bone [31, 33]. Further, low oxygen availability in regions containing sinusoidal vasculature likely further dictates regional oxygen gradients as historical studies have predicted that sinusoidal blood flow is roughly one-tenth that of arteriole flow [34]. Local oxygen tension in the bone has been shown to play a critical role in coordinating osteogenesis and angiogenesis as well as in maintaining hematopoietic stem cells [35–39]. However, due to technical challenges the regional oxygen gradients in the bone remain largely unknown.

Advancements in tissue processing, immunostaining, and imaging techniques have recently allowed for the investigation of the bone vasculature at high resolution. Although the vasculature does not indicate absolute oxygenation, it can give valuable insight into the oxygen availability in a particular region of the bone. As previously proposed [40], arteries enter the bone through the cortex, run centrally along the diaphysis and branch into smaller arterial vessels near the endosteal surface [41, 35]. Here arterial vessels give rise to sinusoids that coalesce as a central sinus at the center of the diaphysis. Recently, two distinct subtypes of capillaries were identified: type H (CD31^{hi}/Emcn^{hi}) and type L (CD31^{lo}/Emcn^{lo}) vessels [35]. The metaphysis contains type H vessels which have direct arterial connections and are organized into densely packed vessel columns. In contrast, the diaphysis contains few, mainly unbranched arteries and L vessels, which form a dense, highly branched sinusoidal network that connects to the large central vein [35]. Although distinct subtypes, these vessels form a single vasculature network that interconnect at the interface between the metaphysis and diaphysis. Interestingly, oxygenated blood is exclusively delivered by arteries to type H vessels. Thus, arterial oxygen is preferentially fed into the metaphysis resulting in a relatively less hypoxic region compared to the diaphysis. Additional studies have supported these observations using hypoxia markers such as pimonidazole, HIF1 α , and HIF target proteins, MCT4 and Glut1 [36, 35]. These markers were abundant in the diaphysis, indicating low oxygenation, but not in the metaphysis. While these studies provide novel insights into the organization of the bone vasculature, regional oxygen delivery and gradients can currently only be assumed for these regions of the long bone.

Two-photon phosphorescence lifetime microscopy was recently used to directly measure oxygen tension in the calvaria of live mice [30]. The highest oxygen tension values were

obtained for the periosteum (~7% or 50 mm Hg) indicating a rather non-hypoxic environment. This level of oxygen is postulated to be due to the relatively low cellularity and resultant low oxygen consumption as compared to the high metabolic demand of the bone marrow. In accordance with previous studies [31, 32], the oxygen tension in the bone marrow ranged between ~ 1% and 4% (~7 mm Hg and 29 mm Hg). The endosteum has historically been described as a region of little vasculature and low oxygenation that serves as a supportive niche for hematopoietic stem cells [41, 42, 36]. In contrast to the endosteum being extremely hypoxic, direct measurement of oxygen tension revealed that pO₂ decreased with increasing distance from the endosteum towards the bone marrow [30]. More specifically, oxygen levels near the endosteum were ~1.8% (13.5 mm Hg) whereas oxygen levels in the deeper sinusoidal region were ~1.3% (9.9 mm Hg). Of note, these oxygen measurements were performed in the calvaria of mice. Previous comparison of the calvaria and femora revealed no major differences in blood vessel distribution, vessel size, or perfusion efficiency [43]. However, of potential importance, pimonidazole staining revealed that hypoxic cells are less frequent in the calvaria than the long bones. Therefore, it remains unclear whether the oxygen levels in the calvaria are comparable to those in the long bones.

Currently, the thickness of the cortical bone prevents investigation of the vasculature and oxygen tensions using high-resolution imaging. Therefore, mathematical modeling has been used to approximate the oxygen tension of the cortical bone to be around 4.2% (30 mm Hg) [44]. Similar to the periosteum, the oxygen level distribution in the cortical bone is postulated to be a result of low cellularity and oxygen consumption. Spencer et al. recently reported comparable oxygen levels of 4.8% (~35 mm Hg) in the cortical bone of the calvaria [30]. Trabecular bone forms a honeycomb-like network and is found throughout the metaphysis. Oxygen tensions inside the mature trabeculum are predicted to be 2.6%-2.4% (18.9-17 mm Hg moving from the surface of the trabeculum to the inner osteocyte layer) when trabeculae are located approximately 200µm from a capillary [44], as in the case of trabeculae in the sinusoidal regions. When oxygen is assumed to diffuse across the bone similar to water (same diffusion coefficient), oxygen levels are predicted to be even lower (2.4%-0.08% or 17-0.6 mm Hg moving from the surface of the trabeculum to the inner osteocyte layer). [44] Since both scenarios are predicted from mathematical modeling, the exact oxygen tension in the trabeculae remains unknown; however, both models suggest that osteocytes embedded in the core of the trabeculum may experience a hypoxic environment. Several studies have provided evidence that the low oxygen tensions in the trabeculum result in preferential homing of HSCs [45–47], however the absolute oxygen gradient is currently unclear.

Taken together, regional oxygen tensions in the bone are governed by the level of cellularity, oxygen consumption and supply of oxygenated blood (Figure 1B). It is important to note that the majority of studies thus far have investigated oxygen tensions via hypoxia markers such as pimonidazole, HIF1α, or HIF targets (MCT4 and Glut1). While these methods offer indirect estimations of hypoxia, direct measurements of oxygen tension in various regions of the bone remain technically challenging and are thus limited. Future studies using advanced imaging techniques *in vivo* are critical to our understanding of regional oxygen gradients and their biological importance.

III. Hypoxia in tumor metastasis to bone

Hypoxia and activation of HIF1 α and HIF2 α promote tumor progression and metastasis to a number of organs [48–51]; however, the impact of hypoxia on tumor metastasis to the bone specifically has not been well studied. Clinically, patients with a greater number of disseminated tumor cells in the bone marrow have 3-fold higher levels of HIF1 α in their primary tumors, with a corresponding down-regulation in VHL [52]. Although these studies were not functionally tested, it suggests that HIF1 α may promote tumor dissemination to bone through a VHL-mediated mechanism. It has also been shown that HIF1 α deletion in the mammary fat pad (using MMTV-Cre recombinase) reduces spontaneous lung metastases when crossed with the PyMT mouse [53], which spontaneously develops mammary carcinomas, but bone metastases were not evaluated in this study. Interestingly, the PyMT mice have been reported to harbor disseminated tumor cells in the bone marrow [54], and given the effect of HIF1 α deletion on lung metastases, this raises the question of whether HIF1 α deletion in the mammary fat pad may also prevent dissemination to the bone marrow.

Previous work by Hiraga et al. demonstrated that forced expression of a constitutively active HIF1 α construct in MDA-MB-231 bone metastatic breast cancer cells enhanced tumor cell bone colonization following intracardiac inoculation [55]. Accordingly, knockdown of HIF1 α in these cells reduced bone colonization, indicating that intracrine HIF signaling in breast tumor cells enhances their ability to extravasate and colonize the bone marrow. A similar effect was observed by Dunn et al. when HIF1 α was knocked down in MDA-MB-231 cells [56]. In this study, the effect of HIF1 α knockdown on tumor gene expression was similar to that of TGF- β signaling blockade through expression of a truncated TGF- β type II receptor. Genetic inhibition of either pathway reduced osteolytic bone destruction but there was no additive effect; however, blockade of both pathways with small molecular inhibition had an additive benefit in blocking tumor burden in bone and improving bone micro-architecture [56]. These data suggested that the effect of HIF1 α signaling in promoting tumor colonization of the bone marrow may be in part through effects on the bone microenvironment. While these studies have provided important information on the role for HIF1 α in tumor extravasation and colonization into the bone marrow, the limitations of our models in the field do not address whether HIF1 α at the tumor origin controls spontaneous metastasis to bone (Table 1).

Hypoxia has also been linked to tumor initiating ability of multiple myeloma (MM) cells. Muz et al. showed that incubating MM cells in hypoxic conditions (1% pO₂ for 24 hours) prior to tail vein injection resulted in enhanced tumor initiation as compared to cells cultured in normoxic conditions [57]. At 5 weeks post tumor cell injection, MM cells pre-conditioned in hypoxia led to increased tumor burden compared to normoxic conditioned cells. Previous work by this group attributes these results to more efficient homing of hypoxic MM cells to the bone marrow via a HIF1 α and CXCR4-dependent mechanism [58]. Recently, optical-resolution photoacoustic microscopy was used to show that oxygen saturation (sO₂) in regions of the cerebral bone marrow harboring MM cells was significantly lower than non-tumor affected regions [59]. Further, at 4 weeks post tumor cell injection, sO₂ levels were decreased by 50% in tumor bearing regions compared to the initial sO₂ levels. As the authors indicate, these results suggest that the proliferation of MM cells promotes the

development of hypoxia; however, it remains unclear whether the mechanism is due to higher cellularity and increased metabolic demands or due to signaling alterations in the microenvironment. These data are consistent with our unpublished observations that tumor-bearing bone marrow stains stronger for the hypoxia probe pimonidazole than naïve bone marrow.

HIF signaling in breast cancer cells also promotes lysyl oxidase (LOX) production, and expression of LOX in 4T1 mouse mammary tumor cells promotes tumor-induced osteolysis in spontaneous bone metastases and following intracardiac injection through enhanced osteoclastogenesis and remodeling of the pre-metastatic niche [60]. These results were confirmed in a colorectal cancer cell line, suggesting the effects of HIF-induced LOX in promoting bone metastasis may not be tumor type specific. There is, however, a remarkable lack of data in determining whether HIF activation converts a less aggressive tumor cell (e.g. one with low metastatic potential) to a more aggressive tumor cell (e.g. one with high metastatic potential), since all of the studies of HIF in bone metastases have relied on more aggressive tumor models. Similarly, it remains unclear whether activation of HIF1 α or HIF2 α in the primary tumor site is able to promote bone metastasis through mechanisms that are independent of LOX production.

IV. Hypoxia in tumor cell dormancy

HIF drives urokinase-type plasminogen activator receptor (u-PAR) gene expression (encoded by the gene PLAUR) [61, 62] and u-PAR expression is correlated with an increase in DTCs in the bone marrow [63–65]. Loss of u-PAR converts human epidermoid carcinoma cells (HEp3) from tumorigenic to a dormant state [66, 67], thus consistent for a role in which hypoxia stimulates tumor cells to exit a dormant state through modulation of the ECM. In support of this hypoxia-driven exit from dormancy, our lab has found that the leukemia inhibitory factor receptor (LIFR) maintains breast tumor cells in a dormant state in the bone marrow and that hypoxia ($pO_2 < 0.5\%$) down-regulates the LIFR [68]. This is consistent with our unpublished observations that multiple ECM components are differentially regulated downstream of the LIFR in breast cancer cells.

When LIFR signaling was knocked down in breast cancer cells, this converted the cells from a quiescent state to a proliferative, aggressive phenotype [68], and a number of pro-dormancy genes were down-regulated. Since the bone marrow is generally hypoxic [30–32], we propose that when tumor cells end up in a strongly hypoxic region, this may trigger down-regulation of the LIFR and an exit from dormancy. Unfortunately our current technologies do not allow us to directly test this hypothesis in the bone marrow. Interestingly, blockade of STAT3 signaling in tumor cells pheno-copied loss of LIFR *in vivo* [68], suggesting that the effects of LIFR on dormancy may be mediated through STAT3 signaling. Paradoxically, STAT3 has been thought of as an oncogene [69, 70] and has been found activated in human breast tumor samples [71, 72], but if STAT3 also maintains disseminated tumor cells in a dormant tumor state, this may account for the failure of STAT3 inhibitors to improve patient outcome in clinical trials [73].

In contrast to its role in promoting metastasis, it has been proposed for some time that hypoxia may in fact *induce* long-term dormancy at the tumor origin [74]. Recent work from the Aguirre-Ghiso lab indicates that tumor hypoxia, using a desferrioxamine-loaded fabricated NANOVID system for hypoxia, selects for a sub-population of “post-hypoxic” DTCs in head and neck squamous cell carcinoma (HNSCC) and breast cancer that induce an NR2F1 dormancy program, are capable of evading chemotherapy, and may therefore give rise to distant metastases and patient recurrence [75]. This evidence for hypoxia inducing tumor dormancy is particularly intriguing because this indicates a dual role for hypoxia in dormancy, in which tumor cells at the origin may be induced into a dormant state in an NR2F1-dependent manner [75], and driven out of dormancy in the bone marrow through down-regulation of the LIFR and a number of pro-dormancy genes [68]. This study did not investigate whether these post-hypoxic DTCs would give rise to bone metastases specifically; however, prior work has shown that NR2F1 promotes dormancy in lung DTCs, but inhibits dormancy in bone DTCs [76]. Thus, this may be an example of tissue specificity for dormancy programs.

Interestingly, many of the factors that promote dormancy have been found to be highly tissue specific, as in the case of LIFR for breast cancer (bone but not lung) [68], thrombospondin1 for breast cancer (TSP1; lung and bone) [77], BMP4 for breast cancer (lung but not bone or brain) [78], and TGF- β 2 for HNSCC (bone but not lung) [79]. Tissue specificity in tumor dormancy was recently reviewed and nicely summarized by Dasgupta et al. [80]. While there appears to be little overlap in dormancy regulators across tissue types, it is unclear why these tissue differences exist. We can speculate that this is likely due to differences in the microenvironment, but whether these are due to stresses such as oxygen tensions, mechanical signaling, or differences in the ECM remains to be determined.

V. Conclusions

Although the current understanding of regional oxygen gradients in the bone is limited, it is well accepted that the bone is a generally hypoxic environment. The studies described herein suggest a role of tumor and micro-environmental HIF signaling in promoting breast tumor cell dissemination to the bone. Hypoxia has been shown to stimulate tumor cells to exit a dormant state in the bone marrow and exhibit an aggressive phenotype; however, the role of hypoxia and HIF activation at the tumor origin in promoting tumor metastasis to bone remains less clear. It also remains unclear precisely how hypoxia in the bone marrow may temporally and spatially influence tumor dissemination to bone. Thus, further investigation of hypoxia in both the primary and secondary sites is necessary in order to further our understanding of hypoxic signaling in promoting tumor cell metastasis and dormancy in the bone.

Acknowledgments

The authors wish to acknowledge Ms. Lauren Holtslander for Figure 1 graphic design. This manuscript was supported by R00CA194198 (RWJ) and CA67166, CA198291, and CA197713 (AJG).

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•Of importance

••Of major importance

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Figure 1. Tumor-induced bone destruction and the hypoxic bone marrow microenvironment

A: Breast tumor cells secrete factors (e.g. PTHrP, etc.) that stimulate osteoclastogenesis via osteoblast^{RANKL} expression. Growth factors are then released from the bone matrix and further promote tumor growth and secretion of osteolytic factors. **B:** The bone marrow is a naturally hypoxic microenvironment, with anatomical fluctuations in oxygen levels. Gray boxes = oxygen levels derived from calvarial measurements. White box= oxygen levels

derived from mathematical modeling of long bone measurements. * = estimated conversion to pO₂ from mm Hg.

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Table 1

HIF1 α promotes metastasis to the lung and colonization of the lungs, liver, and bone.

	Tumor Type	Spontaneous Metastasis	Colonization
Hypoxia or HIF1α-driven	Melanoma	Lung [46]	?
	Multiple myeloma		Bone marrow [57]
	Breast	Lung [50] Bone [54, *LOX]	Liver [47] Bone [52] [53] [62, *LIFR]
	Hypopharyngeal	?	Lung [45]

* Hypoxia-regulated factors that promote bone metastasis or colonization.