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HIF targets in bone remodeling and metastatic disease

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Abstract

The bone marrow is a hypoxic microenvironment that is rich in growth factors and blood vessels and is readily colonized by tumor cells disseminated from numerous cancers including tumors of the breast, prostate, lung, and skin. The origin of metastatic growth promoting factors for tumor cells disseminated to the bone marrow is derived from multiple sources: the bone matrix, which is a reservoir for growth factors, and cells residing in the marrow and along bone surfaces, such as osteoblasts, osteoclasts, macrophages, and T cells, which secrete cytokines and chemokines. Low oxygen levels within the bone marrow induce hypoxia signaling pathways such as hypoxia inducible factor (HIF), which is regulated by oxygen requiring prolyl hydroxylases (PHDs) and von Hippel–Lindau (VHL) tumor suppressor. These hypoxia signaling pathways have profound effects on bone development and homeostasis. Likewise, hypoxic conditions observed in local breast and prostate tumors point to a role for hypoxia-inducible genes in metastasis to and colonization of the bone marrow. This review will explore the role of hypoxia-regulated factors in bone development and remodeling, and how these elements may contribute to solid tumor metastasis to the bone.

Keywords

Hypoxia; HIF; PHD; Remodeling; Bone metastasis; Bone microenvironment

1. Introduction

The bone and its associated marrow offer a unique microenvironment to tumor cells with access to growth factors, cytokines, blood supply and tumor-supportive cells including macrophages, T cells and stromal cells. Osteoblasts (bone forming cells) and osteoclasts

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

(bone resorbing cells) play a pivotal role in skeletal development and remodeling, and osteoclasts in particular impact metastatic tumor cells in the bone marrow through resorption of the bone matrix and release of growth factors. Upon dissemination to the bone marrow, cancer cells secrete parathyroid hormone-related protein (PTHrP), which binds to the parathyroid hormone (PTH) type 1 receptor (PTH1R) on osteoblast lineage cells and stimulates production of receptor activator of NF κ -B (RANK) ligand (RANKL) by osteoblasts (Mundy, 1997; Sterling et al., 2011) (Fig. 1). RANKL binds to its receptor RANK on osteoclast precursors, promoting osteoclast maturation and resorption of the bone matrix. The bone matrix is a reservoir for many cytokines and growth factors, and harbors the largest pool of latent TGF- β 1 in the body (Bonewald & Mundy, 1990). Osteoclast resorption of the bone matrix releases TGF- β in its active form and provides a paracrine signal to tumor cells to increase PTHrP production and proliferate, further increasing osteoclast-mediated bone resorption and release of TGF- β (Mundy, 1997; Sterling et al., 2011). This results in osteolytic bone destruction, which is common in breast and lung cancer bone metastasis, and observed less often in prostate cancer bone metastases. Many of the primary therapies for bone metastatic breast cancer target osteoclasts in an effort to block tumor cell proliferation and osteolytic bone destruction. Melanoma (Patten et al., 1990; Suva et al., 2011), thyroid (Coleman, 2006), breast (Abrams, 1950; Shirazi et al., 1974; Boxer et al., 1989; Coleman, 2006), prostate (Coleman, 2006), and lung (Coleman, 2006; Katakami et al., 2014; Riihimaki et al., 2014) tumors have the highest predilection of solid tumors for the skeleton (Table 1), and renal cell carcinoma (RCC) metastasizes to bone at a frequency of up to 35% (Coleman, 2006; Santini et al., 2013). RCC patients with bone metastases or lung metastases also have a poorer prognosis than patients without bone metastases (McKay et al., 2014; Park et al., 2012). A recent report indicates that metastatic or recurrent gastric cancer also metastasizes to bone at an approximate frequency of 10% (Kim et al., 2014) and was prognostic of significantly shorter overall survival.

The structure of the long bones is such that the proximal and distal ends of the bone (metaphyses) are wider than the mid-shaft (diaphysis) of the bone, and there is a high degree of vascularization in the distal femoral metaphysis and proximal tibial metaphysis. The bone marrow microenvironment harbors strongly hypoxic regions (Parmar et al., 2007; Rankin et al., 2011) and a hypoxia gradient that increases with distance from the metaphysis (Parmar et al., 2007; Kusumbe et al., 2014). This is not surprising given the extent of vascularization present in the metaphyseal region. The diaphysis, in contrast, contains few blood vessels and thus is characterized by immunostaining for nuclear hypoxia inducible factor-1 α (HIF-1 α) staining and HIF target proteins including MCT4 and Glut1 (Kusumbe et al., 2014). The effect of oxygen tension on vascularity (and lack thereof with regards to the diaphysis) is directly linked to osteogenesis and angiogenesis and the mechanisms that couple these processes.

The hypoxia inducible factor (HIF) proteins are the major mediators of the cellular response to low-oxygen, or hypoxic conditions. In normal oxygen conditions (normoxia), HIF-1 α and HIF-2 α are hydroxylated by a family of prolyl hydroxylases (PHD), consisting of PHD1, -2, and -3. HIF α hydroxylation provides a scaffold for von Hippel-Lindau (VHL) tumor suppressor protein, which results in polyubiquitination and subsequent degradation of the HIF proteins (Huang et al., 1998; Maxwell et al., 1999; Hon et al., 2002). Under hypoxic

conditions, PHD activity decreases and the alpha subunits of HIF proteins accumulate, translocate to the nucleus, and bind to hypoxia-responsive elements (HREs) on HIF target genes (Fine & Norman, 2002). Transcriptional activation of hypoxia-responsive genes impacts a number of cell processes that drive tumor cell progression, including angiogenesis and glycolysis.

Oxygen gradients fluctuate in solid tumors, meaning specific regions of the tumor may rapidly transition between high and low oxygen levels (Dewhirst et al., 2008) and may not display uniform oxygen tensions. Mechanistically, this is a consequence of leaky and atypical vasculature that is characteristic of large solid tumors (Shah-Yukich & Nelson, 1988). Hypoxia is associated with poor patient prognosis (Vaupel & Hockel, 2001; Brown & Wilson, 2004; Toustrup et al., 2012) and HIF-1 α is increased approximately 3-fold in breast cancer patients who are positive for disseminated tumor cells in bone marrow aspirates (Woelfle et al., 2003), suggesting that the hypoxic conditions the tumor cells encounter in the primary tumor site may promote tumor cell metastasis to bone, and that the hypoxic microenvironment of the bone marrow may promote subsequent bone colonization. This review will discuss the role of HIF and its molecular regulators in bone homeostasis and hypoxic microenvironments with an emphasis on how these processes may contribute to tumor metastasis to and growth in bone. As in other tumor microenvironments, vascularization is pivotal to enable tumor colonization, and the same molecular mediators (VHL, HIF-1 α , VEGF) drive these critical processes. Bone remodeling and bone development in particular are in large part fueled by the spatial and temporal regulation of hypoxia-driven factors that promote vascularization, and these key physiological factors are emerging as key players in tumor metastasis to bone and tumor–bone bi-directional signaling.

2. HIF, PHDs, and VHL as mediators of bone development and remodeling

Bone can form through two different mechanisms, intramembranous or endochondral ossification. While the flat bones of the skull develop from mesenchymal cells that directly differentiate into osteoblasts (intramembranous bone formation), the other skeletal elements derive from a chondrocyte anlage that is replaced by bone (Karsenty, 2003; Kronenberg, 2003; Lefebvre & Smits, 2005; Provot & Schipani, 2005). This latter process is called endochondral bone development. During endochondral bone development, mesenchymal cells first condense; cells within these condensations next differentiate into chondrocytes and generate the fetal growth plate. Growth plate chondrocytes are highly proliferative, and while they divide, they also pile up to form a columnar layer. The most distal cells of the columnar layer stop proliferating, exit the cell cycle, and differentiate into hypertrophic chondrocytes, which mineralize their surrounding matrix. The cartilaginous mold is then invaded by blood vessels and replaced by bone at two sites, the primary spongiosa and the secondary ossification center, respectively (Gerber et al., 1999; Kronenberg, 2003; Zelzer & Olsen, 2005).

The hypoxia signaling pathway is critical for endochondral bone development. The fetal growth plate is a unique mesenchymal tissue because it is avascular, albeit it requires the angiogenic switch in order to be replaced by bone (Maes et al., 2012b). Consistent with its

avascularity, the fetal growth plate has an inner hypoxic region (Schipani et al., 2001; Provot & Schipani, 2007; Maes et al., 2012a). Genetic experiments have shown that HIF-1 α is a survival factor for these hypoxic chondrocytes in vivo, as its deficiency causes massive inner cell death in the inner hypoxic region of the fetal growth plate (Maes et al., 2012a; Provot & Schipani, 2007; Schipani et al., 2001). Moreover, mesenchymal condensations in the limb bud are also hypoxic, and loss of HIF-1 α in limb bud mesenchymal cells delays their differentiation into chondrocytes in vivo (Provot & Schipani, 2007). Of note, in contrast to what has been reported by other investigators (Robins et al., 2005; Amarilio et al., 2007), in our system we found no clear evidence that the delayed chondrogenesis of HIF-1 α null limb buds is associated with impaired expression of SOX9 (Provot & Schipani, 2007), a master transcription factor of chondrogenesis (Akiyama & Lefebvre, 2011).

Vascular endothelial growth factor-A (VEGF) is a classical target of HIF-1 α and HIF-2 α and it is expressed not only in hypertrophic chondrocytes, where it regulates the angiogenic switch and the replacement of cartilage by bone, but also in the inner hypoxic zone of the fetal growth plate, albeit at low levels (Pfander et al., 2004; Zelzer et al., 2004). Conditional knockout of VEGF in murine growth plates results in a cell death phenotype that closely mimics what was observed in HIF-1 α null developing cartilage (Zelzer et al., 2004; Maes et al., 2012a). Moreover, expression of VEGF in proliferating chondrocytes is required to ensure an adequate O₂ supply to cartilage, which is achieved by induction of angiogenesis in the perichondrium (Maes et al., 2012a). Hence, the survival-promoting functions of HIF-1 α in hypoxic chondrocytes could potentially be mediated, fully or in part, through its downstream target VEGF (Maes et al., 2012b). However, testing of this hypothesis revealed that the lethal effect of HIF-1 α knockout in cartilage could only be partially rescued by transgenic expression of VEGF, which implied the involvement of VEGF-independent cell autonomous mechanisms (Maes et al., 2012a). In particular, overexpression of VEGF in chondrocytes fully prevented the inner cell death and the increased hypoxia of VEGF null chondrocytes, most likely by increasing the number of blood vessels in the perichondrium (Maes et al., 2012a). However, it only marginally corrected the inner cell death of HIF-1 α null growth plates and their extreme hypoxia, despite a large increase in the number of blood vessels in the perichondrium and in the surrounding soft tissue (Maes et al., 2012a). These findings indicate that VEGF is unlikely to be the key player downstream of HIF-1 α as a survival factor in the developing growth plate, as other factors are likely to be involved.

Distinct from HIF-1 α , HIF-2 α is dispensable for growth plate development (Araldi et al., 2011). Moreover, conditional knockout of both HIF-1 α and HIF-2 α in limb bud mesenchyme generates growth plate abnormalities that are identical to the ones observed in specimens lacking only HIF-1 α (Schipani, personal communication).

In summary, at this stage we know the following: 1) mesenchymal condensations of the limb bud and fetal growth plates have an inner hypoxic region; 2) HIF-1 α is an early differentiation factor for cells of the limb bud mesenchyme, and a survival factor for chondrocytes; 3) VEGF is not the key mediator of HIF-1 α survival and/or differentiation functions in the developing growth plate; 5) HIF-2 α is not necessary, and it does not compensate for HIF-1 α in endochondral bone development.

A role for VHL, the E3 ubiquitin ligase that targets HIFs to the proteasome, has also been reported. In particular, loss of VHL in mesenchymal progenitors of the limb bud or in chondrocytes (where VHL deletion leads to severe dwarfism) considerably alters the size and shape of skeletal elements via mechanisms that could involve regulation of the unfolded protein response (Pfander et al., 2004; Mangiavini et al., 2014).

Notably, it has also been shown that hypoxia signaling pathways significantly modulate cartilage regeneration in vitro (Khan et al., 2007; Kanichai et al., 2008; Merceron et al., 2010). More recently, an association between HIF-1 α and cartilaginous tumors in humans has been proposed (Visser et al., 2011). In addition, it has been suggested that HIF-2 α is involved in the pathogenesis of osteoarthritis in humans (Saito et al., 2010; Maes et al., 2012b).

In light of the findings summarized above, identification of the molecular mechanisms that mediate the essential and non-redundant role of the hypoxia signaling pathways, and in particular of HIFs, in cartilage development could open new avenues for the treatment of cartilage diseases, and lead to novel approaches for the field of cartilage regeneration.

Despite its high degree of vascularization, a gradient of oxygenation is present in the bone marrow. The endosteal surface of cortical bone has been reported among the most hypoxic areas as revealed by staining with the marker of hypoxia pimonidazole (Parmar et al., 2007; Winkler et al., 2010; Rankin et al., 2011). The high degree of bone marrow cellularity, the high levels of O₂ consumption by leukocytes as well as the sluggish blood flow in the sinusoids are all thought to be contributing factors to the generation of a gradient of oxygenation within the bone marrow (Chow et al., 2001; Rankin et al., 2011).

Loss of VHL in fully differentiated osteoblasts, resulting in the stabilization of HIF-1 α and HIF-2 α and increased activity of HIF signaling in these cells, leads to a high bone mass phenotype in the bones that is formed through the endochondral replacement process (Wang et al., 2007; Rankin et al., 2011). Strikingly, this increased accumulation of trabecular bone in the long bones is associated with an augmented surface and volume of blood vessels in the bone marrow cavity (Wang et al., 2007; Rankin et al., 2012). Therefore, activation of the HIF signaling pathway in osteoblasts is sufficient to modulate bone marrow angiogenesis. More recently, these findings were confirmed and expanded in another mouse model lacking VHL in osteoprogenitors. Loss of VHL in osteoprogenitors (osterix-expressing, OSX-VHL) resulted in dramatically greater trabecular bone mass (Rankin et al., 2012). In particular, OSX-VHL mutant mice exhibited excessive accumulation of trabecular bone in the metaphyseal and diaphyseal regions of the long bones (Rankin et al., 2012). Surrounding the numerous trabeculae were abundant stromal cells and strikingly dilated blood vessels (angiectasis) (Rankin et al., 2012). Loss of HIF-1 α and HIF-2 α in VHL-deficient osteoprogenitors fully corrects the high bone mass phenotype, the increased number of hematopoietic stem cells (HSCs), the angiectasis and the aberrant expansion of the bone marrow stroma secondary to loss of VHL (Rankin et al., 2012). Of note, HIF activity in bone marrow endothelial cells is also an important modulator of bone mass and bone homeostasis (Ramasamy et al., 2014).

These findings highlight the critical role of the HIF signaling pathway in regulating both the osteoblastic niche and the vascular niche. It will now be important to identify the molecular mechanisms that mediate osteoblast-blood vessel cross talk, and to define whether increased angiogenesis is a necessary pre-requisite for the high bone mass phenotype observed upon activation of HIFs in cells of the osteoblast lineage.

Some progress has been made toward this end in showing that a particular CD31 and endomucin double-positive blood vessel subtype couples angiogenesis and osteogenesis (Kusumbe et al., 2014; Ramasamy et al., 2014; Xie et al., 2014). Moreover, it has been reported that conditional deletion of PDGF-BB under the control of tartrate-resistant acid phosphatase (TRAP)-Cre resulted in lower trabecular bone volume and cortical thickness and reduced bone formation rate on the endocortical and periosteal surfaces (Xie et al., 2014).

3. Hypoxic microenvironments and their impact on tumor metastasis and bone colonization

It is well established that both solid tumors and the bone microenvironment harbor regions of hypoxia, but how do hypoxic conditions in the primary tumor impact, if at all, metastasis to the bone? Breast cancer patients who harbor disseminated tumor cells in the bone marrow have 3-fold higher levels of HIF-1 α at the primary tumor site (Woelfle et al., 2003). This study also reported that VHL and cullin-2, a scaffold protein for E3 ligases, are down-regulated in the primary tumor of these patients, suggesting that induction of HIF-1 α in the primary tumor promotes tumor intravasation and dissemination to the bone marrow in a VHL-dependent manner; however, these data have not been reported elsewhere and need to be further validated. Dominant negative (DN)-HIF-1 α in MDA-MB-231 cells markedly reduces tumor homing to bone following intracardiac injection, and constitutively active (CA)-HIF-1 α results in greater tumor burden in bone (Hiraga et al., 2007). Thus it is possible that HIF-1 α induces tumor cell autonomous effects that promote bone homing. In these same studies, exposure of C3H10T1/2 mouse embryonic fibroblasts and mouse primary calvarial osteoblasts to hypoxia or CA-HIF-1 α inhibited their differentiation, while promoting osteoclastogenesis. A study from our group reported that while hypoxia had no effect on osteoblast differentiation in vitro, anoxia (N0.5% oxygen) inhibited osteoblast differentiation (Salim et al., 2004). These in vitro studies are in direct contrast to more recently published in vivo data reporting that conditional deletion of VHL in the osteoblast lineage augments bone mass (Rankin et al., 2012) and over-expression of HIF-1 α in the osteoblast lineage results in greater trabecular bone volume and number of osteoblasts (Regan et al., 2014). Thus the physiological role of VHL and HIF-1 α is to promote osteoblast differentiation and bone accrual. The role of HIF-1 α , VHL, and the PHDs in osteoblasts and osteoclasts in the pathological setting, such as in tumor-induced osteolysis, is unknown.

Genetic models of mouse mammary carcinoma have provided some information on the impact of hypoxia inducible factors in breast cancer metastasis to bone. For example, disseminated tumor cells (DTCs) are detectable in the bone marrow of mouse mammary tumor virus (MMTV)-Polyoma Virus middle T antigen (PyMT) transgenic mice by 4–6

weeks of age as evidenced by anti-cytokeratin (CK) staining (Husemann et al., 2008). At this age, the mammary tissue of MMTV-PyMT mice exhibit atypical ductal hyperplasia (ADH) or ductal carcinoma in situ (DCIS), suggesting that dissemination of breast cancer cells begins early in the progression of the tumor. This is notable because at these early stages the oxygen levels within the breast are similar to normal breast tissue, and thus reduced oxygen levels within the breast may not be necessary for tumor dissemination to the bone. It remains possible that a reduction in oxygen tension in later stages of tumor progression may promote dissemination, and this fits with the increase in HIF-1 α observed in mice and patients as their tumors progress (Bos et al., 2001; Schwab et al., 2012).

HIF-1 α specifically promotes tumor growth of MMTV-PyMT-derived tumor cells implanted into the mammary fat pad (MFP) and enhances the tumor-initiating activity of breast cancer cells (Schwab et al., 2012). Mammary epithelial tumor cells derived from Cre-activated HIF-1 α floxed MMTV-PyMT mice produce smaller and slower-growing xenograft tumors in the mammary fat pad and these mice have increased survival in a lung metastasis model. Tumor initiating activity of HIF-1 α floxed tumor cells was significantly reduced in an in vitro spheroid assay and in vivo with limiting dilutions of tumor cells. HIF-1 α is thus required for full growth potential of breast tumors at the primary site. Interestingly, hypoxia induces PTHrP secretion and gene expression in prostate, breast, and colon cancer cells (Manisterski et al., 2010), and PTHrP is a critical driver of osteolytic bone destruction by disseminated breast cancer cells (Mundy, 1997; Sterling et al., 2011). Chromatin binding studies showed that both HIF-1 α and HIF-2 α bind to the PTHrP P2 promoter, but that HIF-2 α alone drives PTHrP transcription. Thus a model was proposed in which HIF-1 α and HIF-2 α compete for the promoter binding site in acute hypoxia, but in chronic exposure, where HIF-2 α preferentially accumulates and HIF-1 α is degraded by the proteasome, HIF-2 α binds to the PTHrP promoter driving its transcription (Fig. 2). It would be challenging to test this in vivo, but these in vitro experiments provide valuable insight into hypoxic regulation of a key driver of bone destruction. Disseminated tumor cells experience chronic hypoxia in the bone marrow, which may specifically induce PTHrP secretion by the tumor cells via HIF-2 α accumulation and nuclear translocation. A long-standing question in the bone metastasis field is at what point in the metastatic cascade is PTHrP expressed and secreted by metastatic tumor cells. Is PTHrP induced prior to extravasation, during blood circulation, or post-dissemination? These studies provide evidence for the latter.

Building on the observation that PyMT mice harbor circulating and disseminated tumor cells (Husemann et al., 2008) and that HIF-1 α promotes PyMT mammary tumor growth (Schwab et al., 2012), it is reasonable to propose that deletion of the VHL tumor suppressor, which negatively regulates HIF-1 α , may result in mouse mammary tumor formation and perhaps even tumor cell dissemination to the bone marrow. Interestingly, VHL knockout mice have significant impairment of alveologenesis but deletion of VHL is not sufficient to induce mammary tumorigenesis (Seagroves et al., 2010). Normal ductal branching occurs in mammary-specific VHL^{-/-} mice but alveoli in pregnant VHL^{-/-} mice are undifferentiated, altering their secretory function and resulting in failure to lactate. Interestingly, these mice exhibited no signs of hyperplasia or palpable tumors out to 24 months of age. Mild dysplasia was reported during pregnancy, but deletion of the VHL tumor suppressor, which stabilizes

HIF-1 α , is not sufficient to induce tumorigenesis. Thus HIFs alone may also be insufficient to initiate tumor formation.

Interestingly, co-deletion of HIF-1 α in VHL^{-/-} mice was naturally proposed to rescue the alveologenesis phenotype, but instead caused a further reduction in alveoli (Seagroves et al., 2010). Thus there are other factors regulated by VHL that must contribute to mammary gland pathology. In an independent study, deletion of HIF-1 α slowed breast tumor growth in vivo (Schwab et al., 2012), which fits with the published data showing that HIF-1 α staining in breast cancer biopsies is a poor prognostic factor for patients (Yamamoto et al., 2008). Deletion of VHL stabilizes both HIF-1 α and HIF-2 α , which have been proposed to compete for binding to the PTHrP promoter (Manisterski et al., 2010). Therefore it is possible that genetic deletion of VHL reduces transcription of select HIF-1 α target genes that impact tumor progression and dissemination due to competition with HIF-2 α .

Another mechanism by which VHL may impact bone metastasis is through negative regulation of the chemokine receptor C-X-C motif receptor-4 (CXCR4) expressed on cancer cells (Staller et al., 2003). CXCR4 acts as the receptor for stromal-derived factor-1 (SDF-1) (Bleul et al., 1996; Oberlin et al., 1996) and is a key driver of breast cancer cell homing (Muller et al., 2001). CXCR4 is a target of HIF-1 α and in normoxic conditions VHL negatively regulates CXCR4 by degradation of HIF-1 α . In hypoxic conditions HIF-1 α activates CXCR4 transcription. SDF-1 is abundantly expressed in the bone marrow (Dar et al., 2006; Gelmini et al., 2008) and CXCR4 is a predictive marker for bone metastasis in breast cancer patients with visceral metastases and no evidence of disease (Ibrahim et al., 2011). CXCR4 is also a target of BACH1, which was identified as a master regulator of bone metastasis (Liang et al., 2012), and thus targeting VHL or HIF-1 α may also disrupt tumor cell homing to bone through downstream effects on CXCR4. There are chemokines other than CXCR4 that are regulated by hypoxia, and a number of these chemokines stimulate angiogenesis (Strieter et al., 1995). A particular Glu-Leu-Arg amino acid motif is commonly found in the N terminus of angiogenesis-promoting chemokines, such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8 (interleukin-8, IL-8) and may be important for their functionality (Strieter et al., 1995; Belperio et al., 2000). CXCL12, although it lacks this motif, also stimulates VEGF production, and VEGF stimulates CXCR4 production within the vascular tumor microenvironment (Salcedo et al., 1999; Guleng et al., 2005). Hypoxia stimulates IL-8 secretion and CXCR1 and CXCR2 receptor expression in HeLa cells (Liu et al., 2014), suggesting that the hypoxic bone marrow microenvironment may possess a greater number of up-regulated chemokines and receptors to act as chemoattractants for tumor cells. One of the effects of hypoxia on chemokines may simply be to drive organotropism.

4. Therapeutic potential of HIF targets in bone metastasis

4.1. Current therapies directed toward bone metastatic disease

The standard treatment for bone metastases resulting from breast, lung, and prostate cancer is bisphosphonate (BP) infusion every 3–4 weeks, which effectively blocks osteoclast activity (Rodan & Martin, 2000), or subcutaneous injection of denosumab every 4 weeks, a fully humanized monoclonal antibody against RANKL (Bekker et al., 2004). Both therapies

are also used for the treatment of low bone mass diseases such as osteoporosis (Table 2). In a standalone trial, denosumab significantly increased bone metastasis free survival and delayed the onset of bone metastases in non-metastatic prostate cancer patients (Smith et al., 2012), and in a later study this effect was shown to be most evident in patients with prostate specific antigen (PSA) doubling times of 8 months or less (2014; Smith et al., 2014). In direct comparison to BP infusion, denosumab was demonstrated to reduce the risk of developing symptomatic skeletal events in castrate resistant prostate cancer (CRPC) patients harboring bone metastases more effectively than intravenous zoledronic acid (Smith et al., 2015). Prior to this study, there had been three phase III trials that directly compared BPs and denosumab in cancer patients with bone metastasis (Stopeck et al., 2010; Fizazi et al., 2011; Henry et al., 2011). The first study concluded that denosumab was superior to zoledronic acid in delaying the time to a skeletal-related event (SRE) and subsequent SREs in patients with metastatic breast cancer, but the drugs had similar effects on overall survival and disease progression (Stopeck et al., 2010). The second study concluded that denosumab was similarly effective in delaying the time to a SRE in patients with bone metastases, excluding all bone metastatic breast and prostate cancer patients (Henry et al., 2011). Interestingly, this study showed that lung cancer patients performed similar regardless of treatment group except in patients with non-small cell lung carcinoma (NSCLC), who had a significant increase in median overall survival on denosumab compared to BPs (Henry et al., 2011). The third study concluded that denosumab was superior to BPs in delaying time to first SRE in bone metastatic prostate cancer patients (Fizazi et al., 2011). Importantly, in all of these studies the efficacy of denosumab over BPs is only evident in delaying time to an SRE. While this is of obvious clinical importance, only in a subset of lung cancer patients (NSCLC) in one clinical trial has denosumab been demonstrated to be more effective at improving *overall patient survival* over BPs. Of the five clinical trials that are ongoing/completed comparing denosumab and BPs (or combination treatment) in breast cancer patients with bone metastases, none of these trials lists overall survival (OS) or progression free survival (PFS) as a primary or secondary outcome measure, and the same holds true for the four ongoing/completed trials in prostate cancer patients with bone metastases (ClinicalTrials.gov). It is thus unlikely that future trials will find a survival benefit with denosumab treatment in bone metastasis patients.

There is still debate about whether BPs have a direct anti-tumor effect (Gnant & Clezardin, 2012), but recent data suggest that if there are any effects of BPs on the tumor beyond direct osteoclast action these are likely occurring through other cell types in the microenvironment. A recent study by Junankar et al. demonstrated that BPs are taken up by tumor-associated macrophages via the leaky vasculature characteristic of large breast tumors (Junankar et al., 2015). This effect is striking when viewed by real-time intravital imaging and fits well with data showing that BPs may exert anti-angiogenic effects in patients with bone metastatic breast cancer (Santini et al., 2002; Santini et al., 2003). In these studies circulating VEGF and PDGF levels were lower in patients after a single dose of the BP zoledronic acid or pamidronate and deemed to have potential anti-angiogenic properties in vivo. In fact the anti-angiogenic properties associated with BP treatment have been linked to the etiology of osteonecrosis of the jaw (ONJ), the most common side effect of BP treatment, and lower circulating VEGF levels are predictive of ONJ onset in patients receiving BP treatment

(Vincenzi et al., 2012). Similar to BP treatment, ONJ has been reported in patients receiving denosumab (Smith et al., 2012), and the overall risk of ONJ was found to be slightly (but not significantly) elevated in patients receiving denosumab versus BP treatment for bone metastatic breast and prostate cancer (Van den Wyngaert et al., 2011), but the direct effects of denosumab on circulating VEGF levels in these patients have not been evaluated. Interestingly, patients receiving anti-angiogenic therapies are also at increased risk of developing ONJ (Greuter et al., 2008; Aragon-Ching et al., 2009; Christodoulou et al., 2009), likely due to angiogenic and osteogenic coupling. ONJ is a significant side effect, but also has distinct risk factors, such as poor dental health, and thus is preventable with appropriate clinical screening for these risk factors prior to administration (Clarke, 2014). ONJ closely resembles phosphorous necrosis of the jaw (“fossy jaw”), which was common in matchstick factory workers in the 1800s (Purcell & Boyd, 2005), and has been eradicated as a result of regulatory practices and modern dental hygiene. The mechanism leading to fossy jaw was never identified, but is likely similar to the BP mechanism of action.

While BPs and denosumab effectively delay the onset of bone metastases in breast and prostate cancer patients (Stopeck et al., 2010; Fizazi et al., 2011; Henry et al., 2011; Gartrell et al., 2014), there are significant toxicities associated with these therapies, including fatigue, nausea/vomiting, and diarrhea (Table 2). In addition to increased risk of developing ONJ, hypocalcemia (Stopeck et al., 2010; Gartrell et al., 2014) is an observed side effect and is more frequently reported in patients receiving denosumab. Renal insufficiency is also reported in patients receiving BPs (Stopeck et al., 2010; Gartrell et al., 2014), and eczema and cellulitis have been reported in patients receiving denosumab (Dempster et al., 2012).

The pharmacokinetics of denosumab and BPs differ due to the method of delivery and mechanisms of action. BPs bind strongly to and selectively concentrate on the surface of bone and are absorbed by mature osteoclasts to block osteoclast activity through biochemical mechanisms (Pillai et al., 2004; Baron et al., 2011; Marathe et al., 2011), where they are then eliminated linearly (Pillai et al., 2004; Marathe et al., 2011). Thus BPs inhibit bone resorption, but do not completely abolish osteoclasts in the bone compartment. Conversely, denosumab is injected subcutaneously where it is taken up into the plasma, but similar to BPs is also eliminated linearly (Bekker et al., 2004). Denosumab blocks the RANK–RANKL interaction between osteoblasts and osteoclast precursors, preventing the formation of osteoclasts, as well as their function (Baron et al., 2011). Of note, the recovery time after treatment ceases is much shorter following denosumab, suggesting that any anti-angiogenic effects induced by denosumab treatment may be negated once the treatment regimen is ended. There is no evidence supporting sustained denosumab presence within the bone marrow, but immunohistochemical staining of human RANKL knock-in mice treated with denosumab shows distinct staining within the blood vessels of the marrow and cortex, as well as some residual staining of the bone marrow with no apparent pattern (Kostenuik et al., 2009). The half-life of denosumab is approximately 26 days (Bekker et al., 2004), but BPs exhibit a much longer half-life that is largely affected by the rate of bone remodeling (Lin, 1996; Rodan et al., 2004). Importantly, both of these drugs reach the bone compartment at concentrations capable of eliciting biochemical effects in patients (Reitsma et al., 1980; Bekker et al., 2004). An important hurdle for new therapies targeted to tumor cells in the bone is efficient delivery to the bone compartment, which as demonstrated by

subcutaneous injection of denosumab (Bekker et al., 2004), may be achievable with a stable therapeutic that is taken up into the serum and circulation.

With regards to BP and denosumab effects on hypoxia-regulated genes, the BPs clodronate and pamidronate promote the degradation of insulin-like growth factor-1 (IGF-1)-induced HIF-1 α protein levels in human breast cancer cells and inhibit VEGF secretion by abrogating the PI-3K/AKT/mTOR signaling pathway (Tang et al., 2010), but the effect, if any, of denosumab on HIF α or any of its downstream targets has yet to be explored.

4.2. Current therapies targeting HIF/HIF targets

Small molecule inhibitors and chemical compounds targeting the HIF target gene VEGF are widely used across cancer types for their anti-angiogenic properties (Abrams et al., 2003a,b; O'Farrell et al., 2003). Sunitinib, a small molecule inhibitor targeting VEGF receptor (VEGFR) and platelet-derived growth factor receptor β (PDGFR β), is more effective than interferon- α or sorafenib, a chemical kinase inhibitor that targets VEGFR and PDGFR β , at blocking the formation and time to occurrence of osteolytic lesions in bone metastatic renal cell carcinoma (RCC) patients (Zolnierak et al., 2010), likely due to the high extent of vascularization observed in RCC bone metastases. A retrospective study reported that the RCC patient long-term responders to sunitinib had lower odds of having a bone metastasis (Molina et al., 2013), and thus these patients already had a better prognosis and less aggressive disease. These data suggest that sunitinib may only be effective in patients that develop non-skeletal metastases. In a retrospective study of bone metastatic RCC patients taking sunitinib, BPs improved PFS and overall survival OS (Keizman et al., 2012), suggesting that combination therapy targeting angiogenic factors that promote tumor growth in the bone as well as the bone microenvironment is advantageous in the context of RCC. Similar data has been reported in a bone metastatic lung cancer mouse model in which treatment with sunitinib prior to intracardiac inoculation of lung cancer cells increased survival and prevented bone colonization (Catena et al., 2011); however, sunitinib had no effect on the formation of osteolytic lesions in bone unless combined with the BP zoledronic acid. Sunitinib use in triple-negative metastatic breast cancer, the sub-type with the highest predilection for skeletal homing, is currently in clinical trials (ClinicalTrials.gov), and pre-clinical mouse models suggest that sunitinib effectively inhibits breast cancer cell growth in bone, particularly in combination with localized radiotherapy (Zwolak et al., 2008).

Common side effects of sunitinib reported in patients with metastatic RCC are fatigue, diarrhea, dyspepsia, hypertension, nausea, anorexia, and mucosal inflammation, and severe side effects include neutropenia, increased lipase, fatigue, and thrombocytopenia (Motzer et al., 2006) (Table 2). Sunitinib is administered orally 4-weeks on/2-weeks off, but a subsequent clinical trial reported that a 2-week on/1-week off regimen is as effective as the conventional dosing schedule and elicits fewer side effects (Neri et al., 2013). Similarly, sorafenib is administered orally twice/day, and has a similar toxicity profile. In a clinical trial directly comparing sunitinib and sorafenib treatment 7.2% of patients receiving sunitinib reported severe skin toxicity compared with 22.7% of patients receiving sorafenib (Poprach et al., 2012). In patients receiving sorafenib only this skin toxicity was clinically associated with diarrhea. Thus resolving the diarrhea symptoms may relieve associated skin

toxicity in these patients. Unfortunately for patients treated with sunitinib, dermatologic toxicity is significantly correlated with OS and PFS; there is no significant correlation between dermatologic toxicity and OS or PFS in patients treated with sorafenib (Poprach et al., 2012). It will be important for the ongoing clinical trials of sunitinib and sorafenib in patients with advanced breast cancer to report dermatologic toxicity and any associated correlations with OS, PFS, and occurrence of bone metastasis. Since these drugs are orally administered there is considerable potential for low penetration of these drugs into the bone marrow. Studies to determine the efficacy of these drugs in the bone marrow will be critical for their use in patients with metastatic breast cancer, since up to 70% of metastatic breast cancer patients harbor bone metastases (Boxer et al., 1989).

5. Conclusion

Currently there is a drug dichotomy between classical cancer types associated with a high predilection for skeletal metastases (e.g. breast, prostate, and lung tumors) and those that metastasize to bone less frequently (e.g. RCC and colon cancer). The standard treatment for bone metastases associated with breast, prostate, and lung cancer are intravenous BPs or subcutaneous denosumab, but these drugs appear to be underutilized in RCC patients bearing bone metastases (Table 2). Likewise, breast, prostate and lung cancer are highly vascularized tumors that harbor hypoxic regions and disorganized vasculature, similar to RCC tumors. Sunitinib and sorafenib are currently in clinical trials (ClinicalTrials.gov) for their use in advanced breast cancer, and there is a proposed clinical trial suggesting that these drugs could impact breast cancer cell dissemination. These drugs in combination with BPs or denosumab may be of great relevance to breast, prostate, or lung cancer patients with bone metastatic disease, although the administration route of these drugs warrants further investigation of their ability to penetrate into the bone marrow compartment. Importantly, the toxicity profile of sunitinib and sorafenib in particular leaves much to be desired for patient quality of life. Thus novel therapeutic approaches for the treatment of breast, prostate, lung, melanoma and RCC bone metastatic tumors in particular should be explored alone and in combination with current anti-resorptive therapies.

Abbreviations

BP(s)	bisphosphonate(s)
CXCL1	-2, -3, -5, -6, -7, -8, -12, C-X-C motif ligand
CXCR1	-2, -4, C-X-C motif receptor
HIF	hypoxia inducible factor
HRE	hypoxia responsive element
MMTV	mouse mammary tumor virus
ONJ	osteonecrosis of the jaw
OS	overall survival
PDGF-BB	platelet-derived growth factor-BB

PDGFRβ	platelet-derived growth factor receptor β
PFS	progression free survival
PHD	prolyl hydroxylase
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related protein
PyMT	polyoma virus middle T antigen
RANK	receptor activator of NF κ B
RANKL	receptor activator of NF κ B ligand
RCC	renal cell carcinoma
SRE	skeletal-related event
TGF-β	transforming growth factor- β
VEGF	vascular endothelial growth factor
VHL	von Hippel–Lindau

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Fig. 1.

Mechanisms of tumor-induced bone destruction. Tumor cells disseminated to the bone marrow secrete parathyroid hormone-related protein (PTHrP), which binds to the PTH receptor type 1 (PTH1R) on cells of the osteoblast lineage. PTHrP:PTH1R binding increases receptor activator of NF κ -B (RANK) ligand (RANKL) expression on osteoblasts, which binds RANK on osteoclast precursors to drive osteoclast formation. Osteoclasts resorb the bone matrix releasing active transforming growth factor- β (TGF- β) from the matrix, which stimulates tumor cell proliferation and PTHrP production, thus creating a perpetual cycle of osteolysis.

Fig. 2.

PTHrP promoter regulation by HIF-1α and HIF-2α. The parathyroid hormone-related protein (PTHrP) gene has 3 alternative promoters (P1, P2, and P3) to induce transcription (Sellers et al., 2004). Hypoxia responsive elements (HREs) have been identified in the P2 promoter, but not the P1 or P3 promoters (Manisterski et al., 2010). In acute hypoxia conditions, a model is proposed where HIF-1α and HIF-2α compete for binding to HRE1 in the P2 promoter. When HIF-1α binds to HRE1 PTHrP transcription is inhibited. HIF-2α, but not HIF-1α binds HRE2 in the P2 promoter. Under chronic hypoxia conditions, HIF-1α is degraded and HIF-2α accumulates (Holmquist-Mengelbier et al., 2006; Luo et al., 2010), leading to PTHrP transcription.

Table 1

Incidence of bone metastasis by cancer type upon autopsy, except where indicated.

Cancer type	Incidence of bone metastasis
Melanoma	35% (Suva et al. (2011)) 17% (Patten et al. (1990))
Breast cancer	79% (Boxer et al. (1989)) 73% (Coleman (2006); Galasko, 1981) up to 70% (Shirazi et al. (1974); Abrams, 1950)
Prostate cancer	68% (Coleman (2006); Galasko, 1981)
Lung cancer	39% (Riihimaki et al. (2014)) adenocarcinoma, 25% SCLC [48% (Katakami et al. (2014)), NSCLC; 40%, SCLC] at initial diagnosis 36% (Coleman (2006); Galasko, 1981)
Renal cell carcinoma	22% (Santini et al. (2013)) 35% (Coleman (2006); Galasko, 1981)
Gastric cancer	10% (Kim et al. (2014)) 5% (Coleman (2006); Galasko, 1981)
Head and neck cancer	<1% (Bhandari, 2013), squamous cell carcinoma only 12% (León et al., 2000)
Thyroid	42% (Coleman (2006); Galasko, 1981)
Ovarian	0.1% (Suva et al. (2011))

Table 2

Therapies commonly used in the treatment of bone metastases associated with breast, prostate, and lung carcinomas (bisphosphonates and denosumab) and kinase inhibitors frequently used in metastatic cancer with a lower incidence of bone metastasis (sunitinib and sorafenib).

Therapy	Molecular mechanism	Administration route	Current oncological use	Common Side effects
Bisphosphonates (e.g. zoledronate, pamidronate)	Binds to bone and blocks osteoclast activity	Intravenous infusion every 3–4 weeks	Metastatic breast cancer, prostate cancer, lung cancer, multiple myeloma, metastatic renal cell carcinoma	Fatigue, fever, nausea/vomiting, anemia, bone/joint pain, ONJ (rare)
Denosumab (e.g. Xgeva)	RANK ligand (RANKL) monoclonal antibody blocks osteoclast formation and activity	Subcutaneous injection every 4 weeks	Metastatic breast cancer, prostate cancer, lung cancer, multiple myeloma	Nausea, diarrhea, fatigue, ONJ (rare)
Sunitinib (e.g. Sutent)	Small molecule tyrosine kinase inhibitor of VEGFR and PDGFR- β	Oral capsule daily or 4 weeks on/2 weeks off	Metastatic renal cell carcinoma, gastrointestinal stromal tumors, pancreatic neuroendocrine tumors	Diarrhea, nausea/vomiting, fatigue, constipation, loss of appetite/anorexia, hypertension, dermatologic toxicity
Sorafenib (e.g. Nexavar)	Chemical kinase inhibitor of VEGFR and PDGFR- β	Oral tablet twice/day	Metastatic renal cell carcinoma, liver cancer, thyroid cancer	Diarrhea, fatigue, dermatologic toxicity