Molecular Mechanisms of Breast Tumor Progression, Dormancy, and Bone Metastasis

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To my parents and sister for their unconditional love and unwavering support

And

To my husband and best friend for his love and belief in me as we walk this journey together.

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ABBREVIATIONS

ABCSG	Austrian Breast & Colorectal Study Group
ADP	Adenosine Diphosphate
Akt	RAC-alpha Serine/Threonine-Protein Kinase
AML	Acute Myeloid Leukemia
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BCAC	Breast Cancer Association Consortium
Bcl-2	B-cell Lymphoma 2
Bcl-XL	B-cell Lymphoma-Extra Large
BRD4	Bromodomain-Containing Protein 4
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CBP	CREB Binding Protein
CD11b	Integrin alpha M (cluster of differentiation molecule 11B)
CD298	Sodium/potassium-transporting ATPase Subunit Beta-3
CD4	T-Cell Surface Glycoprotein CD4 (cluster of differentiation 4)
CD8	T-Lymphocyte Differentiation Antigen T8/Leu-2 (cluster of differentiation 8)
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
Cre	Carbapenam-Resistant Enterobacteriaceae
CREB	cAMP Response Element Binding Protein
CSC	Cancer Stem Cell
CTLA4	Cytotoxic4 T-lymphocte Associated Protein
CXCL12	C-X-C Motif Chemokine Receptor 12
CXCR4	C-X-C Motif Chemokine Receptor 4
DAB	3-diaminobenzidine
DAB2	Disabled Homolog 2
DAB2IP	DAB2 Interacting protein
DAPI	4′,6-diamidino-2-phenylindole

DCIS	Ductal Carcinoma In Situ
DHS	Donkey Horse Serum
DMEM	Dulbecco's Modified Eagle's Media
DNA	Deoxyribonucleic Acid
DTC	Disseminated Tumor Cell
E2F8	E2F Transcription Factor 8
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
eIF3G	Eukaryotic Translation Initiation Factor 3G
eIF4E	Eukaryotic Translation Initiation Factor 4E
eIF4G1	Eukaryotic Translation Initiation Factor 4G1
EMT	Epitheltial to Mesencymal Transition
ER	Estrogen Receptor
ERa	Estrogen Receptor Alpha
ERB	Estrogen Receptor Beta
ERK	Extracellular Signal-Regulated Kinase
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF3	Fibroblast Growth Factor-3
Foxp3	Forkeahd Box P3 Protein
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
Gcn5	General control non-depressible 5 (GCN5)/ lysine acetyltransferase 2A (KAT2A)
GSEA	Gene Set Enrichment Analysis
GTP	Guanosine-5'-triphosphate
HA	Hemagglutinin
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HER2	Erb-B2 Receptor Tyrosine Kinase 2
HHM	humoral hypercalcemia of malignancy
HIF	Hypoxia Inducible Factor
HIF1-a	Hypoxia-Inducible Factor 1-Alpha
Hsp90	Heat Shock Protein 90
IACUC	Institutional Animal Care and Use Committee

IFNγ	Interferon Gamma
IL-1β	Interleukin-1 Beta
IL-2	Interleukin 2
JAK1	Janus Kinase 1
LIFR	Leukemia Inhibitory Factor Receptor
LoxP	Locus of Crossover in P1
MAPK	Mitogen Activated Protein Kinase
MAPK11	Mitogen Activated Protein Kinase 11
MAPK14	Mitogen Activated Protein Kinase 14
MDSC	Myeloid-derived Suppressor Cell
MM	Multuple Myeloma
MMTV	Mouse Mammary Tumor Virus
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamaycin
NAD	Nicotinamide Adenine Dinucleotide
$NF_{\kappa}B$	Nuclear Factor–Kappa B
NK	Natural Killer
NLS	Nuclear Localization Signal
OSM	Oncostatin M
P/S	Penicillin/ Streptomycin
PARP	Poly ADP Ribose Polymerase
PBS	Phosphate Buffered Saline
PCAF	P300/CBP-associated factor
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death 1 Ligand
PD-L2	Programmed Death 2 Ligand
PGE2	Prostaglandin E2
PIMO	Pimonidazole
PKA	Protein Kinase A
PMA	Phorbol-12-myristate-13-acetate
PTH	Parathyroid Hormone
PTH1R	PTH Receptor Type 1
PTHLH	Parathyroid Hormone Like Hormone
PTHrP	Parathyroid hormone-related protein

PyMT	Polyoma Middle T Antigen
RANKL	Receptor Activator of NFκB Ligand
RECIST	Response Evaluation Criteria in Solid Tumors
ROS	Reactive Oxygen Species
RRM2	Ribonucleotide Reductase Regulatory Subunit M2
SDS	Sodium Dodecyl Sulfate
Selenbp1	Selenium Binding Protein 1
shRNA	Small Hairpin RNA
SIRT	Sirtuin
SNAI2	Snail Family Transcriptional Repressor 2
SOCS3	Suppressor of cytokine signaling 3
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
TCF4	T-cell Factor 4
TGFβ2	Transforming Growth Factor-beta 2
TIBD	Tumor-Induced Bone Disease
TNBC	Triple Negative Breast Cancer
TPM1	Tropomyosin-1
TRAP	Tartrate Resistant Acid Phosphatase
uPAR	urokinase plasminogen activator receptor
VEGFA	Vascular Endothelial Growth Factor A

CHAPTER I

INTRODUCTION

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Overview

Breast cancer is the most diagnosed cancer among women in the United States [1]. Despite remarkable advances in the detection and treatment of the primary tumor that have dramatically improved patient outcomes, metastatic disease is a leading cause of morbidity and mortality [2, 3]. Indeed, breast cancer remains the second leading cause of cancer-related deaths in women in the United States, due to metastatic spread [1]. Disseminated tumor cells (DTCs) may home to distant organs and proliferate into a macrometastasis or enter a prolonged quiescent state before developing into a clinically detectable metastasis [4]. Dormant tumor cells may persist as quiescent solitary cells, or as micrometastases with limited growth capacity due to balanced proliferation and apoptosis [5-7]. Clinically, patients with estrogen receptor positive (ER+) breast tumors and no nodal involvement at diagnosis have an approximate 20% risk of developing distant metastases 5-20 years after primary diagnosis, suggesting prolonged periods before dormant DTCs resume proliferating [8].

The bone and lung are two of the most common sites of breast cancer metastasis, and approximately 70% of breast cancer and prostate cancer patients present with bone metastases upon autopsy [9]. Lung cancer, melanoma, renal cell carcinoma, and thyroid cancers also metastasize to bone with relatively high (>20%) frequency [9] and form osteolytic lesions [10]. Cancers that most commonly metastasize to the lung include colorectal, breast, head and neck,

urologic (renal, prostate) and osteosarcoma [11, 12]. Thus, therapeutically targeting tumor cells that have metastasized to the bone and lung, among other distant sites holds great clinical importance.

Patients with bone metastases may experience severe pain, impaired mobility, pathologic fractures, spinal cord compression and hypercalcemia [13]. Bisphosphonates and denosumab, inhibitors of osteoclast activity and bone resorption, are commonly utilized to manage metastasis-related symptoms and can prevent the development of bone metastases and improve survival, but only in select patient populations [14]. The Early Breast Cancer Trialists' Collaborative Group reported that adjuvant bisphosphonates reduce the rate of breast cancer recurrence in the bone and improve survival in women who were postmenopausal, but not premenopausal at the time of treatment initiation [15]. These findings have been confirmed by several follow-up studies [16-18]. A trial by the Austrian Breast & Colorectal Study Group (ABCSG) determined that adjuvant use of denosumab with an aromatase inhibitor in postmenopausal patients with hormone receptor-positive breast cancer improves disease-free survival [19]; however, the D-CARE study found no benefit of denosumab on breast cancer patient survival [20]. Currently, there is no cure or prevention for breast cancer metastasis. Thus, there is an urgent need to better understand the mechanisms that regulate DTC behavior and identify therapies that improve breast cancer patient survival, particularly for individuals with metastatic disease.

Histone Modifications in Breast Tumorigenesis

In the search for new therapeutic targets, genetic mutations in the nucleotide sequence that alter the expression of oncogenes and tumor suppressor genes have been investigated as potential drivers of tumorigenesis and metastasis. These studies have also uncovered an important role for epigenetic modifications in controlling the expression of genes that regulate the initiation and progression of cancer [21]. These epigenetic modifications are heritable changes in gene expression that do not alter the nucleotide sequence [22]. Among many other layers of epigenetic regulation of gene expression discussed thoroughly elsewhere [22, 23], chromatin remodeling accomplished by histone modifications is a central mechanism by which transcription machinery gains access to condensed genomic DNA. The human genome is compacted into chromatin, a DNA-protein complex comprised of nucleosomes containing ~147bp of DNA wrapped around an octamer of four core histone proteins (H2A, H2B, H3 and H4) [24]. Nucleosomes are linked together by histone H1 to form a chromosome. Histone tails are targets for posttranslational modifications (PTMs) including acetylation, methylation, phosphorylation, and ubiquitination. Together, these modifications comprise what is known as a "histone code" that

either directly regulates gene expression by altering the positioning of nucleosomes to dynamically switch chromatin between a closed versus open state or indirectly by recruiting other effector molecules that regulate transcription [24, 25]. Histone methylation and acetylation will be reviewed in greater detail below and discussions of the other histone PTMs have been published previously [26].

Histone methylation mostly occurs on the basic amino acids arginine and lysine, but histidine has also been reported to be methylated [27]. Lysines are commonly mono-, di- or trimethylated on their ε-amino group while arginines can be mono- or dimethylated. Though previously believed to be an irreversible modification, methyl groups have actually been found to be turned over more slowly than other PTMs [28]. Histone methyltransferases catalyze the transfer of methyl groups from *S*-adenosylmethionine to histones, while demethylases remove these PTMs. Histone methylation can activate or repress transcription depending on the position of the modification. Generally, methylation of H3K4, H3K36 and H3K79 is considered to activate transcription, whereas H3K9, H3K27, and H4K20 methylation silences transcription [29]. Regulation of gene expression by histone methylation is also dependent on the recruitment of effector proteins that specifically bind methylation sites. For instance, methyl-lysine binding proteins containing one of three domains (the chromodomain, the tudor domain or the WD40-repeat domain) repress gene expression via their interactions with other components of the transcriptional machinery [30].

Reversible acetylation of the ε-amino group of lysine residues within histones is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) [31]. Lysine acetylation on histone tails by HATs generally promotes gene expression by weakening the interaction between the histone and neighboring nucleosomes to relax the chromatin structure and make target sequences accessible for transcription. Deacetylation of histones by HDACs strengthens this interaction to repress gene transcription [32]. Histone acetylation also activates transcription by serving as a site for the recruitment of bromodomain-containing proteins which recognize acetyl-lysine. For instance, BRD4, a member of the Bromodomain and Extraterminal (BET) protein family, accumulates on hyperacetylated chromatin regions and serves as the center for assembly of additional protein complexes that bind and stabilize RNA polymerase II for transcription [33]. In some cases, histone acetylation can repress transcription. For example, acetylation of H4K16 is associated with the transcription start sites of minimally expressed genes in humans and co-localizes with the transcription repressor, neuron-restrictive silencer factor/repressor element 1-silencing transcription (NRSF/REST) NRSF/REST [34]. Importantly, lysine acetylation, in particular, regulates the expression of numerous genes critical in tumor

development and metastasis [35]. Furthermore, many nonhistone proteins including transcription factors, hormone receptors, chaperones, and cytoskeletal proteins are also reversibly acetylated, resulting in altered function, stability, localization, and protein-protein interactions [36].

Histone Deacetylases in Metastatic Cancer

HDACs have been grouped into four classes based on their homology, sequence similarity, and expression patterns (Table 1) [37]. The class I enzymes are ubiquitously expressed in all human tissues [38]. The expression of class IIa enzymes is generally restricted to the heart, skeletal muscle, and brain, while class IIb expression is restricted to the liver, kidney and placenta. HDAC11, the only class IV enzyme, shares sequence similarity to both Class I and Class II proteins. Class I, II, and IV HDACs all belong to the arginase/ deacetylase superfamily of proteins containing arginase-like amidino hydrolases and histone deacetylases [37, 39]. Class III enzymes, the sirtuins, possess deacetylase activity but are functionally unrelated to the other HDACs and utilize nicotinamide adenine dinucleotide (NAD) instead of zinc as a cofactor [40]. The functional classification of these enzymes is of particular importance given that different HDAC inhibitors target different classes of HDACs. Thus, while there is potentially some specificity for targeting groups of HDACs, it is particularly challenging to target an individual HDAC, since these enzymes can have compensatory functions within each class [41, 42].

Class I Enzymes

Aberrant expression of class I HDACs has been identified in breast cancer and other tumor types that have a high predilection for metastasizing to the bone and lung. In breast cancer, tumors from patients with invasive ductal cell carcinoma and ductal carcinoma in situ (DCIS) have elevated expression of HDAC1, 3 & 8 [43]. Immunohistochemical analysis of tissue microarrays from patients with primary invasive breast cancer revealed that elevated HDAC2 & 3 protein expression correlates with negative hormone receptor status, while high HDAC2 expression is associated with HER2 overexpression and lymph node metastasis [44]. HDAC1 & 3 are also significantly upregulated in prostate cancer with higher HDAC1 levels in metastatic tumors [45]. HDAC1 and 2 expression in prostate cancer also correlates with Gleason scores and tumor dedifferentiation, with high-grade tumors expressing higher levels of both isoforms [46]. This suggests that these enzymes may play a role in prostate cancer progression and metastasis. HDAC2 is upregulated in non-small cell lung cancer cells and promotes migration and invasion, cellular characteristics critical for metastasis [47]. In renal cell carcinoma, *HDAC1* mRNA is upregulated in 4% of patients in The Cancer Genome Atlas data set and its expression is

Table 1. Histone deacetylase classification and cellular localization

Class	Isoforms	Cellular Localization
I	HDAC1, 2, 3, 8	nucleus
IIa IIb	HDAC4, 5, 7, 9 HDAC6, 10	cytoplasm and nucleus
III	SIRT1-7	nucleus (SIRT1, 3, 6, 7) cytoplasm (SIRT2) mitochondria (SIRT3-5)
IV	HDAC11	nucleus

associated with worse overall survival [48]. Lastly, elevated HDAC1 and HDAC2 expression has been observed in osteosarcoma cells, which have a high predilection for metastasizing to the lung [49]. In this study, siRNA-mediated silencing of HDAC1 and 2 significantly reduces osteosarcoma cell growth *in vitro*.

Class II Enzymes

Aberrant expression of class II HDACs has also been implicated in tumor development among bone and lung metastatic cancers. HDAC7 downregulation is associated with decreased histone 3 lysine 27 acetylation (H3K27ac) at transcription start sites and super enhancers in stem-like breast cancer cells [50]. Notably, these transcriptional changes repress expression of oncogenes including *C-MYC, VEGFA, SNAI2* and *SMAD* as well as multiple stem cell transcription factor genes. Self-renewing cancer stem cells (CSCs) are important drivers of tumor initiation, progression, metastasis and therapy resistance [51]. Thus, targeting HDAC7 may present an important avenue for inhibiting the CSC phenotype and activation of multiple oncogenes in breast cancer to prevent tumor development and metastasis. In prostate cancer, HDAC4 and 5 are up-regulated in primary and metastatic tumors *in vivo* and their expression enhances cell invasion *in vitro* [45]. In clear cell renal cell carcinoma HDAC6 up-regulation has been reported in a subset of patients with metastatic disease [48, 52]. Both HDAC1 and HDAC6 overexpression also increase renal cell carcinoma tumor cell invasion *in vitro* by increasing matrix metalloproteinase expression [48, 52]. In non-small cell lung cancer, HDAC10 is upregulated in primary and metastatic tumors and preferentially localizes to the cytoplasm of cancer cells but not in adjacent normal lung epithelial cells [53]. The same study also found that *in vitro* overexpression of HDAC10 and a nuclear localization-defective HDAC10 mutant significantly increases cell growth and G1/S phase cell cycle transition while HDAC10 knockdown induces G1 arrest via upregulation of the cell cycle inhibitors p21 and p27. These data indicate that alterations in the subcellular localization of class II HDACs may also contribute to tumorigenesis and metastasis. Indeed, HDACs have multiple functions outside of the nucleus. In cancer cells, HDACs may be shuttled from the nucleus to the cytoplasm, which reduces the ability of HDACs to transcriptionally repress oncogenes and deacetylate nonhistone proteins in the cytoplasm that regulate tumorigenesis and metastasis. For example, excessive acetylation of α-tubulin, which is a substrate of HDAC6, stabilizes microtubules, resulting in cell cytotoxicity [54], an effect that has been leveraged in the use of taxanes as standard of care therapies for multiple cancer types. By deacetylating α-tubulin, cytoplasmic HDAC6 also regulates microtubule-mediated processes including cell division and migration that drive tumor development and metastasis [55]. The Hsp90

chaperone protein is also a substrate of HDACs acting in the cytoplasm. Increased acetylation of Hsp90 by either HDAC6 knockdown or HDAC inhibition inactivates its chaperone activity and leads to degradation of its target proteins including HER2 ErbB1, ErbB2, Akt, and c-Raf [56]. Thus, aberrant deacetylation of cytoplasmic chaperone proteins alters the expression of numerous oncogenic factors that drive tumorigenesis. The full scope of the cytoplasmic roles of HDACs in cancer remains to be fully uncovered and continued studies are needed to better understand how dysregulation of their extra-nuclear actions drives tumor progression.

While few studies have directly investigated the role of HDAC expression in the process of bone or lung metastasis, the class II enzymes, in particular, may drive dissemination to these sites through regulation of pathways known to promote metastasis. One of the key pathways known to regulate tumor progression that is targeted by HDACs is hypoxia inducible factor (HIF) signaling [57-59] (Figure 1). Tumor cells are subject to fluctuating hypoxic conditions as solid tumors grow beyond several millimeters, which activates HIF signaling. Importantly, HIF signaling is activated by tumor cells in the bone marrow since this microenvironment is known to have regions with physiologically low oxygen tensions, despite extensive vasculature [9, 60]. HIF activation also promotes bone colonization by tumor cells. Expression of constitutively active HIF1-α in MDA-MB-231 bone metastatic breast cancer cells enhances bone colonization *in vivo*, while bone metastasis is significantly reduced in mice inoculated with cells expressing dominant-negative HIF1-α [61, 62]. Similarly, knockdown of HIF1-α reduces MDA-MB-231 colonization of the bone marrow [63]. Breast cancer patients with a greater number of disseminated tumor cells in the bone marrow have 3-fold higher expression of HIF1-α in their primary tumors [58]. HIF1-α expression also predisposes the lungs for metastasis [39, 64, 65], and expression of dominant negative HIF1-α or treatment with 2-methoxyestradiol reduces lung colonization by breast cancer cells. Furthermore, knockdown of HIF1-α in the mammary fat pad, reduces lung metastasis [66].

Multiple studies have explored the effects of HDAC inhibition and silencing on HIF1-α expression. Silencing of HDAC6 reduces HIF1-α levels by disrupting its association with the chaperone Hsp90, leading to subsequent proteasomal degradation [67]. Genetic or pharmacologic HDAC9 inhibition downregulates HIF1-α in a process dependent on the eukaryotic translation initiation machinery, especially eIF4E, 4G1 and 3G subunits [68]. Lastly, HDAC7 has also been identified as a transcriptional activator of HIF1-α signaling by translocating to the nucleus under hypoxic conditions and forming a complex with HIF1-α and p300 [69]. Thus, targeting HDACs through the use of HDAC inhibitors may impact multiple pathways, including HIF, that promote tumor colonization of the bone and lungs.

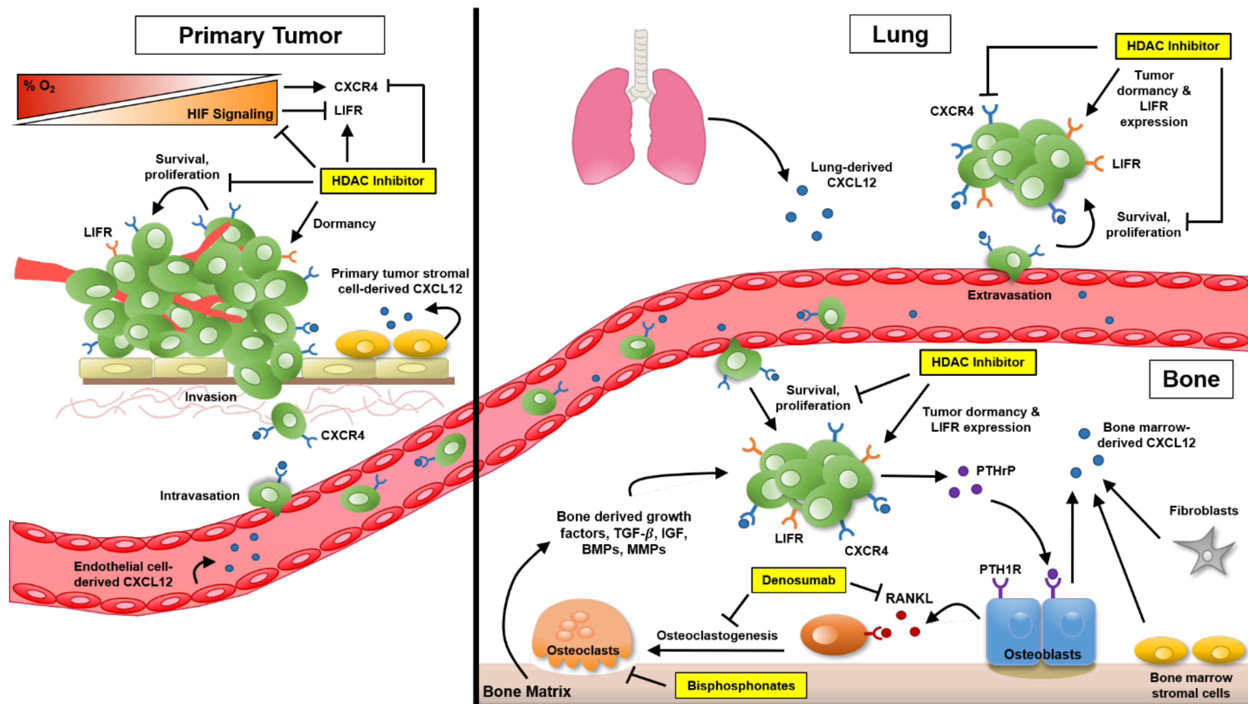

Figure 1. HDAC inhibitors in the treatment of lung and bone metastasis. Hypoxia-inducible factor (HIF) signaling plays a critical role in cancer metastasis. Hypoxia (low-oxygen tension) and HIF expression promote metastasis to the lung, bone and other organs via multiple mechanisms including upregulating the expression of CXCR4 by tumor cells. Signaling via CXCR4 and its ligand, CXCL12, plays a key role in tumor cell dissemination to distant sites by enhancing tumor growth, invasion, angiogenesis as well as adhesion to endothelial cells to promote the early stages of metastasis. CXCL12 secreted by cells in the bone, lung and other distant organs acts as a chemoattractant to promote homing of cancer cells to metastatic sites. At the end organ, CXCR4 signaling triggers adhesion of cancer cells to endothelial cells to promote extravasation as well as proliferation. In the bone specifically, disseminated tumor cells can induce bone destruction to support their own growth by releasing PTHrP which stimulates production of RANKL by osteoblasts, resulting in osteoclastic bone resorption that releases bone derived growth factors which further stimulates tumor growth and exacerbates bone destruction. This process can be targeted with bisphosphonate or denosumab treatment to inhibit osteoclast-mediated bone resorption. Treatment with HDAC inhibitors can directly target tumor cells by reducing HIF signaling and CXCR4 expression, as well as inducing the expression of pro-dormancy genes (leukemia inhibitory factor receptor, LIFR), making them potentially effective therapeutics for the treatment and prevention of lung and bone metastasis.

Class III Enzymes / Sirtuins

The role of sirtuins has also been investigated in bone and lung metastatic tumors [70]. In prostate tumor cells, SIRT7 overexpression induces epithelial-mesenchymal transition (EMT) to promote cell migration and invasion *in vitro* while its depletion reduces lung metastasis *in vivo* [71]. SIRT7 inactivation reverses the EMT phenotype as evidenced by decreased levels of the EMT-inducing transcription factor Slug (SNAI2) and vimentin (a mesenchymal marker), as well as increased expression of E-cadherin (an epithelial marker and cell-cell adhesion molecule) and DAB2 interacting protein (DAB2IP), a tumor suppressor whose loss promotes EMT and metastasis. In breast cancer, SIRT1 plays both tumor suppressing and tumor promoting roles. Increased expression of SIRT1 in triple negative breast cancer patients is associated with lymph node metastasis, and SIRT1 knockdown in MDA-MB-231 cells, a model of metastatic cancer, suppresses invasion *in vitro* [72]. Latifkar et al. proposes that loss of SIRT1 expression promotes metastasis by reducing lysosomal acidification and protein degradation, which may support the release of exosomes containing extracellular matrix hydrolases that increase invasion [73]. In contrast, in hormone receptor and HER2 positive breast cancer, SIRT1 expression suppresses TGF-β driven EMT and is associated with lower risk of lymph node metastasis [74]. These studies indicate that inhibiting sirtuins may be beneficial in blocking tumor progression in prostate cancer and hormone receptor negative breast cancer but may have the opposite effect in hormone receptor positive breast cancer. Of note, these studies did not examine bone metastasis, so it remains unknown whether these sirtuins regulate bone metastasis similar to their effects on lung and lymph node metastasis. Lastly, in renal cell carcinoma, SIRT1, 3, & 6 levels are significantly downregulated [75]. In particular, high SIRT3 expression is associated with better overall survival and greater metastasis free survival in patients. In a separate analysis of clear cell renal cell carcinoma, SIRT1, SIRT3, and SIRT5 expression is lower in tumor specimens with advanced TNM stage and poor histological grade, while SIRT6 and SIRT7 expression is higher [76]. Additional *in vivo* and *in vitro* experiments are needed to further validate the functional role of these sirtuins in renal cell carcinoma. The role for sirtuins in tumor progression varies widely across tumor types, and their role in bone metastasis remains unclear. It will therefore be important to examine the effect of individual inhibitors that target the class III enzymes for each tumor type and subtype.

Class IV Enzymes

Though much less heavily studied, biphasic roles in tumor progression and metastasis have been identified for the only class IV enzyme, HDAC11. In breast cancer, HDAC11

expression promotes tumor progression within the lymph nodes through a mechanism involving the downregulation of factors that induce cell cycle arrest [77] including *RRM2* [78] and *E2F8* [79]. HDAC11 knockdown significantly decreases the percentage and size of tumors formed by breast cancer cells injected into the axillary lymph node. Unexpectedly, this study also revealed that HDAC11 shRNA knockdown and treatment with quisinostat, the most potent HDAC11 inhibitor [80], significantly increases metastasis to the lung *in vivo* and cell migration *in vitro*. Interestingly, quisinostat still significantly inhibited axillary lymph node tumor growth *in vivo.* These results suggest that increased HDAC11 expression in the lymph node promotes tumor cell survival and proliferation. However, a decline in HDAC11 expression promotes a migratory phenotype allowing tumor cell dissemination from the lymph node to distant organs. While hematogenous dissemination is the main route for tumor cell dissemination to the bone marrow, axillary lymph node metastases are recognized as an independent risk factor for bone metastases in breast cancer patients [81]. Due to the spectrum of HDAC11 expression along the metastatic cascade, caution should be exercised in the utilization of HDAC11 inhibition for cancer therapy since serious unintended consequences on tumor metastasis may result due to untimely initiation of treatment or poorly selected patient candidates.

Histone Acetyltransferases in Metastatic Cancer

The HATs are classified into five major subfamilies (Table 2) [82, 83]. Like the HDACs, studies have not directly investigated the role of HAT expression or activity in lung or bone metastasis. However, numerous studies have found evidence highlighting the role of the HAT1 subfamily in tumor progression and metastasis among the highly bone and lung metastatic cancers. Compared with surrounding normal epithelium, lung tumors express lower levels of HAT1 as well as Fas, a death receptor required for apoptosis [84]. This study also demonstrated that restoration of HAT1 promotes Fas expression and significantly increases cancer cell death, suggesting that HAT1 may serve as a suppressor of lung tumor progression. The Gcn5/PCAF family has also been implicated in tumor proliferation. In non-small cell lung cancer, GCN5 is upregulated and induces cell proliferation and G_1/S phase cell cycle transition via increased histone H3 and H4 acetylation at the *cyclin D1, cyclin E1,* and *E2F1* promoters [85]. These data indicate that histone modifications by HATs drive tumorigenesis by inducing the transcription of genes that promote cell cycle progression and proliferation. Reduced PCAF expression has also been shown to dysregulate cell cycle progression by impairing the acetylation of p53 and downstream p21 transcription, resulting in increased cyclin D1, phosphorylation of retinoblastoma 1, and progression through the G1/S transition [86].

Table 2. Histone acetyltransferase classification and origin of subfamily names

Subfamily	Naming Origin
HAT1	Founding member histone acetyltransferase 1
Gcn5/PCAF	Founding member yeast Gcn5 and human paralog, PCAF
MYST	Founding members MOZ, Ybf2/Sas3, Sas2, and TIP60
P300/CBP	Human paralogs p300 and CBP
Rtt109	Initial identification as regulator of Ty1 transposition gene product 109

Altered expression of the MYST family of HATs has also been studied in some bone and lung metastatic cancers. MYST3 is amplified in 11% and up-regulated in 15% of primary breast tumors with an even higher frequency (22%) detected in the more aggressive luminal B subtype (HER2-) in patient datasets from The Cancer Genome Atlas [87]. High MYST3 expression correlates with reduced progression-free and overall survival in patients with estrogen receptor-positive (ER+) breast cancers. Furthermore, MYST3 depletion significantly reduces proliferation of ER+/MYST3-high breast cancer cells in vitro. These data suggest that MYST3 expression may promote breast tumor progression and a more aggressive cancer phenotype. In contrast, homozygous deletion of MYST4 has been identified in lung cancer cell lines and primary lung tumors [88]. The same study also found that depletion of MYST4 in vitro enhances cancer cell growth and viability while MYST4 depletion in vivo increases tumor growth and liver metastasis, indicating that this histone acetyltransferase likely serves as a tumor and metastasis suppressor in lung cancer.

Lastly, abnormal expression of the p300/CREB-binding protein (CBP) subfamily is observed in some bone and lung metastatic cancers. Breast tumors express higher levels of p300 in comparison to normal surrounding breast tissue [89]. Expression of p300 correlates with higher histological grade, advanced stage at diagnosis, tumor recurrence, and shortened overall survival. Another study demonstrated that in vitro inhibition of p300 acetyltransferase activity induces apoptosis and reduces migration and invasion of breast cancer cells [90]. In this study, in vivo inhibition also reduces metastatic lung tumor burden as well as mitotic index and Ki67 levels, indicating that p300 activity promotes breast cancer lung metastasis. Ring et al. found that expression of CBP is higher in triple negative breast cancer than other less aggressive breast tumor subtypes [91]. Targeting CBP in vivo also decreases breast tumor growth more than with paclitaxel alone [92]. These data indicate that CBP expression is associated with the development of a more aggressive tumor phenotype and that targeting p300/CPB may enhance sensitivity to standard-of-care chemotherapies. CBP is also a known transcriptional activator of β-catenin [93], a key signal transducer in the Wnt signaling pathway which is known to promote EMT and metastasis [94-97]. Wnt activation by β-catenin/T-cell factor 4 (TCF4) overexpression in lung and breast cancer cells also increases the expression of the transcription Gli2, which in turn promotes production of parathyroid hormone-related protein (PTHrP), a key driver of osteolysis in bone metastatic tumors [98]. Furthermore, Wnt signaling is also upregulated in prostate tumors that have metastasized to the bone [99, 100] and breast tumors that have metastasized to the lung [101, 102] . Collectively these data suggest that targeting p300/CPB may be beneficial in blocking tumor metastasis to the bone and lung, particularly in the case of breast cancer.

Much like the HDACs, overexpression of HATs has been implicated in tumor development and metastasis in multiple cancer types. Inhibitors of these enzymes could prove clinically beneficial for the treatment of metastatic cancer. However, unlike the HDAC inhibitors, HAT inhibitors have not produced consistent and promising results that translate from in vitro to in vivo and clinical studies [103]. This may be due, in part, to challenges such as HATs functioning in large, multi-protein complexes that regulate the enzymatic activity and substrate specificity of the acetyltransferase. It is necessary to accurately recapitulate these protein-protein interactions in vitro, otherwise the recombinant complexes may not reflect their in vivo enzymatic activity and the ability to develop effective inhibitors will be limited. Poor cell permeability and stability in vivo as well as lack of selectivity also contribute to the limited development and use of HAT inhibitors. Lastly, while p300/CPB appear to be a promising therapeutic target in breast cancer, some HATs such as MYST4 [88] display both tumor promoting and suppressive roles, indicating that the selectivity of any HAT inhibitors in clinical development will need to be rigorously examined in pre-clinical studies for each tumor type.

Clinical Use of HDAC Inhibitors in Metastatic Cancer

The HDAC inhibitors are broken into four different classes based on their chemical structures: hydroxamates, aliphatic/ short chain fatty acids, benzamides, and cyclic peptides (Table 3). HDAC inhibitors are currently FDA approved for hematologic malignancies like multiple myeloma, which often has a bone osteolysis component [104], and lymphomas [105]. The HDAC inhibitor valproic acid (i.e. valproate) is also FDA-approved for the treatment of epilepsy, bipolar disorder, and migraines. Despite their success in treating hematologic malignancies, single-agent HDAC inhibitor therapy has not shown the same clinical efficacy in solid tumors. In a phase II clinical trial in patients with metastatic breast cancer, vorinostat monotherapy did not induce complete or partial responses based on Response Evaluation Criteria in Solid Tumors (RECIST) [106]. Additional phase II clinical trials of vorinostat also demonstrated minimal activity for the treatment of relapsed non-small cell lung cancer, recurrent ovarian cancer [107], metastatic head and neck cancer [108] and only modest activity in patients with recurrent glioblastoma multiforme [109]. Yet another phase II trial of vorinostat in patients with metastatic castration-resistant prostate cancer who had already been pretreated with chemotherapy found no significant clinical activity as measured by rate of progression at six months [110]. In fact, all 29 participants had to be taken off therapy (400mg orally daily) before six months due to significant toxicities or disease progression. Other clinical trials of single-agent HDAC inhibitor therapy have also failed to identify any clinically meaningful anti-tumor activity. Panobinostat had no objective antitumor response

Table 4. HDAC inhibitors and their targets. Light yellow = HDACs targeted by each inhibitor at the IC50; dark yellow box = HDACs targeted at ten times the IC50; gray = HDACs not targeted at either concentration; asterisk (*)= FDA approved

Class	Inhibitor name	FDA Use	Concentrations (lowest = IC50)	HDAC1	HDAC2	HDAC3	HDAC4	HDAC5	HDAC6	HDAC7	HDAC8	HDAC9	HDAC10	HDAC11
I: Hydroxamate	*Belinostat	T-cell lymphoma	27nM [114]; 0.27μM											
	*Panobinostat	Multiple myeloma	5nM [115]; 50nM											
	*Vorinostat	T-cell lymphoma	1μM [116]; 5μM											
II: Short chain fatty acid	*Valproic acid	Epilepsy, bipolar disorder, migraines	1mM [117]; 10mM											
III: Benzamide	Entinostat	Phase III for breast cancer, phase II for lung cancer	0.5μM [118]; 5μM											
IV: Cyclic tetrapeptide	*Romidepsin	T-cell lymphoma	5nM [119]; 50nM											

in a phase I trial in patients with metastatic melanoma [111] or a phase II study in patients with castration-resistant prostate cancer [112]. A similar lack of efficacy has also been demonstrated with romidepsin [113].

Findings from these studies demonstrating poor efficacy of single-agent HDAC inhibitor therapy are likely due to multiple factors. Notably, a lack of efficient drug delivery is *not* likely as immunohistochemical analysis of tumor sections revealed increases in histone acetylation following drug treatment [109]. Rather, the poor efficacy could be attributed to the effects of prior adjuvant treatments received by study participants as in the aforementioned trials on vorinostat in platinum-resistant ovarian cancer [107] and conventional therapy resistant metastatic head and neck cancer [108]. Prior failure on adjuvant therapy has been associated with decreased response rates and worse outcomes with subsequent chemotherapy treatments [114], possibly due to additional acquired mutations. For this reason, there is a shift to exploring combination HDAC inhibitor therapy to overcome resistance to conventional treatments. Emerging evidence suggests that rather than acting solely as a traditional cytotoxic agent, HDAC inhibitors may function better as biological response modifiers, particularly in modulating the immune system's response against cancer growth [115]. Combination treatment with HDAC inhibitors and immunotherapy will be discussed further in the next sections. Lastly, commonly recognized mechanisms of resistance to HDAC inhibitors likely contribute to their poor efficacy in clinical trials. Changes in drug efflux mechanisms [116], increased expression of the antiapoptotic protein Bcl-2 [117] as well as elevated levels of thioredoxin leading to lower reactive-oxygen species (ROS)-mediated DNA damage [118] have been cited as just a few factors driving resistance to HDAC inhibitors.

The results of these clinical trials have prompted assessment of combination HDAC inhibitor therapy since studies have identified synergistic or additive interactions between the inhibitors and cytotoxic agents including microtubule inhibitors, antifolates, and nucleoside analogs [119, 120]. Combination treatment with DNA-damaging agents has also been heavily investigated since HDAC inhibitors induce chromatin decondensation, which facilitates access of these agents to their DNA substrates to induce apoptosis [121, 122]. Abrogation of the DNA repair response seen with many cytotoxic agents, especially poly ADP ribose polymerase (PARP) inhibitors, has also been exploited as a benefit of combination vorinostat therapy [123]. In a separate study on multiple myeloma (MM), co-treatment with the mTOR inhibitor everolimus overcomes resistance to panobinostat by synergistically downregulating multiple DNA repair genes, anti-apoptotic factors, and G2/M mitotic factors, thereby suppressing DNA damage repair, inhibiting cell cycle progression, and inducing cell death [124]. Mechanistically, MM cell resistance

to panobinostat monotherapy is mediated by overexpression of C-X-C motif chemokine receptor 4 (CXCR4) which in turn activates pro-tumorigenic AKT/mTOR signaling. CXCR4 normally promotes lymphocyte trafficking, hematopoietic stem cell homing to the bone marrow, and endothelial cell precursor recruitment to sites of ischemia [125-127]. However, CXCR4 is overexpressed in numerous cancers. This promotes tumor cell dissemination by enhancing chemotaxis to tissues that normally secrete high levels of its ligand, C-X-C motif chemokine ligand 12 (CXCL12), including the bone, lung, brain, and liver which are common sites of metastasis (Figure 1). Of note, CXCR4 is one of the most enriched genes in MDA-MB-231 cells human breast cancer cells that form osteolytic bone metastases *in vivo* and is recognized as a key driver of bone metastasis [128]. In prostate cancer, bone-disseminated tumor cells express higher CXCR4 levels than tumor cells derived from the primary tumor or other soft tissue metastases [129]. Activation of CXCL12/CXCR4 expression and signaling also enhances the development of lung metastasis in melanoma [130] and breast cancer [131]. Panobinostat has previously been shown to deplete CXCR4 expression and downregulate AKT and ERK1/2- mediated pro-survival signaling in acute myeloid leukemia (AML) cells [132]. Thus, combination HDAC inhibitor therapy targeting CXCR4 may be particularly advantageous for treating bone and lung metastases by reducing receptor expression and chemotaxis to distant organs as well as inhibiting pro-tumorigenic signaling.

Preclinical studies have also revealed rationale for the combination of HDAC inhibitors with hormonal therapy due to the transcriptional regulation of estrogen receptor expression and signaling by several HDACs [133]. Promoter hypoacetylation and hypermethylation silences ERα expression, but this can be reversed by HDAC and DNA methyltransferase inhibition [134, 135]. In ER-negative breast cancer, re-expression of ERα induced by the HDAC inhibitor trichostatin A sensitizes the tumor cells to aromatase inhibitors and other antihormonal therapies [136, 137]. Interestingly, selective genetic ablation or HDAC inhibition in ER-positive cells transcriptionally downregulates ERα expression but upregulates expression of ERβ, which acts as a tumor suppressor in multiple cancer types [138-141]. In a preclinical study, the selective ER β agonist LY500307 suppresses triple negative breast cancer and melanoma metastasis to the lung by inducing tumor cell IL-1 β release to increase intratumoral neutrophil infiltration [142]. Co-treatment with an HDAC inhibitor and ER β agonist may prove beneficial for the treatment of hormone therapy insensitive metastatic breast cancer by upregulating ERβ expression and activity. This is of particular clinical importance since breast cancer patients originally diagnosed with ERα positive breast cancer frequently present with ERα negative metastases due to ERα

downregulation and this is especially prevalent in the case of bone metastasis [143, 144]. HDAC inhibitor therapy may be particularly useful in patients whose metastatic tumors have converted from positive to negative ER status, greatly expanding the available therapy options.

Preclinical studies have revealed mechanistic rationale for the combination of HDAC inhibitors with immunotherapies. Multiple class I HDAC inhibitors including vorinostat modulate the expression of programmed death 1 ligand (PD-L1) by melanoma tumor cells and augment the antitumor response to PD-1 blockade *in vivo* [121]. *In vivo* studies of lung adenocarcinoma demonstrated that treatment with romidepsin augments PD-1 immunotherapy response by increasing the expression of multiple T-cell chemokines, enhancing T-cell tumor infiltration, and promoting T-cell dependent tumor regression [145]. Another study demonstrated an interesting mechanism whereby PD-1 blockade enhances T-cell function and the subsequent production of interferon-gamma (IFNγ) and other pro-inflammatory cytokines as expected. However, the cytokines, in turn, activate a negative feedback response that induces melanoma tumor cell expression of PD-L1 and PD-L2 and promotes pro-tumorigenic M2 macrophage infiltration [146]. In this study's model, selective HDAC6 inhibition in combination with anti-PD-1 antibodies increases infiltration of CD8+ T-cells and natural killer cells and diminishes intratumoral M2 macrophages populations. Another study demonstrated that belinostat upregulates IFNγ and decreases expression of immunosuppressive regulatory T cells (Tregs) to enhance the antitumor activity of anti-CTLA-4 therapy [147]. Lastly, combining HDAC inhibitors with high dose interleukin 2 (IL-2) has also demonstrated synergistic activity by downregulating Foxp3 expression and function of Tregs as well as myeloid derived suppressor cells, both of which suppress immune clearance of tumor cells [148]. Given that neither immune checkpoint inhibitors nor HDAC inhibitors have been successful as monotherapies in breast tumors, the potential benefit of combination therapy is promising, but will need to be extensively tested in preclinical models since both drugs can cause significant patient toxicity as monotherapies [149, 150].

Systemic Effects of HDAC Inhibitors and their Impact on Bone Metastasis

Since HDAC inhibitors are administered systemically, it is important to understand their potential adverse effects. The most common events reported from single agent trials include nausea, vomiting, and anorexia [150]. Transient thrombocytopenia, neutropenia, and anemia have been reported. HDAC inhibitor-induced thrombocytopenia, in particular, is a major dose limiting toxicity [151]. Patients on chronic valproic acid therapy also have an increased risk of osteoporosis and osteomalacia [152, 153]. This is corroborated by several *in vivo* studies demonstrating that HDAC inhibitors negatively impact bone volume. One study found that while

vorinostat significantly lowers intratibial tumor burden in SCID/NCr mouse models of mammary carcinoma and prostate cancer, the contralateral limbs of tumor bearing mice and femurs of non-tumor bearing mice treated with vorinostat exhibit 50% loss of trabecular bone density compared with controls [154]. Histochemical staining showed increased numbers of active, tartrate resistant acid phosphatase (TRAP)-positive osteoclasts in non-tumor bearing, vorinostat treated mice, indicating that HDAC inhibitors also negatively impact the activity of normal bone marrow resident cells to alter bone density. A separate study demonstrated that vorinostat causes substantial trabecular bone loss in C57BL/6 mice by inducing DNA damage and cell cycle arrest in bone marrow stromal cells, which significantly decreases mature osteoblast numbers [155]. From this study, the effect appears to be primarily due to osteoblast formation since osteoclast numbers in this study were reduced, though not statistically significant, and RANKL production was not substantially altered. Given that the HDAC inhibitors are not cell-type specific, the finding that vorinostat affects bone resident cells is not surprising and needs to be considered in preclinical and clinical studies going forward. Contrary to these findings, a separate study found that non-tumor bearing mice administered vorinostat less frequently (100 mg/kg, i.p. every other day for 3 weeks) did not exhibit any bone loss [156]. The mice also did not exhibit an increase in osteoclasts or a decrease in osteogenic colonies, serum osteocalcin, or osteoblast numbers. The discrepant *in vivo* effects of vorinostat on bone loss are likely related to the frequency of treatment since in the previously mentioned studies, mice were treated at 100 mg/kg, daily for 3 or 4 weeks [154, 155]. This draws an interesting parallel with the known effects of intermittent versus continuous parathyroid hormone (PTH) effects on bone, where intermittent PTH induces bone formation [157], while continuous PTH leads to bone loss due to sustained RANKL activation [158]. Thus, careful consideration should be given to the frequency of HDAC inhibitor administration in patients.

To combat the effects of HDAC inhibitor-induced bone loss, co-administration of an anti-resorptive agent with HDAC inhibitors may be necessary. Bisphosphonates and denosumab are already FDA approved and frequently administered to patients to reduce the risk and severity of skeletal related events due to metastatic bone disease. Thus, adding these drugs to an HDAC inhibitor treatment regimen is clinically feasible. Antiresorptive agents have been shown in multiple studies to improve outcomes for patients with breast, prostate and other cancers [159, 160]. In addition to protecting against HDAC inhibitor-induced bone loss, combination therapy regimens may also enhance the anti-tumor activity of HDAC inhibitors. Vorinostat acts synergistically with the bisphosphonate zoledronic acid to induce prostate cancer cell apoptosis by disrupting the mitochondrial transmembrane potential to activate caspase-3 and DNA

fragmentation [161]. Panobinostat synergizes with zoledronic acid in prostate cancer and multiple myeloma to inhibit proliferation and induce apoptosis by increasing reactive oxygen species production and inhibiting p38-MAPK activation [162], the latter of which has been shown to mediate acquired resistance to zoledronic acid [163]. Combined treatment with panobinostat and zoledronic acid also significantly inhibits prostate tumor growth *in vivo* [162]. Thus, the combinatory use of a bisphosphonate may protect against HDAC-inhibitor induced bone loss and reduce tumor growth in the bone, making this a beneficial therapy regimen for patients with bone-disseminated cancer. Importantly, bisphosphonates have a known safety profile and are well tolerated in patients who take HDAC inhibitors for both cancer and non-cancer indications [164, 165].

Therapeutic Targeting of Dormant Tumor Cells

Another evolving area of focus in the treatment of metastatic disease is targeting tumor dormancy. Patients may present with clinically detectable metastases decades following primary tumor resection. This late tumor relapse / recurrence is thought to be caused by the emergence of tumor cells from a dormant state at distant metastatic sites [166]. In general, a non-proliferative (e.g. Ki67 or BrdU negative) disseminated tumor cell that has not grown into a micrometastasis is considered dormant [5-7, 167]. There are currently no available therapies to prevent tumor cell exit from dormancy in the bone or lung. While the mechanisms that regulate breast tumor dormancy remain incompletely understood [168], several key factors in the bone have been identified including leukemia inhibitor factor (LIFR) [169]. Loss of LIFR expression and signaling in MCF7 human breast cancer cells, which lie dormant *in vivo,* results in greater tumor-induced bone destruction due to increased tumor cell proliferation and reduced expression of genes that promote a dormancy phenotype including transforming growth factor-β2 (TGF-β2) [170] and tropomyosin-1 (TPM1) [171], among others. These data strongly suggest that LIFR signaling is key in regulating breast tumor dormancy in the bone, however the mechanisms that regulate LIFR expression have not been fully elucidated.

Previous studies determined that LIFR signaling is downregulated in hypoxia (low oxygen tensions), in part, due to epigenetic mechanisms involving histone acetylation. Consequently, treatment with the pan-HDAC inhibitor valproic acid significantly increases expression of LIFR and other pro-dormancy genes in MCF7 cells cultured in normoxia (normal oxygen tensions) and hypoxia. This of importance in the context of bone and lung metastatic disease since the bone marrow is a physiologically hypoxic microenvironment [172, 173] and hypoxia is evident in most solid tumors larger than a few millimeters [174]. Yet another mechanism by which LIFR is

downregulated in bone DTCs is through overexpression of parathyroid hormone-related protein [175], which drives tumor-induced bone disease (TIBD) [176]. The biology of PTHrP and its implications in cancer progression will be discussed in greater detail below. However, it is important to consider the potential for HDAC inhibitors to target dormant tumor cells by also altering the intracellular factors that regulate LIFR expression. Understanding the various mechanisms by which HDAC inhibitors affect breast tumor cell signaling and behavior can provide insight into why HDAC inhibitors are less efficacious in some clinical contexts.

Finally, a previous study found that while valproate increases LIFR expression on breast cancer cells, LIFR/STAT3 signaling may also result in drug resistance to HDAC inhibitors over time, which can be overcome with the addition of a JAK1 or bromodomain containing 4 (BRD4) inhibitor [177]. These findings suggest that HDAC inhibition may present an interesting viable option for maintaining disseminated tumor cells in a dormant state to prevent tumor recurrence but will need to be evaluated for the potential for therapeutic resistance. The combination of these findings suggests that HDAC [164, 165] inhibitors may help promote dormancy through LIFR, but if LIFR signaling must be blocked in order to prevent therapeutic resistance then the net effect on dormancy may be lost. Further studies are needed to determine whether mitigating HDAC inhibitor therapeutic resistance also lessens its potential beneficial effect in promoting tumor dormancy.

PTHrP and Tumor-Induced Bone Disease

Parathyroid hormone- related protein (PTHrP) was initially identified as the cause of humoral hypercalcemia of malignancy (HHM), a paraneoplastic syndrome in which elevated levels of PTHrP lead to increased osteoclastic bone resorption and serum calcium levels [178, 179]. HHM is most often diagnosed in patients with advanced-stage lung, renal, and neuroendocrine tumors. Though less frequently, breast cancers can also cause HHM. The role of PTHrP in cancer now extends well beyond its role in HHM. PTHrP is a well-established critical mediator of tumor-induced osteolysis, especially in breast cancer, which has a high tropism for disseminating to the bone marrow [180]. Bone-disseminated tumor cells secrete PTHrP [181-183], which drives bone destruction via stimulation of RANKL-mediated differentiation and activation of osteoclasts [176]. Osteoclasts resorb the bone matrix, releasing numerous pro-tumorigenic factors such as transforming growth factor beta (TGFβ), matrix metalloproteinases, and other growth factors that subsequently fuel tumor cell proliferation and more PTHrP secretion [184, 185].

PTHrP expression by bone-disseminated tumor cells is also uniquely fueled by the microenvironment. The rigidity of the bone matrix activates TGF-β dependent mechanical signals that stimulate expression of both PTHrP and Gli2, a transcription factor in the hedgehog signaling pathway that in turn induces more PTHrP expression [186]. The bone microenvironment provides yet another critical level of regulation of Gli2 and PTHrP expression via the Wnt pathway [187]. Matrix rigidity activates Wnt signaling and induces nuclear β-catenin accumulation, while bone marrow stromal cells secrete canonical (including Wnt3a) and non-canonical Wnt ligands. Both processes further drive Gli2 and PTHrP transcription and eventual bone destruction.

PTHrP Biology

PTHLH, the gene encoding PTHrP, is located on chromosome 12, and has nine exons spanning approximately 15kb with at least three identified promoters. Alternative splicing gives rise to three isoforms containing 139, 141 and 173 amino acids [188]. PTHrP also has multiple domains, each with different biological functions [189]. The first 36 amino acids (-36 to -1) encode a domain that controls intracellular trafficking of PTHrP precursors before being cleaved when the mature molecule is secreted. The next domain (amino acids 1-34) is responsible for PTHrP binding to and activation of the PTH receptor type 1 (PTH1R), a G-protein couple receptor. In fact, eight of the first thirteen residues within this region of PTHrP are identical with PTH, allowing the two polypeptides to exert agonist effects on their shared receptor [189, 190]. The nuclear localization sequence (NLS) spans amino acids 67-94 and regulates intracrine actions that influence cell proliferation, survival, and apoptosis [191]. Lastly, the carboxy-terminal domain beginning at residue 107 is associated with a number of identified biological actions including inhibition of osteoclast-mediated bone resorption and anabolic effects in bone via a region termed “osteostatin” as well as a nuclear export sequence (NES) [188, 192].

Endocrine, autocrine, and paracrine activity of PTHrP

In normal physiology, PTHrP acts as a hormone to control calcium transport across the placenta to the fetus [193] and during lactation when it enters systemic circulation [194]. In HHM, PTHrP secreted by tumors in the breast and lung, for instance, also acts as a hormone distantly to increase bone resorption [178, 179]. PTHrP is highly expressed in human tissues and plays important roles in mammary gland development, tooth eruption, keratinocyte differentiation for hair follicle development, chondrocyte maturation, and endochondral bone formation [189, 191]. Perhaps one of the most well studied paracrine functions of PTHrP is the regulation of normal bone remodeling where it is produced locally by early osteoblast progenitors to promote

differentiation of mature osteoblasts and bone formation [195, 196]. PTHrP also inhibits apoptosis of early and mature osteoblasts and osteocytes. Furthermore, osteoblast-derived PTHrP stimulates osteoclast differentiation to increase bone resorption. These actions of PTHrP must occur in a balanced manner to maintain the integrity of the bone. While physiologic, these paracrine functions of PTHrP can also pathologically fuel osteolysis and the growth of bone disseminated tumors as discussed previously [184, 185]. Lastly, PTHrP plays a well-recognized role as a paracrine regulator of smooth muscle relaxation, particularly in the vasculature [197] where incubation with PTHrP (1-34aa) also activates cAMP production, indicating that this effect is indeed mediated through PTH1R [198, 199]. In vascular smooth muscle cells, treatment with exogenous PTHrP acting through PTH1R inhibits cell proliferation [200, 201].

In addition to binding and activating PTH1R to exert its paracrine/ autocrine functions, PTHrP can translocate into the nucleus when its NLS forms a complex with importin β, a nuclear transport factor, and the GTP-binding protein Ran [202]. Interestingly, in vascular smooth muscle cells, intracrine actions of PTHrP localized to the nucleus paradoxically increases proliferation [201]. Indeed, in A10 smooth muscle cells overexpressing wild-type PTHP, the protein localizes in the nucleus of dividing cells. This is in striking contrast to findings that PTHrP inhibits proliferation and cell cycle progression in the same cells when acting through PTH1R [200, 201]. These effects of PTHrP are particularly important in the discussion of its role as a regulator of tumor dormancy as it has also been demonstrated that PTHrP lacking the NLS arrests cell cycle progression by increasing $p27^{Kip}$, a cyclin dependent kinase inhibitor, and decreasing phosphorylation of Rb [203, 204]. Cell cycle arrest in the G0-G1 phase is a key characteristic of quiescent cells [205, 206] and p27 is elevated in G0 arrested cells [207, 208]. These findings in vascular smooth muscle cells are remarkable as they indicate that PTHrP can have paradoxical roles on mitogenesis depending on the mode of signaling: paracrine / autocrine versus intracrine.

In addition to nuclear localization mediated by importin β, PTHrP can also gain entry into the nucleus via other mechanisms. PTHrP can be secreted but then internalized in an autocrine/paracrine manner via the PTH1R before being shuttled to the nucleus [209]. Secreted PTHrP may also enter the nucleus via endocytosis-dependent translocation initiated by binding with a non-PTH1R cell surface receptor [210]. Another potential mechanism regulating its subcellular localization is if translation is initiated at a codon different from the classic AUG site. As a known example, translation of fibroblast growth factor-3 (FGF3) can be initiated at an AUG codon resulting in direction of the peptide for secretion [211]. If translation begins at an alternative upstream CUG site, FGF3 is directed into the nucleus. Like FGF3, the PTHrP prepro region has an alternative translational start site at a CUG codon [212], which may serve a similar purpose in

regulating PTHrP secretion versus nuclear import. Since the differential localization of PTHrP produces divergent mitogenic cellular effects in vascular smooth muscle cells, the same is likely true in cancer cells, complicating the understanding of PTHrP as a regulator of cell proliferation and tumor dormancy. Consequently, if altering PTHrP nuclear localization is to be leveraged for therapeutic purposes, more investigation is needed to better understand the regulation of PTHrP subcellular localization in cancer cells and how this may change during tumorigenesis.

Roles of PTHrP in Tumorigenesis, Metastasis and Tumor Dormancy

Preclinical evidence for PTHrP regulation of tumor growth and proliferation

Our understanding of the paracrine / autocrine and intracrine actions of PTHrP extends far beyond the physiologic activities described in the bone, vasculature, and various other normal epithelial tissues. PTHrP also modulates growth, progression, and metastasis in various cancer types by regulating: (i) cell survival, (ii) cell proliferation, (iii) apoptosis, and (iv) invasion and migration [213, 214]. For example, human MCF7 breast cancer cells overexpressing PTHrP (-36-139) display significantly greater survival as they are protected from serum starvation-induced apoptosis and express elevated levels of the antiapoptotic proteins Bcl-2 and Bcl-xL [214]. Other studies have demonstrated that PTHrP drives breast tumor growth by promoting proliferation, as demonstrated by increased staining for the proliferative markers Ki67 and cyclin D1 [206]. Human breast cancer cells expressing PTHrP (-36-139) are also enriched in the G2/M cell cycle phase compared with cells overexpressing NLS-mutated PTHrP, indicating an intracrine role for PTHrP in regulating cell cycle progression and cell growth. In prostate cancer cells, PTHrP expression stimulates proliferation and induces intracrine production of Il-8, a known growth-promoting factor [215]. Prostate cancer cells overexpressing full-length PTHrP also show significantly increased cell survival when exposed to various apoptotic agents [216]. Another study determined that treatment with PTHrP neutralizing antibodies dramatically inhibits clear cell renal cell carcinoma cell proliferation *in vitro* and induces regression of implanted tumors by inducing apoptosis *in vivo,* further indicating a role for PTHrP in regulating both proliferation and cell death [217].

PTHrP expression in the primary tumor has also been identified as an important regulator of tumor growth in *in vivo* genetic models. In the PyMT-MMTV (mouse mammary tumor virus-polyoma middle tumor-antigen) model of breast carcinoma where mice spontaneously develop mammary tumors, Cre-loxP-mediated *Pthrp* ablation delays primary tumor initiation and inhibits tumor progression [213]. Mechanistically, the authors found reductions in the expression of Ki67, factor VIII (an angiogenesis marker), Bcl-2 (an antiapoptotic protein), cyclin D1 (a cell-cycle

regulator) and AKT1 (a pro-survival factor). These data indicate that in this model of breast cancer, PTHrP acts as a pro-tumorigenic factor that drives tumor cell growth and proliferation in the primary site. In striking contrast, another *in vivo* study found that Cre-mediated loss of PTHrP in the MMTV-*neu* mouse model increases tumor incidence and reduces survival [218]. In comparing these discrepant results from the studies on the PyMT-MMTV mice [213] versus the MMTV-*neu* mice [218], it is important to note that the *neu*-based model reflects late-onset oncogenesis representing tumors arising in older animals while the PyMT-MMTV-based model reflects earlier onset tumorigenesis. Age can significantly affect tumor behavior [219, 220]. Thus, in these pre-clinical tumor models, age at which cancer develops must be carefully factored into the interpretation of the effects of PTHrP on tumorigenesis. Lastly, authors of the PyMT-MMTV study report that they deleted exon 4, which encodes amino acids 1-137 in mice [189]. While the authors of the MMTV-*neu* study do not explicitly state which portion of the gene was targeted, deletion of a different exon or smaller portion of the gene could explain these opposing observations since targeting different domains of the PTHrP molecule can elicit very distinct cellular responses.

PTHrP's role in regulating tumor cell dormancy

Most pre-clinical data support a pro-tumorigenic role for PTHrP. PTHrP is also likely a negative regulator of tumor cell dormancy due to its actions that modulate proliferation, apoptosis and cell survival. One study that provides some of the most direct and striking evidence to support this found that in ER+ human MCF7 breast cancer cells, which lie dormant *in vivo* following intracardiac injection [175, 221-223], overexpression of PTHrP (1-141) pushes these cells out of quiescence, switches them to a highly osteolytic phenotype and dramatically increases tumor burden in the bone [221]. Consistent with this enhanced bone colonization and exit from dormancy, a later study determined that PTHrP (1-139) overexpression in MCF7 cells also represses expression and downstream signaling of leukemia inhibitory factor receptor (*LIFR*), a known breast tumor suppressor and dormancy factor in the bone [175]. In this study, overexpression of PTHrP and loss of LIFR both enable otherwise dormant breast cancer cells to downregulate several quiescence- associated genes including thrombospondin-1 (TSP1) [224], transforming growth factor-β2 (TGF-β2) [170], tropomyosin-1 (TPM1) [171], and Selenbp1 [225], among others. Common regulation of this group of genes suggests that PTHrP may inhibit pro-dormancy signaling mediated by LIFR. Moreover, intracardiac injection of MCF7 LIFR knockdown cells into mice results in greater osteolysis and tumor cell proliferation [175]. Thus, repression of LIFR either directly or perhaps through PTHrP overexpression can push bone-disseminated breast tumor cells out of dormancy. These data are further supported by the PyMT-MMTV genetic

studies by Li et al. [213], which demonstrated that *Pthrp* ablation reduces primary breast tumor growth with reductions in pro-proliferative factors Ki67 and cyclin D1 as well as the anti-apoptotic protein Bcl-2, all factors known to regulate dormancy.

Interestingly, evidence exists suggesting that multiple breast cancer cell lines express PTH1R at varying levels, but do not activate downstream cAMP signaling in response to PTH or PTHrP, despite functional signaling in response to calcitonin and PGE_2 which serve as positive controls [226]. In this study, there was also no activation of a cAMP response element binding protein (CREB) reporter construct, and RNA sequencing confirmed that only 2 out of 36 genes in a previously described panel of CREB-responsive genes [227] were significantly upregulated in MCF7 PTHrP-overexpressing cells. Taken together, these data provide convincing evidence that in the bone colonization models, the effects of PTHrP overexpression on gene expression, including dormancy-associated factors in MCF7 cells, are independent of PTH1R activation of the cAMP/PKA/CREB pathway. Further studies are warranted to explore non-PTH1R mediated actions, which may reveal novel mechanisms by which PTHrP negatively regulates dormancy in bone-disseminated breast tumor cells.

Lastly, other studies in breast cancer have also revealed that PTHrP may alter adhesion to extracellular matrix (ECM) cell surface receptors, which can trigger intracellular signaling that promotes cell cycle progression and exit from a dormant state [228-230]. Specifically, PTHrP regulates the expression of integrins which mediate interactions between tumor cells and the ECM that can modulate cellular quiescence [231]. For example, downregulation of the urokinase plasminogen activator receptor (uPAR), a known mediator of tumor dormancy *in vivo*, decreases complex formation with α5β1 integrin and cell adhesion to fibronectin [232]. This reduced ECM binding consequently maintains tumor cells in a dormant state by inhibiting activation of extracellular regulated kinase (ERK) signaling, which normally functions to promote cell cycle progression and division [233]. Additional studies have also confirmed that inhibiting ERK signaling via altered uPAR-mediated α5β1 integrin interactions promotes quiescence *in vivo* [234]. This is highly relevant in the evaluation of PTHrP as a regulator of dormancy since overexpression of PTHrP (-36-139) in MDA-MB-231 human breast cancer increases adhesion to fibronectin [235]. PTHrP (-36-139) overexpression in tumor cells also significantly increases mRNA and cell surface expression of various integrins including α_5, α_6, β_1 and β_4. Though it has not been directly studied, PTHrP may push tumor cells out of dormancy by inducing integrin expression, cell adhesion to fibronectin, and activation of ERK signaling. Additional studies are needed to understand how PTHrP alters ECM binding to regulate tumor dormancy.

Prostate tumors, like breast tumors, also exhibit long latency periods before micrometastases become clinically detectable [171, 236]. One study found direct evidence that PTHrP promotes prostate cancer progression in the bone [237]. Overexpression of PTHrP (1-87) and PTHrP (1-173) in the non-invasive DU-145 human prostate cancer cell line converted these cells to an aggressive phenotype resulting in significantly greater bone tumor burden and mixed osteolytic/ osteoblastic lesions following intrafemoral injection. Interestingly, mice injected with PTHrP (1–173) cells had more extensive bone lesions than those injected with PTHrP (1–87) mice despite lower serum PTHrP levels. Not only does this study demonstrate that PTHrP expression can push prostate tumor cells out of dormancy but it also highlights the pleiotropic actions of the protein's different domains, as PTHrP (1–87) lacks the nuclear localization sequence, osteostatin region, and mitogen regulatory sequences contained in the carboxy terminus of the full-length molecule. The effects of the carboxy terminus of PTHrP, in particular, need to be examined more extensively to specifically understand how this region promotes cancer progression in bone and regulates tumor dormancy. Another study of early prostate adenocarcinoma also demonstrated that PTHrP overexpression significantly increases primary tumor growth [238]. This study found no difference in growth rates between human prostate cancer cells transfected with full-length PTHrP and vector controls, but PTHrP overexpression did render the cells less susceptible to phorbol-12-myristate-13-acetate (PMA)- induced apoptosis. Other studies have also identified a role for PTHrP in inhibiting apoptosis [239, 240]. Thus, PTHrP may negatively regulate tumor dormancy by not only increasing cell proliferation, but also by disrupting the balance with cell death.

Interestingly, other *in vitro* studies, particularly on tumor cells in other soft tissues have provided contrasting findings on the role that PTHrP plays in tumor dormancy. Administration of neutralizing antibodies against PTHrP (1-34) to mice inoculated with PTHrP-expressing orthotopic lung carcinomas significantly increases tumor growth [241]. In a later study by the same authors on human lung adenocarcinoma lines that are normally PTHrP-negative, ectopic expression of PTHrP (1-87) induces arrest in or slows progression through G1 compared with control cells [242]. Expression of cyclin D2 and cyclin A2 were also lower while expression of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor, was increased indicating that PTHrP inhibits the proliferation of lung tumor cells and may actually promote dormancy in this tumor model. It is interesting to note that in this study, as in the breast cancer study by Johnson et al. [226] discussed previously, there was no observed increase in cAMP production, making autocrine/ paracrine signaling via PTH1R unlikely. In addition, the plasmid for PTHrP (1-87) encodes a truncated protein lacking the NLS suggesting that this form of the protein may interact with other cytoplasmic factors to regulate

tumor cell proliferation. However, it is worth noting that peptides less than 50-60kDA such as PTHrP (1-87) can still passively enter the nucleus without an NLS [243], thus even truncated forms of PTHrP that lack the NLS may still localize to the nucleus. This further highlights the necessity of more studies to establish whether the mitogenic and dormancy effects of PTHrP depend on autocrine / paracrine, or intracrine mechanisms.

The studies on breast, prostate and lung cancer discussed in the previous sections do present mixed findings regarding the role of PTHrP in regulating tumor growth and dormancy. This would suggest that the actions of PTHrP are highly dependent on the tumor type and microenvironment. In the bone, tumor cell autonomous actions of PTHrP promote emergence from a quiescent state [169, 221, 237]. This may be complemented by paracrine actions of tumor-secreted PTHrP on bone marrow stromal cells like osteoclasts that promote the release additional pro-tumorigenic factors to further increase tumor growth. However, in tumors that arise in other soft tissues, the opposite may be true. This is also evident in another *in vivo* small cell lung cancer study where administration of an anti-PTHrP antibody significantly inhibits bone metastasis formation, but not metastasis to visceral organs (lungs, liver, kidneys, lymph nodes) [244]. This suggests that PTHrP may uniquely drive metastasis formation in the bone, but not other soft tissues. Clinical evidence of PTHrP's role in metastasis to bone versus soft tissues will be discussed further in later sections. This is particularly important as the success of PTHrP targeted therapies will depend on careful selection of patients with tumor types at highest risk for recurrence in organs where its expression actually drives exit from dormancy and metastatic outgrowth.

PTHrP's role in regulating tumor mass dormancy

In addition to modulating cellular dormancy, PTHrP's role in regulating angiogenesis and immunosurveillance, the two key mechanisms that characterize tumor mass dormancy, must also be considered. Angiogenesis is critical as tumors generally cannot exceed 2 to 3 mm in diameter without developing new blood vessels or co-opting pre-existing vasculature to avoid growth-limiting oxygen deprivation due to hypoxia and nutrient deprivation [245]. Importantly, the bone marrow is a physiologically hypoxic microenvironment [172, 173] and hypoxia is evident in most solid tumors [174]. Angiogenic dormancy results when insufficient vascularization induces cell death that counterbalances the rate of proliferation, resulting in no net growth of the tumor mass [205, 246]. Emergence from dormancy and tumor progression may resume after an "angiogenic switch" in which there is a shift in the balance between pro-angiogenic factors (e.g. vascular

endothelial growth factor (VEGF)) and anti-angiogenic factors (e.g. thrombospondin-1) [247]. Consequently, pro-angiogenic signaling dominates and new blood vessels form.

Several studies have investigated the effects of PTHrP on tumor-induced angiogenesis, though the results are conflicting. Early work by Bakre et al. demonstrated that PTHrP inhibits endothelial cell migration *in vitro* and angiogenesis in prostate tumors *in vivo* through activation of protein kinase A [248]. Consistent with this inhibitory effect, PTHrP reduces VEGF production during osteoblast differentiation and endochondral bone formation [249]. These results suggest that PTHrP may prevent tumor growth by inducing angiogenic dormancy. However, numerous other studies have demonstrated that PTHrP stimulates tumor-induced angiogenesis. PTHrP increases expression of pro-angiogenic factors including VEGF [250], and factor VIII [213] in breast cancer bone metastases. In prostate cancer cells PTHrP overexpression stimulates IL8 production, another key pro-angiogenic factor [251]. Malignant pituitary tumor cells that overexpress PTHrP also induce neovascularization in xenografts [252]. Mechanistically, recombinant PTHrP (1-34) increases capillary formation by endothelial cells through PTH1R activation and cAMP signaling.

Overall, these studies indicate that PTHrP promotes tumor-induced angiogenesis, making it plausible that the protein could act as a key negative regulator of tumor dormancy by stimulating new vessel formation. Conflicting findings are likely due to diversity within the tumor microenvironment where there are different target cells of PTHrP that each may individually regulate angiogenesis. Moreover, different domains and biologically active fragments of PTHrP likely will have differing effects on endothelial cells and other stromal cells during angiogenesis, but these studies did not explore differences between the different PTHrP isoforms. Lastly, it is important to note that while angiogenesis and angiogenic dormancy can be regulated by both hypoxia and PTHrP activity, PTHrP is also regulated by hypoxic signaling. Studies in chondrocytes determined that PTHrP expression is induced by hypoxia in a HIF1α (hypoxia inducible factor 1 alpha) and HIF2α dependent manner [253]. However, it has been show in prostate cancer cells that while HIF1α and HIF2α are both able to bind to the *PTHLH* promoter, only HIF2α induces transcription [254]. Since hypoxia has dual roles in both promoting and negatively regulating quiescence [255, 256], PTHrP's complex role in angiogenesis may be yet another mechanism by which low oxygen tensions differentially regulate tumor dormancy.

Immunosurveillance plays a well-characterized role in suppressing tumor growth and maintaining micrometastases in a dormant state [257]. Components of the adaptive immune system including CD4+ [258, 259] and cytotoxic CD8+ [260] T cells limit the outgrowth of dormant disseminated tumor cells [261]. Natural killer (NK) cells are a pivotal component of the innate

immune system that can maintain tumors in a dormant state via both their cytotoxic activity as well as stimulation of anti-tumorigenic cytokine production by CD4+ and CD8+ T cells [262, 263]. In contrast, regulatory T cells (Tregs) are associated with immune suppression and tumor progression in numerous cancer types [264, 265]. Lastly, the myeloid-derived suppressor cells (MDSCs) are a unique subpopulation of immature myeloid cells that can reactivate dormant disseminated tumor cells and support metastatic outgrowth by promoting immune suppression and angiogenesis [266, 267]. While few studies have examined the role of PTHrP in modulating tumor infiltration of each of these immune cell types, a few have specifically examined the MDSCs that are identified by the expression of myeloid cell (CD11b) and granulocytic (Gr-1) markers [268]. One study found that treatment with recombinant PTHrP or overexpression of the protein both promote the recruitment of $CD11b^{+}Gr1^{+}$ MDSCs into prostate tumor tissue where they increase primary tumor growth *in* vivo [269]. In the bone marrow, tumor-derived PTHrP also promotes recruitment and activation of $CD11b^{+}Gr1^{+}$ MDSCs, resulting in increased MDSC-derived MMP-9 expression, which drives prostate cancer invasion and angiogenesis. Similar findings were demonstrated in a separate study of murine mammary carcinoma where intratumoral $CD11b^{+}Gr1^{+}$ cell recruitment enhanced metastatic outgrowth via increased metalloproteinase activity [270]. $CD11b^{+}Gr1^{+}$ MDSCs derived from the bone marrow of breast tumor-bearing mice also have elevated expression of transforming growth factor β (TGFβ), a well-known potent stimulator of PTHrP expression, thus perpetuating the cycle of tumor-induced osteolysis that fuels tumor growth [271]. Taken together, these results suggest that PTHrP may play a critical role in negatively regulating tumor mass dormancy by increasing infiltration of immune suppressive MDSCs [272, 273]. PTHrP actions on recruitment of other immune populations in the tumor microenvironment have been inadequately explored. These studies are critical to gaining a more complete understanding of the role of PTHrP as a regulator of tumor mass dormancy.

Clinical Evidence for PTHrP Effects on Tumor Growth and Metastasis

Much like the *in vitro* and *in vivo* analyses, clinical studies investigating PTHrP as a prognostic factor have produced opposing findings, complicating the understanding of the role of the molecule in tumorigenesis, metastasis, and tumor dormancy. Henderson et al. conducted a large and comprehensive prospective study over 10 years in patients with breast cancer and found that positive immunohistochemical staining for PTHrP in 79% of the primary tumors was associated with significantly improved survival and decreased bone metastasis [274]. These results would suggest that PTHrP decreases the invasive capacity of breast tumor cells and is

protective against tumor growth in the primary site and formation of distant metastases. Interestingly, this study also revealed that of the 19 patients with bone metastases requiring surgical intervention, 7 patients had PTHrP-negative primary tumors. However, the majority of the individuals with PTHrP-negative primary tumors still developed PTHrP-positive bone lesions. All patients in the study with PTHrP-positive primary cancers also had positive expression in their bone metastases. Thus, there is not a clear inverse relationship between PTHrP expression at the primary and bone secondary sites. It is important to note this frequency of bone metastases in patients with PTHrP-negative primary breast cancers is still consistent with known tumorigenic roles for PTHrP when tumor cells colonize the bone later in disease progression [184, 185]. Thus, protective PTHrP actions early in tumorigenesis at the primary site are likely distinct from its deleterious effects once disseminated tumor cells reach the bone.

Another breast cancer study that aligns with the overall conclusions of Henderson et al. [274] found that PTHrP is downregulated in malignant compared with normal breast epithelia, but also low levels of nuclear localized PTHrP correlate with unfavorable clinical outcomes [275]. Mechanistically, the authors found a strong positive correlation between nuclear PTHrP levels and nuclear pStat5. This may explain, in part, why nuclear PTHrP is associated with the unfavorable clinical outcomes since loss of Stat5 expression and activation in breast cancer has consistently been associated with poor prognosis [276, 277]. Again, this observed progressive loss of nuclear PTHrP from well-differentiated mammary epithelia to poorly differentiated, aggressive cancer cells would suggest important context-dependent roles for PTHrP signaling in tumorigenesis. In early stages, intracrine signaling of nuclear PTHrP may be protective against malignant transformation, but in distant sites like the bone, reactivation of PTHrP can still induce extensive osteolysis that would drive metastatic tumor growth.

By contrast, numerous other clinical studies, especially in breast cancer, have concluded that PTHrP supports tumor growth and progression. In a large analysis including two genome-wide association studies from 41 case–control studies through the Breast Cancer Association Consortium (BCAC) and nine breast cancer genome-wide association studies, *PTHLH* was identified as a susceptibility locus in both ER+ and ER- breast cancer [278]. This study of patients with invasive breast cancer and ductal carcinoma *in situ* (DCIS) provides additional evidence implicating PTHrP in breast cancer pathogenesis, independent of its roles in promoting osteolysis. It is important to note that this analysis was performed on data from retrospective case-control studies enrolling multiple smaller patient cohorts. This factor should be kept in mind when comparing these findings with those of the better-powered, prospective study conducted by Henderson et al. [274] that identified PTHrP as a protective factor. In another study on patients

with ER+ and ER- breast cancer, expression of both PTHrP and its receptor correlated with reduced disease-free survival while receptor expression alone correlated with reduced overall survival [279]. In this study, PTHrP (1-34) expression was detected in 68% of primary tumor specimens compared with 100% of bone metastases and the PTHrP receptor was present in 37% of tumors compared with 81% of bone metastasis samples. Thus, PTHrP and its receptor are expressed more frequently in bone metastases than primary tumors. However, the functional relevance of this pattern of receptor expression in bone-disseminated tumor cells is still unclear since *in vitro* data indicate that in ER+ breast cancer cells, activation of PTH1R / cAMP signaling does not regulate dormancy gene expression [227]. Nevertheless, while expression of the receptor may not regulate dormancy in the bone, these clinical data still support the understanding that PTHrP expression by bone disseminated tumor cells is critical to their ability to establish metastatic colonies and exit from dormancy. Other studies have also confirmed a positive association between PTHrP expression in primary breast tumors and bone metastasis as well as shortened overall survival [280, 281].

Though in a different metastatic site, a recent study on early stage triple negative breast cancer (TNBC) found that PTHrP expression is significantly correlated with decreased central nervous system (CNS)-progression free survival [282]. These findings, if validated in other large cohorts of early-stage, newly diagnosed TNBC patients, would raise the hypothesis that monitoring PTHrP expression in TNBC patients could detect the initial stages of CNS metastasis and identify individuals with recurrent tumors earlier than conventional detection techniques. Interestingly, this study did not identify a statistically significant relationship between PTHrP expression and the incidence of bone metastasis. It is important to note that only specimens from patients with early stage TNBC without evidence of metastasis at presentation or multiple primary malignancies were analyzed. Thus, examination of patients with later staged cancer may also reveal a significant association between PTHrP expression and bone metastasis in TNBC. This highlights the importance of examining patients with all subtypes of breast cancer and stages of disease progression when investigating PTHrP as a prognostic factor.

Clinical evidence also exists suggesting a role for PTHrP in tumor growth and metastasis in other tumor types. In prostate cancer, PTHrP expression varies depending on the cancer stage, with expression detected in 33% of benign prostate hyperplasias, 87% of well-differentiated tumors and 100% of poorly differentiated and metastatic tumors [283]. Other studies have similarly found that PTHrP is expressed in prostatic bone metastases [284]. Here it seems that a progressive gain of PTHrP in disease progression is associated with tumorigenesis and distant metastasis. In a study of patients with early-stage lung adenocarcinoma, positive staining for

PTHrP (1-34) is associated with worse overall survival and metastasis-free survival, independent of tumor stage [285].

PTHrP as a Prognostic Factor and Dormancy Regulator

Given the conflicting data from both preclinical and clinical studies, a general consensus has not yet been reached regarding the role of PTHrP in tumorigenesis, metastasis, and tumor dormancy. However, there are numerous factors to consider when reconciling these findings. Stage of disease progression is critically important in this discussion. In general, the clinical data suggest that early in tumorigenesis at the primary site, PTHrP inhibits cancer growth and progression since its expression is associated with improved survival and decreased metastasis in patients with various tumor types [274, 282, 286, 287] (Figure 2). In these cases, tumor cell autonomous actions of PTHrP to alter cell proliferation may account for these findings [213]. Late in disease progression, after dissemination to the bone marrow, the growth of surviving tumor cells is driven by increased PTHrP production to stimulate osteoclast-mediated bone resorption, which releases pro-tumorigenic factors that further drive tumor growth and additional PTHrP secretion [184, 185] (Figure 3). These paracrine actions of PTHrP mediated by PTH1R signaling in osteoblasts are necessary for bone metastasis growth and would explain clinical findings that PTHrP is associated with reduced disease-free survival and metastasis formation [280, 281]. Lastly, the preclinical data clearly indicate that increased PTHrP expression drives breast tumor cells out of their quiescent state [169, 221, 226] via a mechanism independent of canonical PTH1R activation. Again, later in disease progression after long latency periods, increased PTHrP expression would favor exit from tumor dormancy in the bone and likely other metastatic sites (Figure 2). This hypothesis is supported by preclinical findings that PTHrP downregulates pro-dormancy gene expression [169], promotes proliferation, and inhibits apoptosis [206, 237] which are two key cellular responses that must be carefully balanced to regulate tumor dormancy.

As noted earlier, PTHrP is a molecule with multiple biologically active domains that control its autocrine / paracrine and intracrine actions. Each of these individual actions must be considered when interpreting data on PTHrP as a dormancy regulator and prognostic factor. Preclinical studies have directly demonstrated that manipulating the expression of different PTHrP isoforms elicits markedly different biological responses. A striking example of this comes from Deftos et al. [237] where mice injected with dormant prostate cancer cells expressing the full-length PTHrP (1–173) molecule developed more extensive bone lesions than those injected with PTHrP (1–87) which lacks the NLS, osteostatin region, and critical mitogen regulatory sequences contained in the carboxy terminus. Findings such as these can be accounted for by multiple

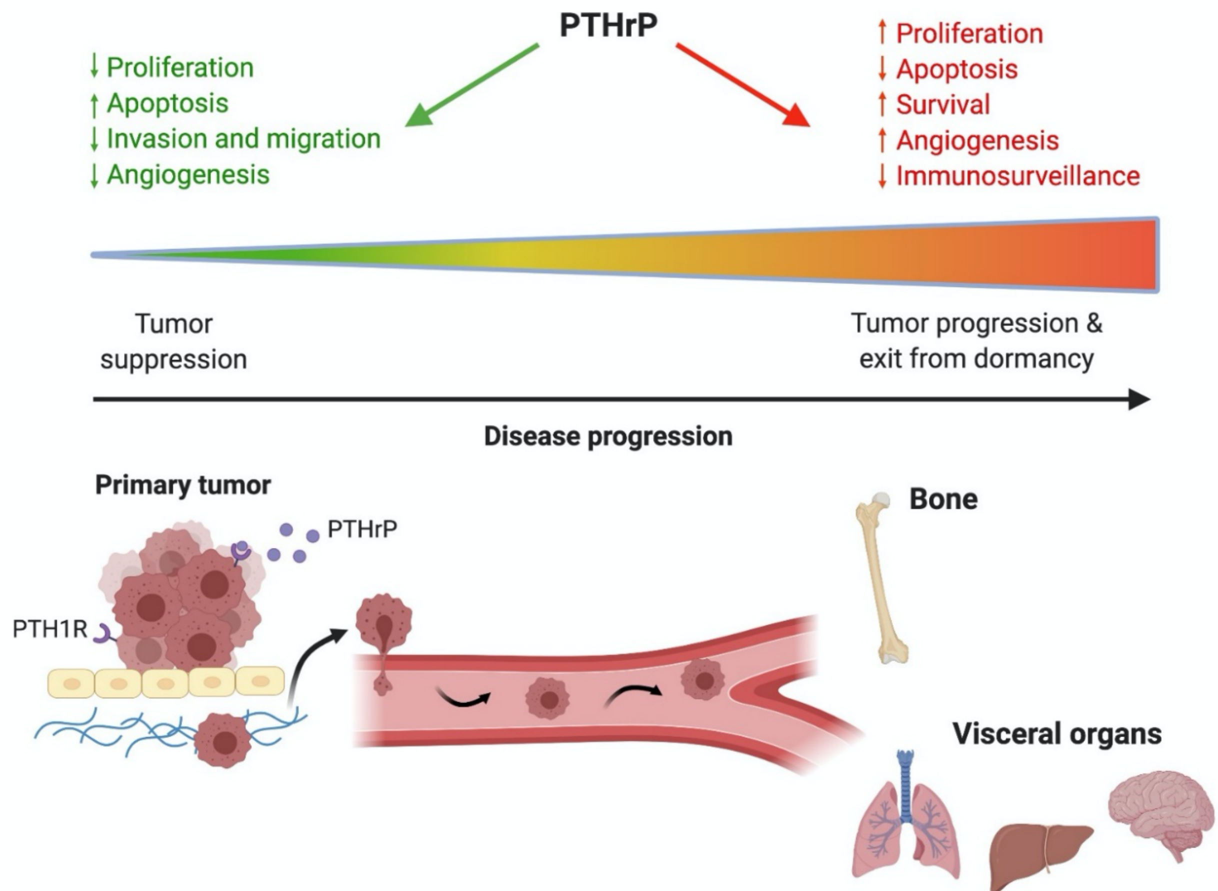

Figure 2. PTHrP has different actions throughout cancer progression. Early in tumorigenesis PTHrP is protective against tumor formation in the primary site by decreasing proliferation, promoting apoptosis, decreasing angiogenesis and reducing tumor cell invasion and migration. Late in disease progression when tumor cells disseminate to distant sites, PTHrP promotes tumor progression and exit from dormancy by stimulating proliferation and angiogenesis while reducing apoptosis and immunosurveillance. These actions in advanced stages of disease contribute to poor patient outcomes and reduced survival. PTHrP = parathyroid hormone-related protein.

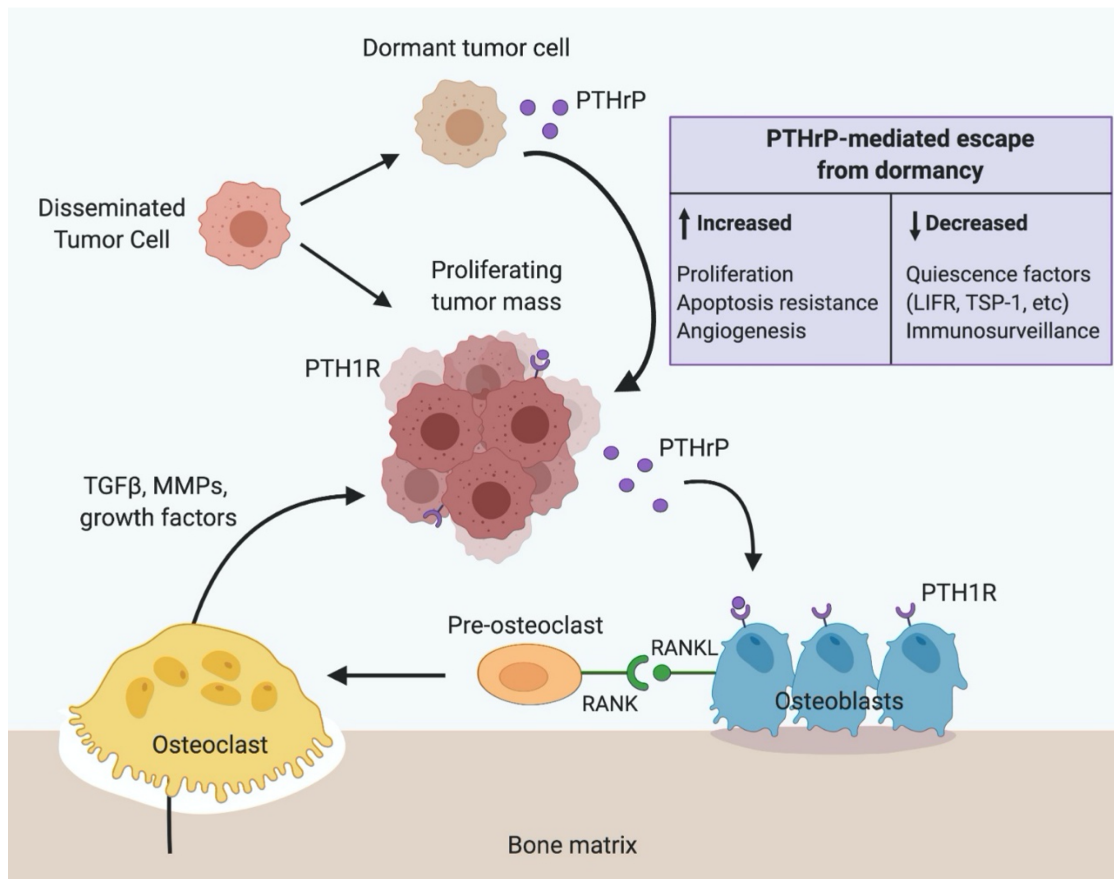

Figure 3. PTHrP dictates disseminated tumor cell fate in the bone to drive metastasis formation. Upon dissemination to the bone, surviving tumor cells can proliferate into a micrometastasis. Tumor cell secretion of PTHrP signals through the PTH1 receptor (PTH1R) on osteoblast lineage cells to stimulate RANKL production and osteoclastogenesis. Osteoclast-mediated resorption releases pro-tumorigenic factors from the bone matrix such as TGF-β, matrix metalloproteinases and other growth factors that further fuel tumor cell colonization, proliferation, and PTHrP production. Alternatively, disseminated tumor cells may instead enter a prolonged dormant state. PTHrP drives tumor cell escape from dormancy and metastatic outgrowth via multiple mechanisms: (1) increased proliferation, (2) apoptosis resistance, (3) increased angiogenesis, (4) decreased immunosurveillance and myeloid-derived suppressor cell recruitment, (5) decreased expression of known quiescence factors (e.g. LIFR). PTHrP = parathyroid hormone-related protein, PTH1R = parathyroid hormone-related protein type 1 receptor, RANKL = receptor activator of nuclear factor–kappa B ($NF_{\kappa}B$) ligand, LIFR = leukemia inhibitory factor receptor (LIFR), TSP-1 = thrombospondin-1.

factors. There are likely important functional elements in the region of PTHrP spanning amino acids 88–173 that uniquely promote tumor progression in bone but have not been fully elucidated. Furthermore, truncated forms of PTHrP may also assume different tertiary structures which alter binding to or interactions with other proteins that may drastically influence tumor cell behavior. Preclinical studies to further elucidate the biological activity of each PTHrP domain will be critically important to understanding the complexity of the molecule's effects in tumor development. In interpreting findings from clinical studies on survival and prognosis in human patients, a third factor to consider is the epitope used to define positive and negative expression, as nearly all of these analyses utilize immunohistochemistry to detect PTHrP. For instance, in their work on non-small cell lung cancer, Montgrain et al. [287] specifically investigated PTHrP (1-34) expression while Monego et al. [285] probed for PTHrP (109-141) and found complete opposite effects with regards to PTHrP as a prognostic indicator. Again, amino-terminal and carboxy-terminal PTHrP regions are known to induce disparate biological effects depending on the cell type and activation of autocrine / paracrine versus intracrine signaling. Due to posttranslational proteolytic processing, the mature PTHrP molecule can also give rise to multiple peptides with different biological activities. Fragments encompassing the amino terminal region (residues 1– 36), mid-molecule regions (38–94), (38– 95), and (38–101), as well as the carboxy terminal (107– 139) have been isolated from plasma [288] and urine of patients with HHM [289]. Thus, antibody selection is important to consider when drawing conclusions from clinical studies relying on immunohistochemistry to analyze PTHrP and any of its cleavage products as a prognostic factor.

PTHrP as a Therapeutic Target

Numerous studies have provided convincing evidence that PTHrP promotes tumor progression, and late recurrence by pushing tumor cells out of dormancy. Thus, PTHrP would seem to be a promising therapeutic target for treating advanced human cancers. Several animal studies have demonstrated reduced distant metastasis to bone with PTHrP small molecule inhibitors [290] and neutralizing antibodies [213, 291, 292]; however, human clinical data are lacking. Furthermore, there are several limitations in our current understanding of the biological activity of PTHrP that greatly complicate the development of safe and efficacious anti-PTHrP therapies. PTHrP is an incredibly complex peptide with multiple distinct domains that can each influence its actions as an endocrine, paracrine, autocrine and intracrine signaling molecule. This coupled with the fact that its different isoforms and fragments can elicit diverse cellular responses could result in PTHrP targeting therapies that inadvertently promote tumor growth and recurrence if used in the wrong patient population or stage of disease progression. This is especially true in

breast cancer, where preclinical and clinical data suggest that PTHrP inhibits early tumor progression, but promotes distant metastasis in advanced stages of disease [293]. Studies fully defining PTHrP's role in different stages of cancer and in tumor dormancy are needed in order to identify the appropriate therapeutic window for targeting PTHrP.

In addition to direct PTHrP inhibition, alternative approaches including targeting upstream regulators of the peptide's expression have been explored. As discussed previously [187], Wnt signaling drives PTHrP expression in highly osteolytic cancer cells and thus presents a potential therapy to prevent tumor-induced bone destruction and metastatic outgrowth. However, there are challenges to targeting Wnt therapeutically due to deleterious off-target, effects since signaling is critical during normal development and tissue homeostasis, especially bone formation [294-296]. However, the anti-tumor activity of Wnt inhibitors has been investigated and shown varying efficacy, primarily in preclinical gastrointestinal cancer models [297, 298]. In recent years, more cancer cell-specific molecular targets such as vacuolar-ATPase (v-ATPase) have been explored in the development of Wnt signaling inhibitors [299, 300]. Bafilomycin and concanamycin, which directly bind to and inhibit v-ATPase, markedly inhibit Wnt/ β-catenin signaling in colorectal cancer cells *in vitro* and reduce tumor cell proliferation *in vivo* without significant toxicity [299]. Selective inhibitors of Porcupine (PORCN), an acyltransferase that catalyzes post-translational modification and activation of WNT ligands, have also shown promising anti-tumor activity *in vivo,* while sparing WNT-dependent tissues [301, 302]. While inhibiting the Wnt pathway may be an effective therapy to decrease PTHrP expression for the treatment of metastatic cancers, more extensive investigation is needed to identify the most selective inhibitors and safest therapeutic window.

Alternative upstream targets include TGF-β which upregulates expression of Gli2 and in turn increases tumor secretion of PTHrP [303, 304]. Gli2 repression significantly reduces tumor-induced bone destruction mediated by TGF-β signaling in human breast cancer MDA-MB-231 cells [303]. Inhibitors against TGF-β and GLI proteins have been evaluated in clinical trials as anti-cancer therapy [305] [clinicaltrials.gov]. Another study demonstrated that the EGF receptor promotes PTHrP production, since treatment with erlotinib, an EGF receptor tyrosine kinase inhibitor, suppresses PTHrP expression in non-small cell lung cancer cells and reduces osteolysis [306]. Other EGF receptor tyrosine kinase inhibitors including gefitinib also reduce PTHrP levels [307]. Lastly, targeting downstream effectors of PTHrP may also provide an efficacious strategy. For instance, as mentioned previously, PTHrP (1-139) overexpression in MCF7 cells also represses expression and downstream signaling of LIFR, a known breast tumor suppressor and dormancy factor in the bone [175]. Consequently, LIFR downregulation promotes human MCF7 breast cancer cell emergence from dormancy in the bone. Treatment with the histone deacetylase

inhibitor valproic acid subsequently increases LIFR expression in human MCF7 breast cancer cells *in vitro,* suggesting that targeting LIFR, a downstream factor in PTHrP signaling may effectively maintain tumor cells in a dormant state to prevent metastatic outgrowth. Multiple strategies should therefore be considered to develop the most selective and effective PTHrP targeting therapies.

Summary and Study Aims

Breast cancer metastasis is a common occurrence and causes considerable morbidity and mortality in patients, but there is still no cure or prevention for metastatic disease. HDAC inhibitors have emerged as promising cancer therapeutics and are FDA-approved for some hematologic malignancies. However, despite some evidence of improved efficacy as combination therapy in preclinical and early clinical studies of solid tumors, they have unfortunately still failed in some late-stage trials. A better understanding of how the inhibitors influence tumor cell behavior in different microenvironments may help to predict the patient populations in which combination HDAC inhibitor therapies will be most efficacious. We sought to address this gap in knowledge in Chapter III by investigating whether and how HDAC inhibition stimulates expression of the tumor dormancy regulator, LIFR, when it is downregulated by hypoxia and PTHrP overexpression. These are two characteristics of the breast tumor and bone microenvironments that critically influence cancer cell signaling.

In Chapter IV, we investigate PTHrP further with the goal of understanding how its nuclear localization signal (NLS) and C-terminal domain alter breast tumor cell proliferation and growth. PTHrP is a complex protein with diverse effects on breast tumor cell behavior mediated by its different biological domains. Late in disease progression, PTHrP drives tumor-induced osteolysis, exit from dormancy, and bone metastasis formation. However, studies suggest that it functions in the opposite manner to inhibit tumor progression in early stages of disease, though the mechanisms are not well understood. Here, we establish that the PTHrP NLS and C-terminal domains differentially regulate breast tumor growth, in part, via a LIFR-mediated signaling cascade that alters cyclin-dependent kinase inhibitor expression. Finally, Chapter V discusses the future implications of Chapters III / IV and how these findings may give insight into additional targets to explore as anticancer therapeutics or help guide patient selection for existing treatments.

CHAPTER II

MATERIALS AND METHODS

Cells. Human MCF7 breast cancer cells were obtained from American Type Culture Collection (ATCC). Human bone-metastatic MDA-MB-231 cells (MDA-MD-231b) were established from a bone clone generated by the Mundy laboratory [308, 309]. Murine 4T1BM2 bone metastatic cells [310] were gifted by Dr. Normand Pouliot at the Peter MacCallum Cancer Centre. MCF7 PTHrP (-36-139) overexpressing cells were established by Pat Ho in the Martin Laboratory at St. Vincent's Institute of Medical Research as previously described for Ocy454 cells [311]. All cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Human T47D breast cancer cells were obtained from ATCC and cultured in RPMI 1640 medium supplemented with 10% FBS and 1% P/S. All cell lines were regularly tested for mycoplasma contamination and recently re-authenticated by ATCC.

PTHrP mutant cell lines were established in the Martin Laboratory at St. Vincent's Institute of Medical Research. Four *Pthlh* constructs were synthesized by Integrated DNA Technologies (IDT) (Coralville, IA, USA): *Pthlh*(-36-139), *Pthlh* (1-139), *Pthlh*(-36-67), *Pthlh*(-36-139DNLS). These were cloned into the plasmid murine stem cell virus (MSCV)-zeo by XhoI1/ EcoR1 enzyme digestion and ligation. The *Pthlh*(-36-139DNLS) construct omitted the sequence encoding residues 68 to 94. Each construct had a human influenza hemagglutinin (HA) epitope tag at the C terminus). DNA sequencing was undertaken by the Australian Genome Research Facility to confirm the sequences. Each mutant plasmid was used to transfect Phoenix cells. Viruses produced were used to infect MCF7 cells and Zeocin was used for selection and establishment of stable lines. Frozen aliquots of cells were thawed and grown first at the permissive temperature (33°C) for 2 to 3 days before transfer to 37°C for differentiation. PTHrP mutant cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). All cell lines were regularly tested for mycoplasma contamination.

Hypoxia. For HDAC inhibitor experiments, cells were seeded at 2×10^5 cells/ well (for RNA) or 1 $\times10^6$ cells/10cm^2 dish (for protein) in normoxia, allowed to adhere overnight, and placed into a hypoxia (0.5% or 1.0% O_2 as indicated) chamber (Invivo$_2$ Hypoxia Workstation 400) for 24 hours prior to initiating treatments as indicated in figure legends. Cells were harvested *in situ* for RNA with TRIzol (Life Technologies) or for protein with RIPA lysis buffer (Sigma) supplemented with protease and phosphatase inhibitors (Roche). For chromatin immunoprecipitation experiments,

cells were plated onto 500 cm^2 plates (~20–25 million cells/plate) and cultured overnight in normoxia before incubating in the hypoxia chamber at 0.5% O_2 for 24 hours.

HDAC inhibitor treatment. Cells were seeded in a 6-well plate ($2x10^5$ cells/well) for RNA analysis or 10cm plate ($1x10^6$ cells) for protein analysis and allowed to adhere overnight. Cells were then transferred into hypoxia (discussed above) for 24 hours prior to initiating drug treatments. Cells were treated with vehicle (dimethyl sulfoxide, DMSO), entinostat (0.5 μM, 5 μM; SelleckChem, Catalog No. S1053), or panobinostat (5 nM, 50 nM; SelleckChem, Catalog No. S1030) for 24 hours in full serum media.

RNA extraction and real-time qPCR. Cells were harvested for real-time qPCR as previously described [169]. Briefly, RNA was extracted from cells in a monolayer using TRIzol (Thermo Fisher) and DNase treated (TURBO DNA-free kit, Thermo Fisher) prior to synthesizing cDNA (1000ng RNA, iScript cDNA Synthesis Kit, Bio-Rad) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using iTaqTM Universal SYBR Green Supermix (Bio-Rad) on a QuantStudio 5 (Thermo Fisher) with the following conditions: 2 min at 50°C, 10 min at 95°C, (15 s at 95°C, 1 min 60°C) x 40 cycles followed by dissociation (15 s 95°C, 1 min 60°C, 15 s 95°C). Human primers for β2M [169], LIFR [169], p27 [225], PTHLH [312] and GAPDH [313] were previously published. The following primers were designed using PrimerBlast (NCBI) against the human genome (Homo sapiens) and validated by dissociation: p38α (F- CCCGAGCGTTACCAGAACC, R- TCGCATGAATGATGGACTGAAAT), p38β (F- AAGCACGAGAACGTCATCGG, R- TCACCAAGTACACTTCGCTGA), p21 (F- TGTCCGTCAGAACCCATGC, R- AAAGTCGAAGTTCCATCGCTC), PTHrP139aa (F- TCTCAGCCGCCGCCTCAAAA, R- AGAGAAGCCTGTTACCGT). Mouse primers for HMBS were previously published [169]. The following primers were designed using PrimerBlast (NCBI) against the mouse genome (Mus musculus) and validated by dissociation: GAPDH (F- AGGTCGGTGTGAACGGATTTG, R- GGGGTCGTTGATGGCAACA). PTHrP mid-region (F- CATCAGCTACTGCATGACAAGG, R- GGTGGTTTTTGGTGTTGGGTG), PTHrP NLS (F- AACAGCCACTCAAGACACCC, R- GACCGAGTCCTTCGCTTCTT), PTHrP C-terminal region (F- AAAAGAAGCGAAGGACTCGG, R- GCGTCCTTAAGCTGGGCT).

Western blotting*.* Cultured cells were rinsed with cold 1X PBS and harvested in RIPA lysis buffer (Sigma) supplemented with protease and phosphatase inhibitors (Roche). A BCA assay (Thermo Fisher) was used to determine the protein concentration and 20-50 µg protein was loaded onto

an SDS-PAGE gel under reducing conditions and transferred to nitrocellulose membranes. Membranes were probed with antibodies against HIF1α (R&D Systems, Catalog No. MAB1536, 1:1000), HA-Tag (Cell Signaling, C29F4, Catalog No. 37T4S, 1:1000), LIFR (Santa Cruz, C-19, Catalog No. sc-659, 1:1000), p21Waf1/Cip1(Cell Signaling, Catalog No. 2947S, 1:1000), p27 Kip1 (Cell Signaling, Catalog No. 3686S, 1:1000), phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling, Catalog No. 4511, 1:1000), p38 MAPK (Cell Signaling, Catalog No. 8690, 1:1000), phospho-ERK1/2 Thr202/Tyr204 (Cell Signaling, Catalog No. 9101, 1:1000), ERK1/2 (Cell Signaling, catalog number 9102, 1:1000), Calnexin (AbCam, Catalog No. ab22595-100UG, 1:900), GAPDH (Cell Signaling, 14C10, Catalog No. 2118S, 1:5000), α-tubulin (Antibody & Protein Resource at Vanderbilt University, Catalog No. VAPRTUB, 1:5000), or Vinculin (Millipore, Catalog No. AB6039, 1:1000).

Chromatin Immunoprecipitation and qPCR. Cells were plated onto 500cm^2 plates (~20-25 million cells per plate) and incubated in hypoxia (0.5%) as described above. Chromatin was prepared as previously described [314]. Briefly, cells were fixed with 7% formaldehyde and quenched with 2.5M glycine *in situ.* Following, glycine was replaced with cold 1X PBS and cells were removed from hypoxia and lysed with Farnham lysis buffer (5 mM HEPES pH 8.0, 85 mM KCL, 0.5% NP-40, PIC) followed by incubation with nuclei lysis buffer (50 mM Tris-HCL pH 8.0, 10 mM EDTA pH 8.0, 1% SDS). Chromatin was sonicated and then diluted with ChIP Dilution Buffer (50 mM Tris-HCl pH 8.0, 0.167 M NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate), RIPA-150, 50X protease inhibitors, and 1M sodium butyrate. Magnetic anti-rabbit Dynabeads (Thermo Scientific) linked to acetylated histone H3 (Lys9) antibody (Millipore, Catalog No. 07-352) or tri-methylated histone H3 (Lys9) antibody (Abcam, Catalog No. ab8898) were incubated with chromatin overnight at 4°C. The following day immunoprecipitates were washed with RIPA-150, RIPA-500, RIPA-LiCl, and TE for 5 minutes each. After elution, immunoprecipitates were treated with RNase A (Qiagen) followed by proteinase-K (Sigma). DNA was purified by phenol-chloroform extraction and ethanol precipitation, then quantified using Qubit, and analyzed by real-time qPCR. The fold enrichment was calculated using 2ΔCt (threshold cycle) and normalized to input DNA Ct values and then to Ct values from IgG-coated beads as a negative control. Previously published primers were used for the *LIFR* promoter [315, 316].

Immunocytochemistry. For analysis of HA-tagged PTHrP peptides, cells were seeded onto a 4-well culture slide at 6x10^5 cells/ well and allowed to adhere overnight. The following day cells were washed twice with 1x PBS and fixed with 10% formalin for 15 minutes. Cells were then washed

three times with 1X PBS for 5 minutes each, permeabilized in 0.25% Triton-X in 1X PBS for 10 minutes and washed twice with 1X PBS for 5 minutes each. Next cells were blocked in a 3% mix of donkey horse serum (DHS)/ bovine serum albumin (BSA) for 1 hour at room temperature, washed twice with 1X PBS for 5 minutes each and finally incubated with HA-Tag antibody (Cell Signaling, C29F4, Catalog No. 37T4S, 1:500) diluted in DHS/ BSA mix for 1 hour at room temperature. Afterwards, cells were washed three times with 1X PBS for 5 minutes each and incubated in goat anti-rabbit IgG (H+L) Alexa Fluor 488 secondary antibody (Thermo Fisher, Catalog No A-11034, 1:1000) diluted in DHS/ BSA mix in the dark for 1 hour at room temperature. Cells were then washed 3 three times with 1X PBS for 5 minutes each. Lastly, the chamber was removed from each slide before mounting coverslips with VECTASHIELD HardSet Antifade Mounting Medium with DAPI (Vector Laboratories). Fixed cells were imaged on a laser scanning confocal microscope Nikon A1r based on a TiE motorized Inverted Microscope using a (1) 60X lens, NA 1.4, run by NIS Elements C software with sections imaged in 0.23μm slices or (2) 100X lens, NA 1.49, run by NIS Elements C software with sections imaged in 0.23μm slices.

For analysis of p21 and p27, $8x10^5$ cells were seeded onto glass coverslips coated with 5 µg/ml human fibronectin (Millipore) 1-2 hours prior. The following day, cells were washed with 1X PBS, fixed with 10% formalin for 15 minutes, washed three times with 1X PBS for five minutes each and permeabilized with 0.25% Triton-X for 10 minutes. Afterwards, cells were washed twice with 1X PBS for 5 minutes each and blocked with DHS/ BSA mix at room temperature for 1 hour at room temperature. Cells were then washed twice with 1X PBS for 5 minutes each and incubated in p21Waf1/Cip1(Cell Signaling, Catalog No. 2947S, 1:1000) or p27 Kip1 (Cell Signaling, Catalog No. 3686S, 1:1000) diluted in DHS/BSA mix for 1.5 hours at room temperature. Afterwards cells were washed three times with 1X PBS for 5 minutes each and incubated in goat anti-rabbit IgG (H+L) Alexa Fluor 488 secondary antibody (Thermo Fisher, Catalog No A-11034, 1:1000) diluted in DHS/ BSA mix in the dark at room temperature. Lastly, cells were washed three times with 1X PBS for 5 minutes each before mounting on glass slides with VECTASHIELD HardSet Antifade Mounting Medium with DAPI (Vector Laboratories). Images were collected on an Olympus BX41 Microscope equipped with an Olympus DP71 camera using the 40X plain objective. For p21 quantitation in Image J, total nuclei and positive staining cells were counted manually to calculate the percent of positive staining cells. For p27, the fluorescence intensity was quantified using ImageJ with manual cell contouring and measurement of the Raw Integrated Density which was averaged across all cells from 3 separate images.

Proliferation assays. Cells were plated onto 6-well plates at $1*10^6$ cells/ plate and allowed to adhere for 4-6 hours. Adherent were then trypsinized and mixed with 0.4% trypan blue solution. Viable cells were determined based on dye exclusion and counted using a TC20 Automated Cell Counter (Bio-Rad). Proliferation of PTHrP mutant cells was monitored daily for four days by repeatedly trypsinizing cells, counting viable cells by trypan blue exclusion, and reseeding of equal cell numbers onto new plates.

Animal studies and imaging

Animals. Experiments were performed under the regulations of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals and approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC). For the intracardiac inoculation studies, 4-6-week-old female athymic nude mice (Jackson, Catalog No. 7850) were injected with $1x10^5$ MCF7 tumor cells as previously described [317] (n=8-10 mice injected per group). The mice were subcutaneously implanted with a slow-release 17β-estradiol pellet (0.36mg/pellet; Innovative Research of America, Catalog No. SE-121) 24 hours prior to tumor cell injection [317].

For the mammary fat pad study, 17β-estradiol pellets (0.36mg/pellet; Innovative Research of America, Catalog No. SE-121) were subcutaneously implanted into female athymic nude mice 24 hours prior to tumor inoculation [317]. The following day, $5x10^5$ tumor cells in 20μl PBS+50% matrigel (Fisher Scientific) were inoculated into the fourth mammary fat pad (n=10 mice injected per group). Tumor volume was assessed by caliper measurement. Multiple mice had to be sacrificed early due to estrogen-induced toxicities resulting in MSCV= 8 mice, FLSEC=7 mice, DNLS= 10 mice, DNLS+CTERM= 9 mice in the final analysis.

Radiography. Radiographic (x-ray) images were obtained as previously described [318]. Briefly, a Faxitron LX-60 (34kV for 8 seconds) was used to acquire x-ray images and images were quantified for osteolytic lesion number and area using ImageJ software.

Histology. Upon sacrifice of the mice, dissected tumors were fixed in 10% formalin for 48 hours and stored in 70% ethanol until being embedded in paraffin sectioned for further analyses. Dissected hind limbs were fixed in 10% formalin for 48 hours and stored in 70% ethanol until decalcification in EDTA (20% pH 7.4) solution for 72 hours. Decalcified bones were embedded in paraffin and 5-μM thick sections were prepared for further analyses.

Tissue sections were deparaffinized by heating the slides to 50°C and placed in xylene for 5 minutes and then 3 minutes. Next, slides were soaked in 100%, 95%, and then 75% ethanol for

3 minutes each. Slides were slowly changed to deionized water and rinsed twice in water. The slides were immersed in 10 mM TRIS (pH 9.0) and 1 mM EDTA heated to 150°C for 20 minutes. After cooling at room temperature for 20 minutes, slides were rinsed twice with water and then three times with 1X PBS followed by blocking with 10% BSA in PBS for 2 hours. Sections were stained with pan-cytokeratin (Sigma Cat# F0397, 1:50), Ki67 (Thermo Fisher; Catalog No. RM9106S0, 1:500), cleaved PARP (Asp214) (Cell Signaling Technology, Catalog No. 5625T, 1:500), HA-Tag (Cell Signaling, C29F4, Catalog No. 37T4S, 1:1000), p21Waf1/Cip1(Cell Signaling, Catalog No. 2947S, 1:1000), or p27 Kip1 (Cell Signaling, Catalog No. 3686S, 1:1000) in 3% BSA in PBS overnight at 4°C. The following day, sections were washed three times with 1X PBS and coverslips mounted using VECTASHIELD HardSet Antifade Mounting Medium with DAPI (Vector Laboratories). All images except for Ki67 were collected on an Olympus BX41 Microscope equipped with an Olympus DP71 camera using the 10X, or 40X, plain objectives. For LIFR quantitation, the 40X images were used and an area measuring 1900x1180 pixels was selected to measure the Raw Integrated Density. The Raw Integrated Density from 3 representative images was averaged for each mouse and these values are reported in the figure. For p21, p27, and cleaved PARP, the quantitation was performed using ImageJ analysis of the 40X images. Positive staining nuclei and cell counts were determined using color thresholding in ImageJ and the number of positive staining nuclei was divided by the total number of nuclei present to calculate the percent positivity. For Ki67 quantification, the 40X images were used for the quantitation. Fixed samples were imaged on a laser scanning confocal microscope Nikon A1r based on a TiE motorized Inverted Microscope using a 60X lens, NA 1.4, run by NIS Elements C software. Sections were imaged in 0.4μm slices. Positive staining nuclei and cell counts were determined using color thresholding in ImageJ and the number of positive staining nuclei was divided by the total number of nuclei present to calculate the Ki67 positivity.

For immunohistochemistry analyses, forty-five minutes prior to sacrifice, mice were intraperitoneally injected with 60mg/kg pimonidazole (PIMO, Hypoxyprobe Kit, Hypoxyprobe, Inc, Catalog Number HP1-1000Kit). Hind limbs were dissected, fixed, and paraffin embedded as described above. Sections were deparaffinized in xylene twice for 2 minutes, then soaked twice in 100% ethanol for 2 minutes each followed by, 95% ethanol, 70% ethanol, and deionized water for 1 minute each. Slides were then immersed in 3% H_2O_2 in water for 15 minutes, slowly changed to deionized water and soaked in water for 5 minutes followed by blocking with DAKO serum free protein blocker (Agilent Technologies (DAKO), Catalog No. X090930-2) for 5 minutes. Sections were then stained with Rabbit anti-PIMO in DAKO overnight at 4°C. The following day, sections were washed three times with 1X PBS for 5 minutes each, incubated with Biotin anti-rabbit 1:200

in DAKO for 30 mins at 37°C, washed again three times with 1X PBS for 5 minutes each, incubated in streptavidin peroxidase for 30 minutes at 37°C and rinsed a final time with 1X PBS. Sections were then incubated with 3-diaminobenzidine (DAB) for 45 seconds to 1 minute. Following, sections were washed in deionized water for 5 minutes, counterstained with hematoxylin for 45 seconds and rinsed 5 times in deionized water. Lastly, sections were dehydrated in 70% ethanol for 1 minute, 90% ethanol for 1 minute, 95% ethanol three times for 1 minute each, 100% ethanol three times for 1 minute each, and xylene 3 times for 1 minute each. Coverslips were mounted with permount (Fisher Scientific, Catalog No. SP15-500). All images were collected on an Olympus BX41 Microscope equipped with an Olympus DP71 camera using the 10X, 20X, or 40X, plain objectives.

Flow Cytometry. One hindlimb was crushed with a mortar and pestle to obtain the bone marrow. PBS (1mL) was added to the crushed bone marrow and were spun down and washed with PBS to remove bone debris. Bone marrow (5 x 10^5 cells) was stained in 100µL of PBS with LIVE/DEAD™ Fixable Green Dead Cell Stain Kit @488nm (Thermo Fisher Scientific, Catalog Number L34970, 1:1000) for 15 minutes on ice at 4°C in the dark. Cells were washed with PBS and resuspended with 100µL of 1% BSA in PBS with CD298 antibody (BioLegend, Cat #341704) for 30 minutes on ice at 4°C in the dark.

Flow Cytometry Analysis. Flow cytometry experiments were performed in the VUMC Flow Cytometry Shared Resource using the 5-laser BD LSRII and 4-laser BD Fortessa LSRII. Data was analyzed using FlowJo software (FlowJo, LLC) where bone marrow samples were gated based on forward scatter and side scatter geometry and PE-CD298 (+) cells were gated using live cells (LIVE/DEAD-Green negative). MCF7 breast cancer cells were used as a positive control for CD298 stain.

Statistics and reproducibility. For all experiments, *n* per group is as indicated by the figure legend and the scatter dot plots indicate the mean of each group and error bars indicate the standard error of the mean. All graphs and statistical analyses were generated using Prism software (Graphpad). Statistical significance for all *in vitro* and *in vivo* assays was analyzed using an unpaired t-test, one-way ANOVA with Sidak's multiple comparisons test or two-way ANOVA with multiple comparisons, as indicated in the figure legends. For each analysis $p < 0.05$ was considered statistically significant, and *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

CHAPTER III

HDAC INHIBITORS STIMULATE LIFR WHEN IT IS REPRESSED BY HYPOXIA OR PTHRP IN BREAST CANCER

The work presented in this chapter is published and adapted from:

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Summary

Breast cancer cells frequently disseminate to the bone marrow, where they either induce osteolysis or enter a dormant state. Downregulation of leukemia inhibitory factor receptor (LIFR), a known breast tumor suppressor, enables otherwise dormant MCF7 human breast cancer cells to become aggressively osteolytic. Hypoxia (low oxygen tensions), which may develop in tumors as a pathological response to the metabolic demands of the proliferating cells and as a physiological state in the bone, downregulates LIFR in breast cancer cells independent of hypoxia-inducible factor (HIF) signaling. However, the mechanism by which LIFR is repressed in hypoxia is unknown. Histone deacetylase (HDAC) inhibitors stimulate LIFR by increasing histone acetylation in the proximal promoter and induce a dormancy phenotype in breast cancer cells inoculated into the mammary fat pad. We therefore aimed to determine whether hypoxia alters histone acetylation in the LIFR promoter, and whether HDAC inhibitors effectively stimulate LIFR in breast cancer cells residing in hypoxic microenvironments. Herein, we confirmed that disseminated MCF7 cells became hypoxic in the bone and that hypoxia increased the epigenetic transcriptional repressor H3K9me3 in the proximal LIFR promoter while H3K9ac, which promotes transcription, was significantly reduced. Furthermore, HDAC inhibitor treatment rescued hypoxic repression and dramatically increased expression of LIFR, p38β, and p21, which regulate tumor dormancy. In a second model of LIFR repression, in which parathyroid hormone-related protein (PTHrP) suppresses LIFR expression, we found that PTHrP localizes to the LIFR promoter, and that PTHrP suppression of LIFR protein is similarly reversed by HDAC inhibitor treatment. Together, these data suggest that HDAC inhibitors stimulate LIFR regardless of the way it is repressed by the microenvironment.

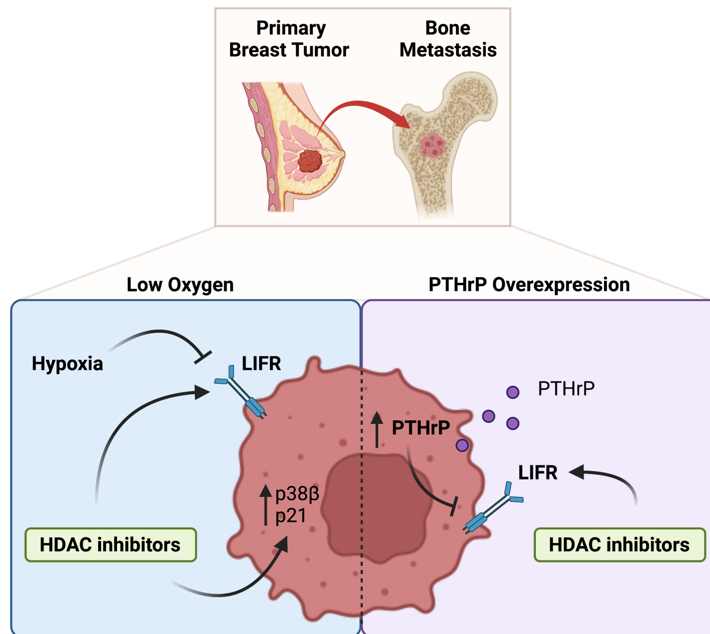


Figure 4. Graphical summary representing stimulation of LIFR repressed by hypoxia and PTHrP overexpression in the primary tumor or bone.

Introduction

Metastasis to the bone is a leading cause of morbidity and mortality in patients with breast cancer [319, 320]. Disseminated tumor cells (DTCs) may home to distant organs including the bone and proliferate into a macrometastasis or enter a prolonged quiescent state before developing into a clinically detectable metastasis [4]. Dormant tumor cells may persist as quiescent solitary cells, or as micrometastases with limited growth capacity due to balanced proliferation and apoptosis [5-7]. Clinically, patients with estrogen receptor positive (ER+) breast tumors and no nodal involvement at diagnosis have an approximate 20% risk of developing distant metastases 5-20 years after primary diagnosis, suggesting prolonged periods before dormant DTCs resume proliferating [8]. Despite improvements in standard-of-care therapies and the use of anti-resorptive agents like bisphosphonates and denosumab, which reduce skeletal related events due to tumor-induced bone disease, there is no cure or prevention for metastatic disease.

Histone deacetylase (HDAC) inhibitors have emerged as attractive cancer therapeutics since they alter the expression of genes that regulate cell cycle progression, apoptosis, angiogenesis, and immune surveillance [321-323]. Multiple HDAC inhibitors are approved by the United States Food and Drug Administration for hematological malignancies [324-326]. While single-agent therapy for solid tumors has limited efficacy [316], when combined with other therapies, HDAC inhibitors showed considerable promise in early preclinical and clinical studies [315, 321, 327]. Entinostat and panobinostat, both pan-HDAC inhibitors, are still being investigated in clinical trials as combination therapy for metastatic breast cancer [clinicaltrials.gov]. Despite some evidence of improved efficacy as combination therapy in preclinical and early clinical trials, a recent phase III study conducted by the ECOG-ACRIN Cancer Research Group investigating entinostat combined with exemestane in aromatase inhibitor-resistant metastatic breast cancer saw no improvement in progression-free or overall survival [328]. This occurred despite confirmation of increased lysine acetylation levels in peripheral blood mononuclear cells. Failure of this study to meet its primary endpoint after favorable phase II trial results highlights the importance of better understanding the mechanism by which HDAC inhibitors influence tumor cell behavior. Such insights could explain why some HDAC inhibitors failed in clinical trials for metastatic breast cancer and help inform current and future clinical trials. This knowledge may also help identify biomarkers to predict which breast cancer patients could benefit from HDAC inhibitor treatment and which combination therapies will be most efficacious.

Leukemia inhibitory factor receptor (LIFR) is a breast tumor dormancy regulator [169, 314], breast tumor suppressor, and lung metastasis suppressor [314, 329, 330]. In preclinical metastasis models, downregulation of LIFR enables otherwise dormant breast tumor cells to become more proliferative [169, 329, 330] and osteolytic in the bone [169]. In patients, loss of LIFR is correlated with poor survival [169]. While the mechanisms have not been fully elucidated, it was previously determined that hypoxia, or low oxygen tensions, represses LIFR mRNA and protein levels as well as promoter activity in breast cancer cells independent of hypoxia-inducible factor (HIF) signaling [169]. Importantly, hypoxia is evident in most solid tumors and in the bone marrow, which has regions as low as 0.5-1% pO_2 despite extensive vascularization [331], suggesting that tumor cells residing in hypoxic microenvironments may express lower levels of the LIFR tumor suppressor. We recently described the direct epigenetic induction of *LIFR* and a pro-dormancy phenotype in breast cancer cells treated with HDAC inhibitors [314], indicating that the inhibitors may be a potential therapy to promote tumor dormancy and prevent recurrence in patients with breast cancer. Here, we sought to explore whether HDAC inhibitors can overcome LIFR repression driven by hypoxia, which tumor cells are likely to encounter in the primary tumor and bone. Such findings may provide insights into whether HDAC inhibitors remain a viable option to induce long-term breast tumor dormancy and prevent recurrence.

In addition to hypoxia, another mechanism by which LIFR is downregulated is through overexpression of parathyroid hormone-related protein (PTHrP, gene name *PTHLH*) which drives tumor-induced bone disease (TIBD) [189, 293, 331]. Bone DTCs secrete PTHrP, which induces osteolysis via paracrine activation of the PTHR1 receptor on osteoblasts [332]. In addition to its role in TIBD, PTHrP has been identified as a novel regulator of breast tumor dormancy. Overexpression of PTHrP in ER+ human MCF7 breast cancer cells, which lie dormant *in vivo* following intracardiac injection [169, 222, 333], switches these cells to a highly osteolytic phenotype and dramatically increases bone tumor burden [221], independent of cAMP signaling [226]. Consistent with the enhanced bone colonization and exit from dormancy in bone [221], PTHrP overexpression also represses LIFR expression and downstream signaling [169]. PTHrP is an important regulator of breast oncogenesis as its expression is detected in most primary breast tumors [274, 279]. Thus, PTHrP repression of LIFR may also influence tumor cell behavior in the primary tumor site, in addition to the bone. It is unknown whether HDAC inhibition can overcome PTHrP-mediated LIFR repression.

Our goal in the present study was to determine whether HDAC inhibition reverses LIFR repression driven by hypoxia and PTHrP overexpression, two characteristics of the breast tumor and bone microenvironments that critically influence cancer cell signaling. Our findings suggest

that the HDAC inhibitors entinostat and panobinostat retain their ability to robustly stimulate LIFR in both settings of repression.

Results

LIFR expression is epigenetically downregulated in breast cancer cells cultured in hypoxia

Since hypoxia downregulates *LIFR* expression in breast cancer cells *in vitro* [169], we first sought to confirm whether disseminated breast tumor cells encounter hypoxia in the bone. Immunostaining for pimonidazole (a hypoxia marker) and cytokeratin (to detect human tumor cells) was performed on serial tibia sections from mice inoculated with human MCF7 breast cancer cells by intracardiac injection. Approximately half of the disseminated breast cancer cells stained positive for pimonidazole (Figure 5 & 6), indicating that bone-disseminated tumor cells may transition to a hypoxic state. Given that hypoxia reduces *LIFR* expression and promoter activity [169] and that HDAC inhibitors have been shown to promote LIFR expression through increased acetylation of histone H3 lysine 9 (H3K9) [314], we next determined whether hypoxia regulates *LIFR* by altering histone acetylation or methylation along the proximal promoter. Lysine acetylation (e.g. H3K9ac) enhances gene expression by weakening the interaction between nucleosomes that comprise chromatin to make target sequences accessible for transcription [32]. Some histone methylation marks, particularly H3K9 trimethylation (H3K9me3), are strongly correlated with gene silencing by recruiting other methyl-binding proteins that inhibit transcriptional elongation [334]. To examine these modifications, we performed ChIP-qPCR for H3K9me3, which represses transcription, and H3K9ac, which activates transcription, along the *LIFR* promoter in MCF7 cells cultured in hypoxia. The LIFR proximal promoter regions were selected from previously published works [314, 316]. H3K9me3 was specifically enriched in region 3 of the *LIFR* proximal promoter [314] in MCF7 cells cultured in hypoxia (0.5% pO_2) compared to MCF7 cells cultured in normoxia (Figure 7A). Simultaneously, H3K9ac was significantly reduced in MCF7 cells cultured in hypoxia in the same promoter regions (Figure 7B). These data suggest that hypoxia initiates multiple histone modifications that result in downregulation of LIFR in breast cancer cells [169].

HDAC inhibitors stimulate expression of LIFR and other pro-dormancy genes in hypoxia

Since we previously demonstrated that HDAC inhibitors induce LIFR mRNA and protein as well as enrich for H3K9ac along the *LIFR* promoter of MCF7 cells in normoxia [314], we next

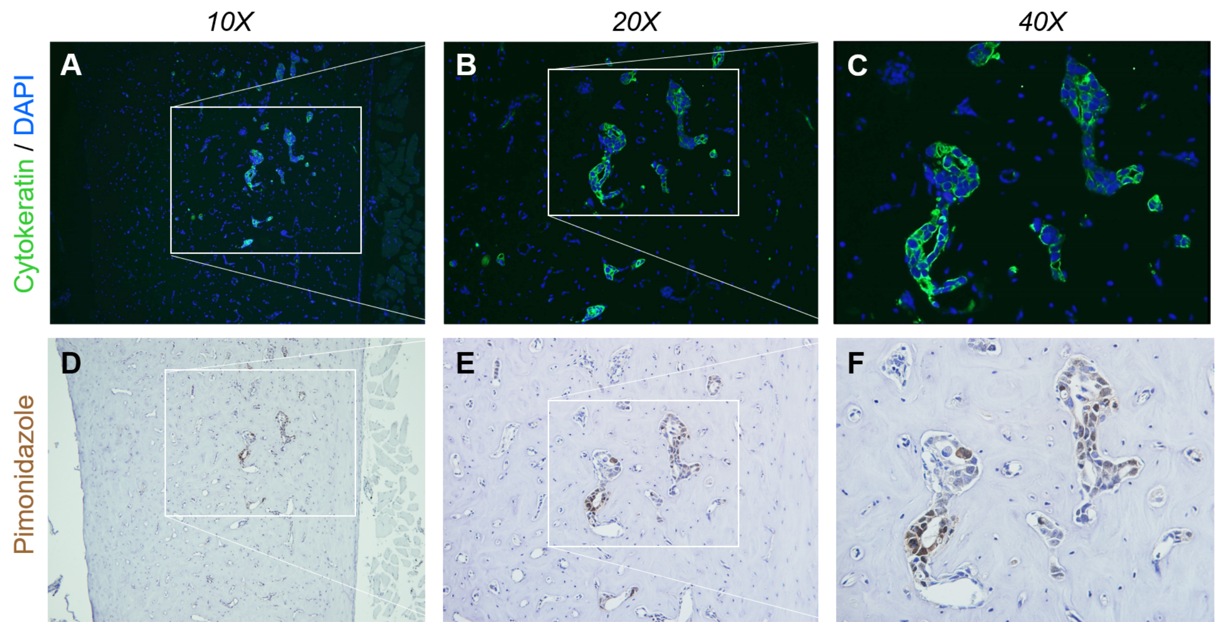


Figure 5. Bone-disseminated breast cancer cells experience hypoxia. MCF7 breast cancer cells in the bone marrow following intracardiac inoculation were stained for (A-C) cytokeratin and DAPI to detect tumor (left to right: 10X, 20X, 40X) and (D-F) pimonidazole for hypoxia (left to right: 10X, 20X, 40X).

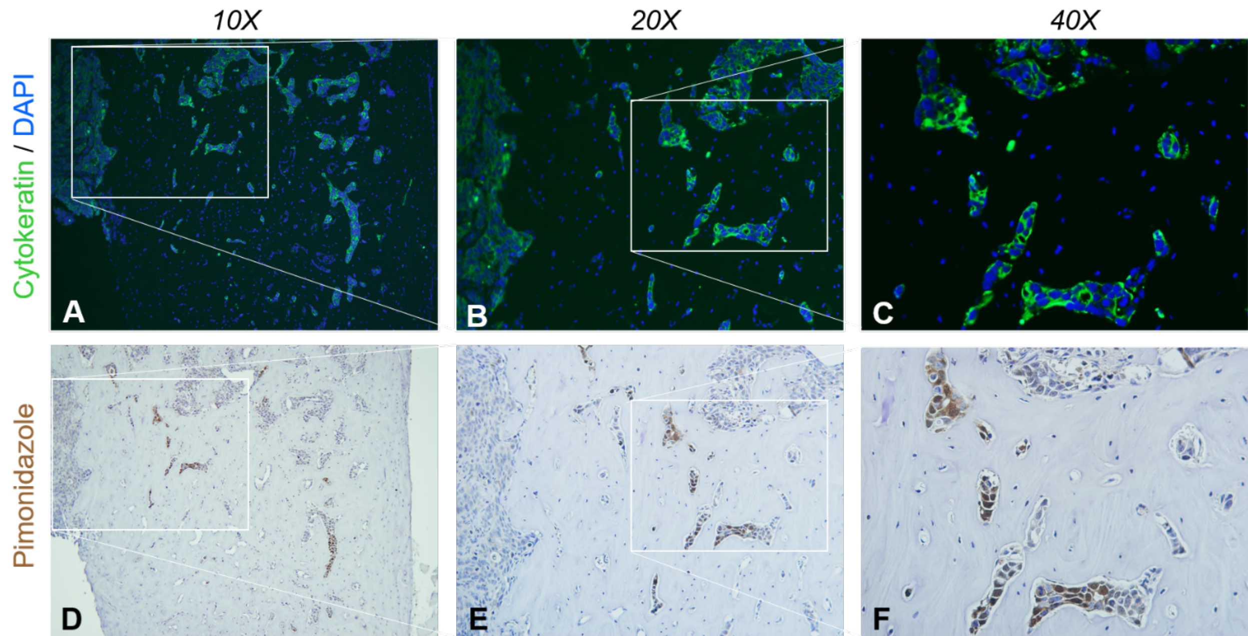


Figure 6. Bone-disseminated breast cancer cells experience hypoxia. Additional example of MCF7 breast cancer cells in the bone marrow following intracardiac inoculation. Bones were stained for (A-C) cytokeratin and DAPI to detect tumor (left to right: 10X, 20X, 40X) and (D-F) pimonidazole for hypoxia (left to right: 10X, 20X, 40X).

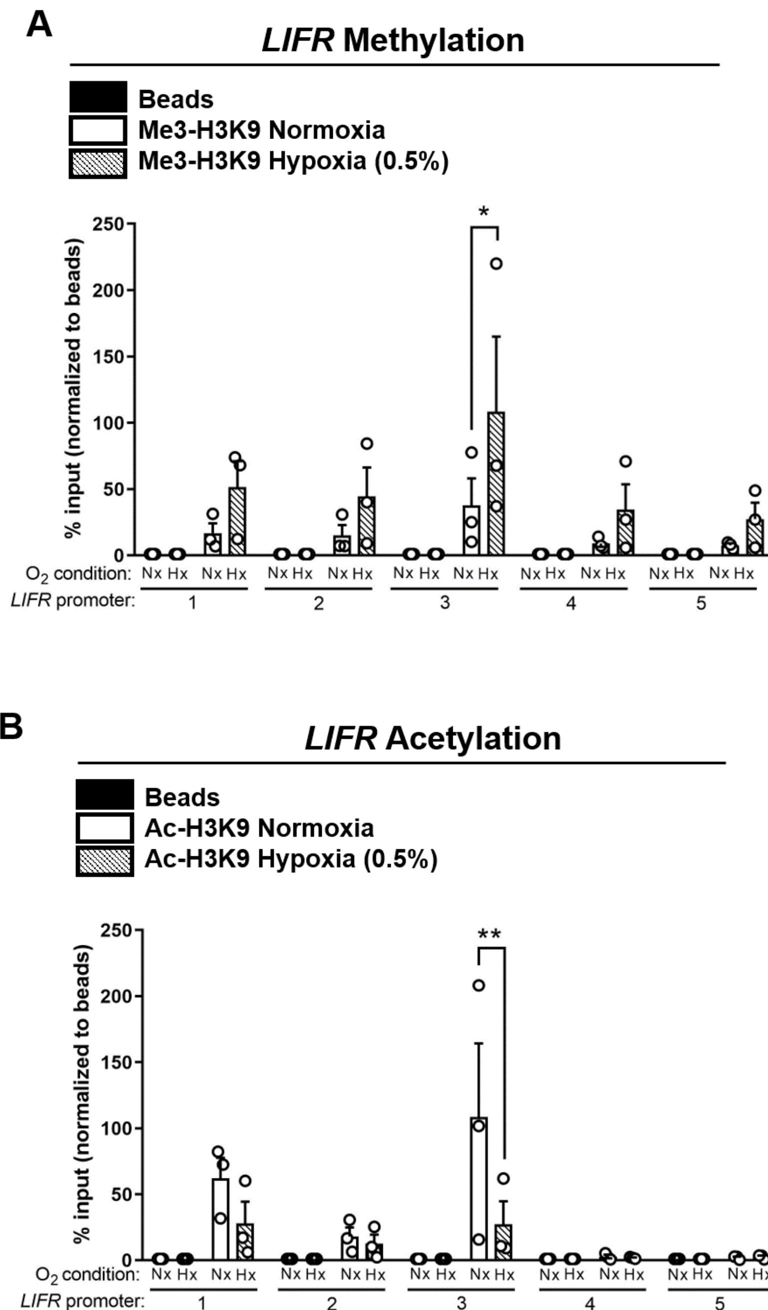


Figure 7. Hypoxia leads to chromatin remodeling in the *LIFR* promoter. Chromatin immunoprecipitation (ChIP) with (A) α-Me3-H3K9 pull-down to detect methylation of histone H3 lysine 9 or (B) α-Ac-H3K9 pull-down to detect acetylation of histone H3 lysine 9 and targeted qPCR for the LIFR proximal promoter in MCF7 human breast cancer cells cultured in normoxia (Nx) or hypoxia (Hx, 0.5% pO2). N=3 biological replicates from independent experiments. $^*p<0.05$ and $^{**}p<0.01$ *vs* Nx by Mann-Whitney test. Bars = mean, Error bars = standard error of the mean.

explored whether the HDAC inhibitors panobinostat and entinostat retain their ability to stimulate LIFR in hypoxia, when promoter histone acetylation is reduced. Entinostat treatment increased LIFR mRNA and protein expression in ER+ human MCF7 and T47D breast cancer cell lines cultured in hypoxia (1% pO_2), and panobinostat treatment increased LIFR protein in both MCF7 and T47D cells cultured in hypoxia (Figure 8 & 9). Similarly, entinostat increased LIFR mRNA and protein levels in triple negative MDA-MB-231 bone-metastatic human breast cancer cells (MDA-MB-231b, [169]) and 4T1 mouse mammary carcinoma cells cultured in hypoxia, while panobinostat increased LIFR protein in both MDA-MB-231b and 4T1 cells cultured in hypoxia (Figure 10 & 11). Entinostat also induced expression of the dormancy and cell cycle regulators p38β and p21 [233, 335] in hypoxia, while levels of p38α and p27, which also regulate tumor dormancy [170], were decreased or unchanged in MCF7 (Figure 12A-D) and MDA-MB-231b (Figure 12E-H) cells. This suggests that HDAC inhibitors may induce dormancy in hypoxia through a p38β and p21-dependent mechanism. Similarly, treatment of 4T1 cells with entinostat stimulated p38β and p21 to varying degrees, and also induced p38α and p27 (Figure 13).

PTHrP directly binds to the LIFR promoter

We previously demonstrated that LIFR mRNA levels are significantly repressed in MCF7 human breast cancer cells overexpressing PTHrP, independent of the PTH receptor, PTH1R [169, 226]. To better understand how PTHrP regulates LIFR, we generated MCF7 cells stably expressing PTHrP (-36-139) containing a C-terminal HA-tag (absent in MCF7 control cells). We confirmed PTHrP overexpression by qPCR analysis of *PTHLH* levels (Figure 14A). To further investigate the mechanism by which *LIFR* is repressed by PTHrP, we performed ChIP-qPCR for the overexpressed HA-tagged PTHrP (-36-139) along the proximal *LIFR* promoter. In this model, we observed that PTHrP localizes to the proximal LIFR promoter in the same region that was epigenetically modified upon culture in hypoxia (Figure 14B), potentially making LIFR the first transcriptional target of PTHrP to be identified.

Treatment with HDAC inhibitors reverses PTHrP-induced repression of LIFR

Next, we determined whether HDAC inhibitors stimulate LIFR in the setting of repression by PTHrP overexpression [226]. Treatment of PTHrP overexpressing cells for 24 hours with entinostat or panobinostat at 5 μM or 50nM, respectively, robustly stimulated LIFR protein (Figure 15A & B). Consistent with these findings, ChIP-qPCR along the proximal *LIFR* promoter revealed an enrichment for H3K9ac, a marker of active transcription, in the MCF7 cells overexpressing PTHrP that were treated with entinostat (Figure 15C). Thus, HDAC inhibitors still stimulate LIFR

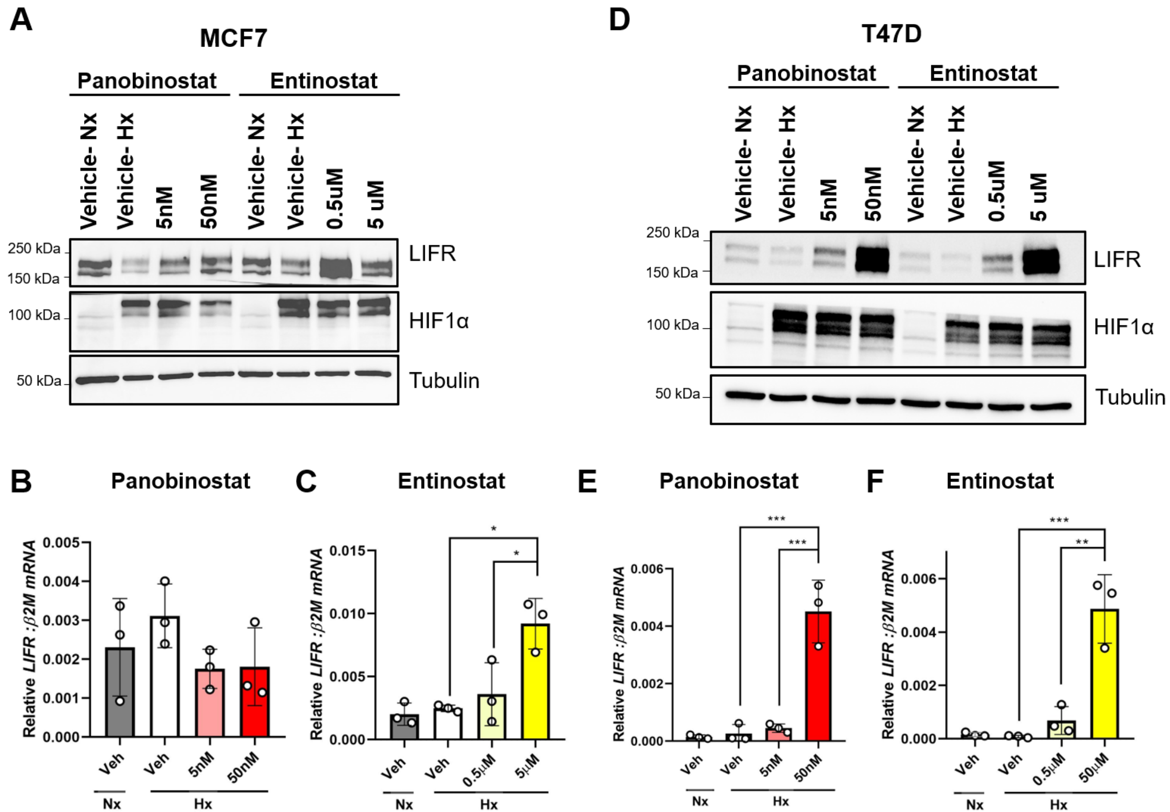


Figure 8. HDAC inhibitors stimulate LIFR expression in ER+ human breast cancer cell lines. Western blot analysis of LIFR protein levels and qPCR analysis of *LIFR* mRNA levels in (A-C) MCF7 and (D-F) T47D cells cultured in normoxia (Nx) or hypoxia (Hx, 1% pO2) and treated with panobinostat (5nM, 50nM) or entinostat (0.5μM, 5μM) for 24 hours (total of 24 hours in hypoxia). (A,D) Representative Western blots. HIF1-α = control for hypoxia assay, Tubulin = loading control. N=3 biological replicates from independent experiments. (C) *$p<0.05$ vs Veh or 0.5μM by one-way ANOVA with multiple comparisons. (E) ***$p<0.001$ vs Veh or 5nM by one-way ANOVA with multiple comparisons. (F) ***$p<0.001$ vs Veh, **$p<0.01$ vs 0.5μM by one-way ANOVA with multiple comparisons. Bars = mean, Error bars = standard error of the mean.

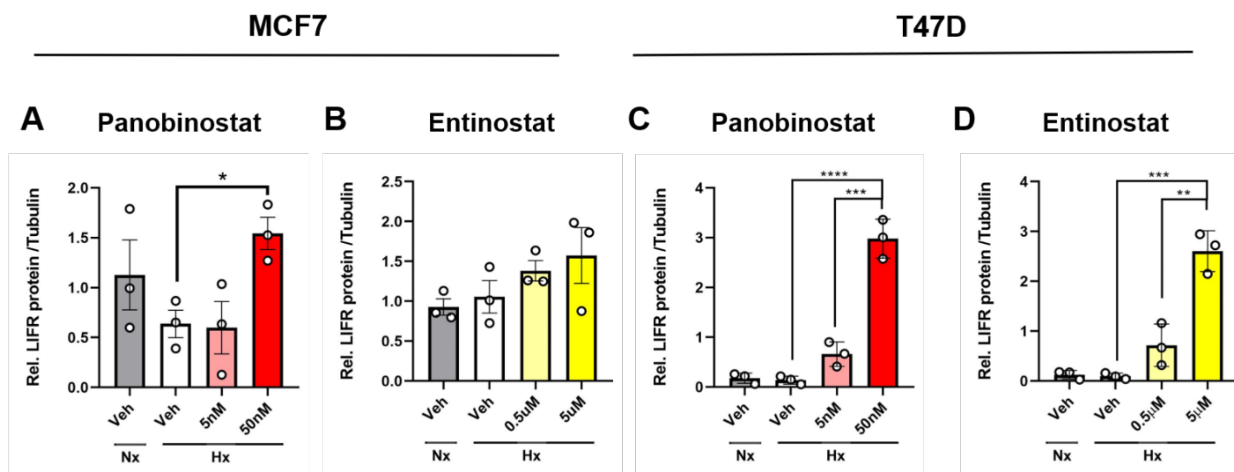


Figure 9. HDAC inhibitors stimulate LIFR protein levels in ER+ human breast cancer cell lines. Densitometry for western blot analysis of LIFR protein levels in (A,B) MCF7 and (C,D) T47D cells cultured in normoxia (Nx) or hypoxia (Hx, 1% pO2) and treated with panobinostat (5nM, 50nM) or entinostat (0.5μM, 5μM) for 24 hours (total of 24 hours in hypoxia). N=3 biological replicates from independent experiments. (A) *$p<0.05$ vs Veh by one-way ANOVA with multiple comparisons. (C) ****$p<0.0001$ vs veh, ***$p<0.001$ vs 5nM by one-way ANOVA with multiple comparisons. (D) ***$p<0.001$ vs Veh, **$p<0.01$ vs 0.5μM by one-way ANOVA with multiple comparisons. Bars = mean, Error bars = standard error of the mean.

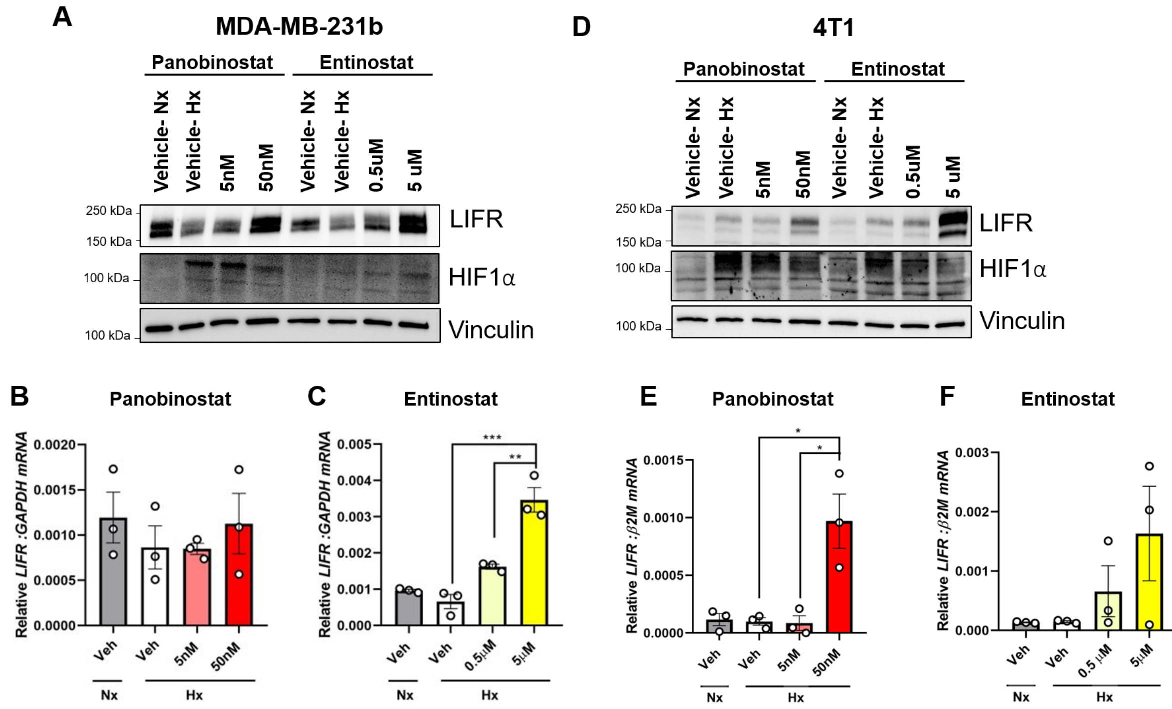


Figure 10. HDAC inhibitors stimulate LIFR expression in aggressive triple negative breast cancer cell lines. Western blot analysis of LIFR protein and mRNA levels in (A-C) MDA-MB-231b and (D-F) 4T1 breast cancer cells cultured in normoxia (Nx) or hypoxia (Hx, 1% pO2) and treated with panobinostat (5nM, 50nM) or entinostat (0.5μM, 5μM) for 24 hours (total of 24 hours in hypoxia). (A, D) Representative western blots. HIF1-α = control for hypoxia assay, Vinculin = loading controls. N=3 biological replicates from independent experiments. (C) ***$p<0.001$ vs Veh, **$p<0.01$ vs 0.5μM by one-way ANOVA with multiple comparisons. (E) *$p<0.05$ vs Veh or 5nM by one-way ANOVA with multiple comparisons. Bars = mean, Error bars = standard error of the mean.

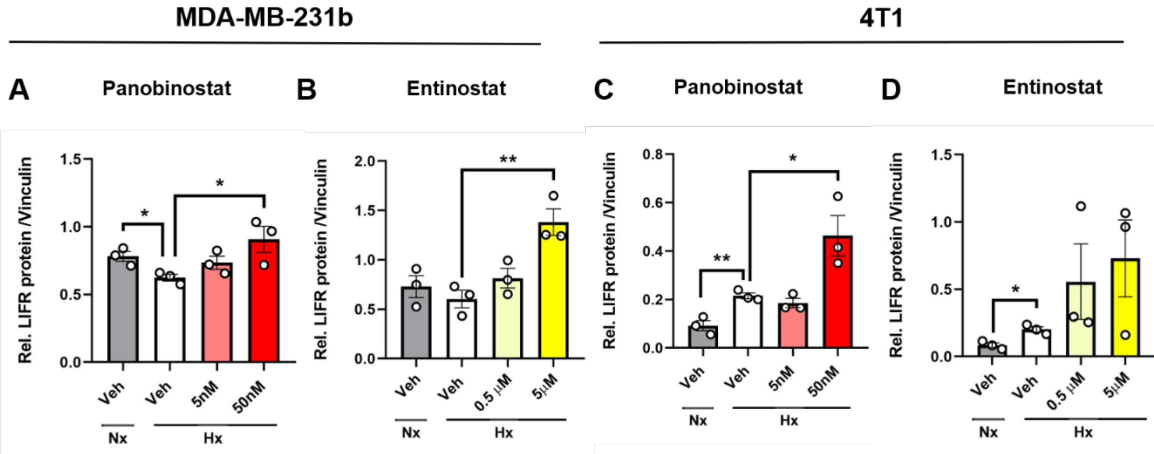
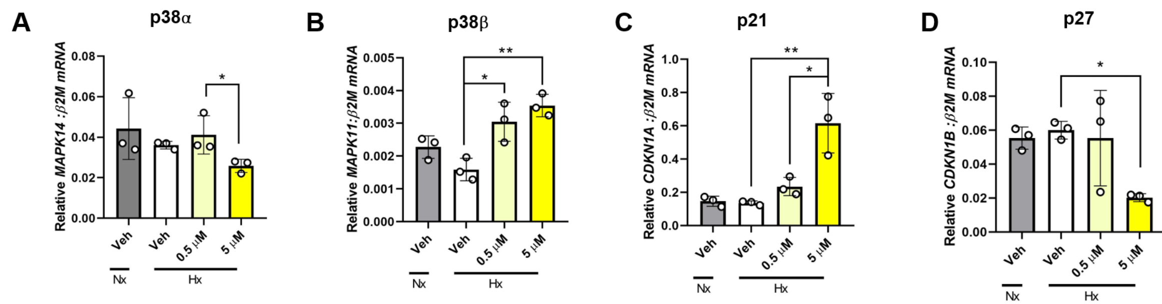


Figure 11. HDAC inhibitors stimulate LIFR protein in aggressive breast cancer cell lines. Densitometry for western blot analysis of LIFR expression in (A & B) MDA-MB-231b and (C & D) 4T1 breast cancer cells cultured in normoxia (Nx) or hypoxia (Hx, 1% pO2) and treated with panobinostat (5nM, 50nM) or entinostat (0.5μM, 5μM) for 24 hours (total of 24 hours in hypoxia). N=3 biological replicates from independent experiments. (A) *p<0.05 vs Veh by unpaired t-test, *p<0.05 vs Veh by one-way ANOVA with multiple comparisons. (B) **p<0.01 vs Veh by one-way ANOVA with multiple comparisons. (C) **p<0.01 vs Veh by unpaired t-test, *p<0.05 vs Veh by one-way ANOVA with multiple comparisons. (D) *p<0.05 vs Veh by unpaired t-test . Bars = mean, Error bars = standard error of the mean.

MCF7



MDA-MB-231b

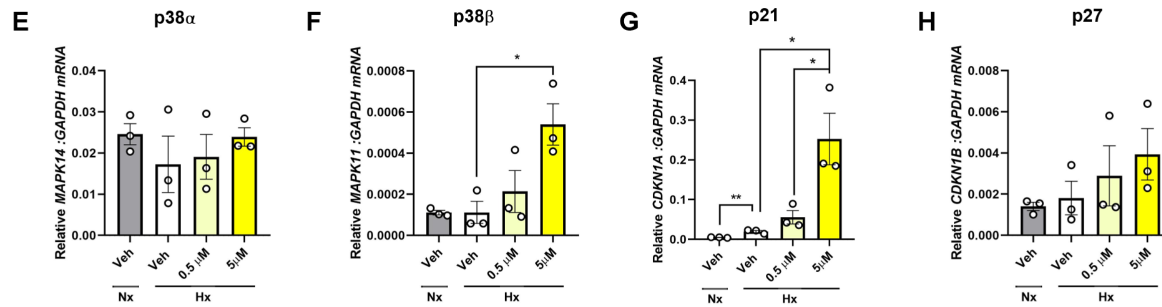


Figure 12. HDAC inhibitors stimulate pro-dormancy genes in ER+ and triple negative breast cancer cells. qPCR analysis for (A,E) MAPK14 (p38α), (B,F) MAPK11 (p38β), (C,G) CDKN1A (p21), (D,H) CDKN1B (p27) in MCF7 (A-D) and MDA-MB-231b (E-H) cells cultured in normoxia (Nx) or hypoxia (Hx, 1% pO2) and treated with entinostat (0.5μM, 5μM) for 24 hours

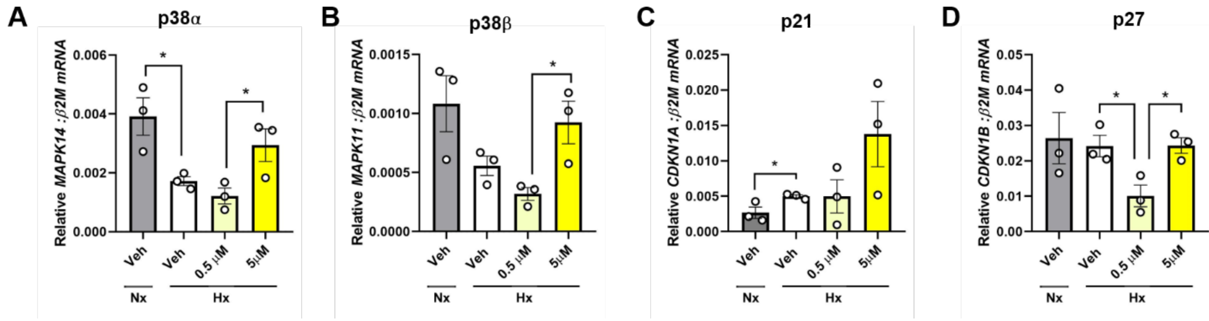


Figure 13. **HDAC inhibitors stimulate pro-dormancy genes in 4T1 mouse mammary carcinoma cells.** qPCR analysis for (A) *MAPK14* (p38α), (B) *MAPK11* (p38β), (C) *CDKN1A* (p21), (D) *CDKN1B* (p27) in 4T1 cells cultured in normoxia (Nx) or hypoxia (Hx, 1% pO2) and treated with entinostat (0.5μM, 5μM) for 24 hours (total of 24 hours in hypoxia). N=3 biological replicates from independent experiments. (A) *$p<0.05$ vs Veh by unpaired t-test, *$p<0.05$ vs 0.5μM by one-way ANOVA with multiple comparisons. (B) *$p<0.05$ vs 0.5μM by one-way ANOVA with multiple comparisons. (C) *$p<0.05$ vs Veh by unpaired t-test. (D) *$p<0.05$ vs Veh or 0.5μM by one-way ANOVA with multiple comparisons. Bars = mean, Error bars = standard error of the mean.

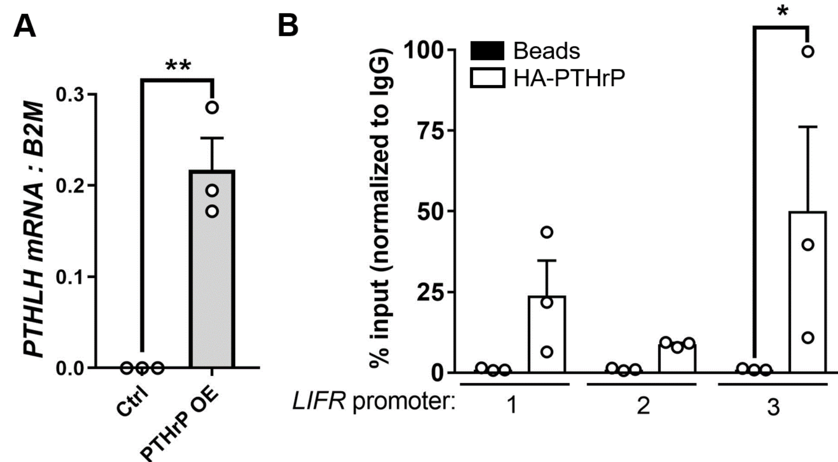


Figure 14. PTHrP binds to the *LIFR* promoter. (A) qPCR analysis for *PTHLH* mRNA levels in MCF7 vector control (Ctrl) versus PTHrP (-36-139) overexpressing (PTHrP OE) human breast cancer cells. (B) Chromatin immunoprecipitation (ChIP) in MCF7 PTHrP overexpressing cells with α-HA pull-down for the PTHrP (-136-139) molecule and targeted qPCR for the LIFR proximal promoter. N=3 biological replicates from independent experiments. (A) $^{**}p<0.01$ vs control by unpaired t-test. (B) $^{*}p<0.05$ vs Beads by unpaired t-test. Bars = mean, Error bars = standard error of the mean.

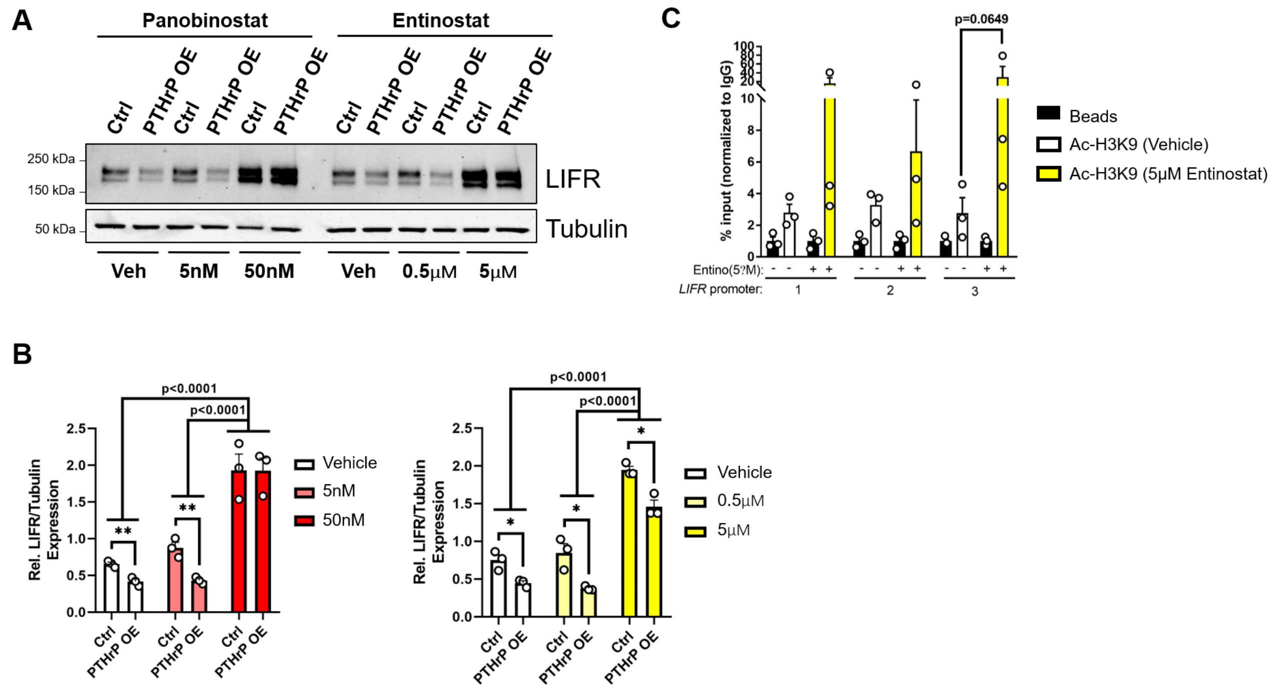


Figure 15. HDAC inhibitors stimulate LIFR even when LIFR is repressed by PTHrP overexpression. (A) Western blot analysis for LIFR protein levels in MCF7 vector control (Ctrl) or PTHrP (-36-139) overexpressing (PTHrP OE) human breast cancer cells treated with panobinostat (5nM, 50nM) or entinostat (0.5µM, 5µM) for 24 hours. Tubulin = loading control. (B) Densitometry for western blot in (A). (C) Chromatin immunoprecipitation (ChIP) in PTHrP (-36-139) overexpressing cells treated with vehicle or 5µM entinostat for 24 hours with α-HA pull-down for the PTHrP (-36-139) molecule and targeted qPCR for the LIFR proximal promoter. N=3 biological replicates from independent experiments. (B) *$p<0.05$ or **$p<0.01$ vs Ctrl by unpaired t-test, $p<0.0001$ by two-way ANOVA with multiple comparisons. Error bars = standard error of the mean.

through increased histone acetylation of the *LIFR* promoter, even when its expression is actively repressed by PTHrP.

Discussion

Hypoxia encountered by bone-disseminated tumor cells epigenetically downregulates LIFR

LIFR slows primary breast tumor progression in both the primary site and bone [169, 314], but the mechanisms that regulate LIFR expression in breast cancer are not well understood. Our previous *in vitro* studies demonstrated that hypoxia, which is evident in most solid tumors and the bone marrow, downregulates LIFR expression and reduces the LIFR:STAT3:SOCS3 signaling pathway in breast cancer cells [169]. Here, we confirmed that bone-disseminated breast cancer cells do indeed reside in hypoxic regions. Rigor of this analysis could be enhanced by performing pimonidazole/ cytokeratin staining of bone-disseminated MDA-MB-231b cells (a human triple negative breast cancer cell line that aggressively colonizes the bone [128]) to investigate whether other breast cancer cell subtypes also become hypoxic in the bone. In our studies, HDAC inhibition also robustly stimulated LIFR expression in MDA-MB-231b cells cultured in hypoxia *in vitro.* Thus, it would be clinically relevant to confirm whether triple negative breast cancer cells also encounter hypoxia *in vivo* since patients with ER- breast cancers commonly develop bone metastases, with the highest risk of recurrence in the initial five years after diagnosis [336, 337]. Furthermore, our data show that low oxygen tensions increase repressive histone modifications (methylation) and reduce transcriptional activation marks (acetylation) within the LIFR promoter, suggesting that this is a mechanism by which hypoxia directly downregulates LIFR. Thus, targeting histone acetylation may be an effective way of stimulating LIFR expression in low oxygen conditions, such as what would be experienced by tumor cells residing in hypoxic regions of the primary tumor or bone.

HDAC inhibition stimulates LIFR and other dormancy genes in hypoxia

We previously demonstrated that HDAC inhibitors epigenetically increase *LIFR* expression and induce a pro-dormancy phenotype characterized by slowed breast cancer cell proliferation *in vitro* and reduced primary tumor growth *in vivo* [314]. Our work here sought to determine whether HDAC inhibitors overcome hypoxic repression of LIFR that may be induced by the primary tumor or bone microenvironment. We confirmed that panobinostat retains its ability to robustly induce LIFR protein levels in breast tumor cells cultured under low oxygen conditions, though stimulation at the mRNA level is less consistent. Lack of induction at the mRNA level, but

an eventual increase in protein may be the result of an indirect effect that panobinostat has on other cellular factors and signaling pathways that regulate LIFR protein. Thus, while panobinostat may directly increase acetylation of the *LIFR* promoter, this effect alone is insufficient to significantly induce transcription and drive the observed increase in protein levels. It is also known that nonhistone proteins can be reversibly acetylated by histone acetyltransferases (HATs) and HDACs [36], so panobinostat may achieve robust induction of LIFR by first acting on another factor that alters the stability of LIFR to increase protein levels. In contrast, entinostat stimulated LIFR at both the mRNA and protein level in hypoxia. Although both panobinostat and entinostat are both considered pan-HDAC inhibitors, they belong to two separate classes of drugs (hydroxamates versus benzamides) and target different HDACs at their IC50 or ten times this concentration (Table 3), which were the two concentrations utilized for our studies. Thus, differences in which HDACs are targeted and the extent of resulting changes in histone acetylation and the chromatin structure may alter the accessibility of the *LIFR* promoter to transcription machinery in different cell types treated with entinostat or panobinostat.

Additionally, in breast cancer cells with both low and high metastatic potential, we observed that HDAC inhibition significantly upregulates expression of p38β and p21, known regulators of tumor dormancy and the cell cycle [233, 335, 338]. It is well established that the preferential activation of p38 MAPK over ERK signaling plays a key role in maintaining tumor cell dormancy [233]. ERK signaling promotes cell cycle progression and cell division to promote exit from dormancy while p38 signaling can inhibit ERK-mediated proliferation, induce G0/G1 arrest, and trigger senescence or apoptosis [338-340]. Paradoxically, hypoxia can both promote quiescence by inducing a low ERK/p38 signaling ratio and promote exit from dormancy by negatively regulating factors like LIFR [169, 341]. Our results indicate that even in low oxygen conditions, such as those encountered in the primary tumor or bone, HDAC inhibitors may play a multifactorial role in promoting breast tumor dormancy by increasing expression of LIFR and other pro-dormancy factors.

While p38β and p21 mRNA levels were increased with entinostat treatment in hypoxia, p38α and p27 mRNA levels remained unchanged or decreased. The exact mechanisms by which HDAC inhibition regulates expression of these dormancy-associated factors in hypoxia remain incompletely understood. Further investigation is warranted to determine if entinostat directly modulates histone acetylation along the p38β and p21 promoters or whether expression of these proteins is altered by other factors downstream of LIFR signaling. In addition, while p38α and p38β are both isoforms in the MAPK family of enzymes, they are encoded by separate genes with distinct promoters. Thus, the differential regulation of their expression by entinostat may be due

to differences in acetylation of their individual promoters. Numerous nonhistone proteins are also reversibly acetylated by HDACs during posttranslational processing [36], so it is plausible that competing non-epigenetic regulatory mechanisms could explain differences in expression of p38α, p38β, p21 and p27 with HDAC inhibition. Ultimately, future studies are needed to determine how HDAC inhibition regulates expression of these factors *in vivo* and whether treatment with the inhibitors reduces bone tumor burden.

HDAC inhibition stimulates LIFR in the setting of PTHrP overexpression

We previously identified PTHrP overexpression as a mechanism by which LIFR is downregulated in breast cancer cells to promote exit from dormancy and metastatic outgrowth [169, 221]. Bone-disseminated breast tumor cells produce elevated levels of PTHrP to drive osteolysis [181-183]. To our knowledge, we show for the first time that PTHrP localizes to the *LIFR* promoter and downregulates its protein expression in breast cancer cells, consistent with our previous finding that PTHrP overexpression reduces LIFR mRNA levels [169]. Here, our *in vitro* studies utilized MCF7 human breast cancer cells that overexpress HA-tagged PTHrP since we have not identified a commercially available antibody suitable for the detection of the endogenous protein. There are limitations to this approach since behavior of the overexpressed recombinant protein could be slightly different from that of the endogenous protein expressed at basal levels. However, our observation that PTHrP (-36-139) co-localizes to the *LIFR* promoter is still a novel and interesting finding to investigate further, as will be discussed in depth in Chapter V below.

A previous study determined that the downregulation of various pro-dormancy factors, including *LIFR,* in MCF7 cells is independent of paracrine activation of PTH1R and downstream signaling via the cAMP/PKA/CREB pathway [226]. Thus, we hypothesized that it must be the intracrine actions of PTHrP that regulate its effect on dormancy gene expression. PTHrP has multiple domains, each with different biological functions that mediate its secretion, interaction with PTHR1, nuclear localization, and cytoplasmic activity through the C-terminal region [189, 342]. It still remains unknown which domains mediate the interaction between PTHrP and the *LIFR* promoter. Although PTHrP does have a known RNA binding motif within its mid-region [343], it has no identified DNA binding domain. Further studies are warranted to confirm whether PTHrP acts as a direct transcriptional regulator of *LIFR* that is able to directly bind DNA, or whether the observations in our ChIP analyses are due to an indirect effect mediated by complex formation with another DNA-binding partner. PTHrP may also act as a direct transcriptional regulator of other dormancy-associated genes, in addition to *LIFR.* Future studies should examine additional

mechanisms by which PTHrP alters quiescence associated gene expression through intracrine non-PTH1R mediated actions.

Concluding Remarks

In summary, these data provide evidence that HDAC inhibitors stimulate LIFR repression regardless of the way it may be downregulated in breast tumors and bone disseminated DTCs. A recent phase III trial did not demonstrate improved survival with combination entinostat and endocrine therapy in metastatic breast cancer, bringing into question the efficacy of HDAC inhibitors in this patient population. Our results suggest that LIFR is likely still stimulated even if patients' tumors are hypoxic or have high PTHrP expression, ruling these factors out as potential reasons for why the drugs failed. HDAC inhibitors may therefore still be useful in inducing tumor dormancy to reduce breast cancer recurrence and improve outcomes, but further studies are required to identify the select patient populations that may benefit from these drug combinations.

CHAPTER IV

INTRACRINE ACTIONS OF THE PTHRP NUCLEAR LOCALIZATION SEQUENCE AND C-TERMINUS REGULATE CYCLIN DEPENDENT KINASE INHIBITORS TO INFLUENCE BREAST TUMOR GROWTH

Introduction

Beyond its well-characterized endocrine and paracrine roles in inducing hypercalcemia of malignancy [344, 345] and tumor-induced bone disease [221, 280, 291, 346], PTHrP has been recognized as an intracrine regulator of tumorigenesis through its effects on cell survival, cell proliferation, apoptosis, invasion, and migration [206, 214, 216, 347]. PTHrP expression can be detected in most primary breast tumors [274, 279] where clinical studies have largely shown that its expression is correlated with improved patient survival and formation of fewer bone metastases. However, preclinical studies using different genetic mouse models have produced directly conflicting findings suggesting that PTHrP can either promote [213, 218] or inhibit breast tumor growth in the primary site [218]. Thus, the prognostic role for PTHrP in primary breast tumor progression remains largely unclear.

We recently identified a novel role for PTHrP as a negative regulator of breast tumor dormancy. PTHrP (1-139) overexpression *in vitro* downregulates leukemia inhibitory factor receptor (*LIFR*) [175], a breast tumor dormancy regulator in bone and breast tumor suppressor [169, 314]. Several other pro-dormancy genes are also downregulated by PTHrP overexpression [175]. Interestingly, these changes in dormancy gene expression occur independent of paracrine activation of the PTH receptor (PTH1R) and downstream canonical cAMP signaling [226].This suggests that PTHrP may promote dormancy escape via its intracellular actions rather than autocrine/ paracrine activation of the PTH1R. In fact, we previously determined that PTHrP binds to the *LIFR* proximal promoter, making *LIFR* the first identified transcriptional target of PTHrP [313].

In contrast to its nebulous role in the primary tumor, PTHrP has deleterious effects on patient outcomes in later stages of disease progression as its expression drives bone colonization and metastatic tumor growth [280, 281]. Bone disseminated breast cancer cells secrete osteolytic factors like PTHrP which induces RANKL-dependent osteoclastogenesis via PTH1R activation in osteoblasts [332]. PTHrP has also been identified as a negative regulator of tumor dormancy in the bone. Overexpression of PTHrP (1-139) in human MCF7 breast cancer cells, which lie

dormant in bone [175, 221-223], switches these cells from a quiescent to a highly osteolytic phenotype and dramatically increases bone tumor burden *in vivo* [221].

The PTHrP molecule contains multiple domains that regulate intracellular trafficking and secretion (amino acids (aa) -36 to -1), paracrine/ autocrine binding and activation of PTH1R (aa 1-34), intracellular distribution through a nuclear localization sequence (NLS) (aa 67-94) and cytoplasmic activity through the carboxy-terminal domain (aa 107-139) [189]. The NLS and C-terminal domain are particularly important for controlling numerous intracrine actions of PTHrP and have been found to alter proliferation in other tissue types [348-350]. However, the function of these domains in controlling breast tumor progression remains incompletely understood. In this study, we sought to determine how the PTHrP NLS and C-terminal domain regulate breast cancer cell signaling and tumor progression *in vivo*. Our findings resolve previous conflicting data in the literature and provide a framework for targeting PTHrP and its downstream signaling mediators in breast cancer.

Results

Human breast cancer cells generated to express full-length PTHrP or truncated peptides

To determine the role for each PTHrP biological domain in breast tumor progression, we generated MCF7 human breast cancer cell lines that stably express different domains of the PTHrP molecule (collectively referred to herein as PTHrP mutant cell lines). The plasmids express full-length secreted PTHrP (termed FLSEC), or truncated forms lacking the NLS alone (termed DNLS) or NLS and C-terminal domain (termed DNLS+CTERM) with a C-terminal HA tag (absent in the MSCV control) (Figure 16A). We were unable to generate a mutant with deletion of the secretion signal since these cells do not survive *in vitro.* We validated plasmid expression at the protein level using an anti-HA antibody and at the mRNA level with qPCR primers targeted to amplify different regions of the *PTHLH* gene (Figures 16B-E). To further verify expression of the plasmids and to characterize the intracellular localization of the PTHrP peptides, we performed immunocytochemical staining for the C-terminal HA tags. We confirmed an absence of HA expression and fluorescence staining in the MSCV control cells as these plasmids do not contain a C-terminal HA tag. Full-length secreted PTHrP localized to both the nucleus and cytoplasm (Figure 16F). Surprisingly, deletion of the NLS alone or NLS and C-terminal domain did not preclude nuclear entry as evidence of both PTHrP mutant proteins was present in the nucleus as well as cytoplasm (Figure 16F & 17). This suggests that truncated PTHrP peptides may utilize alternative mechanisms to gain entry into the nucleus that are not mediated by the

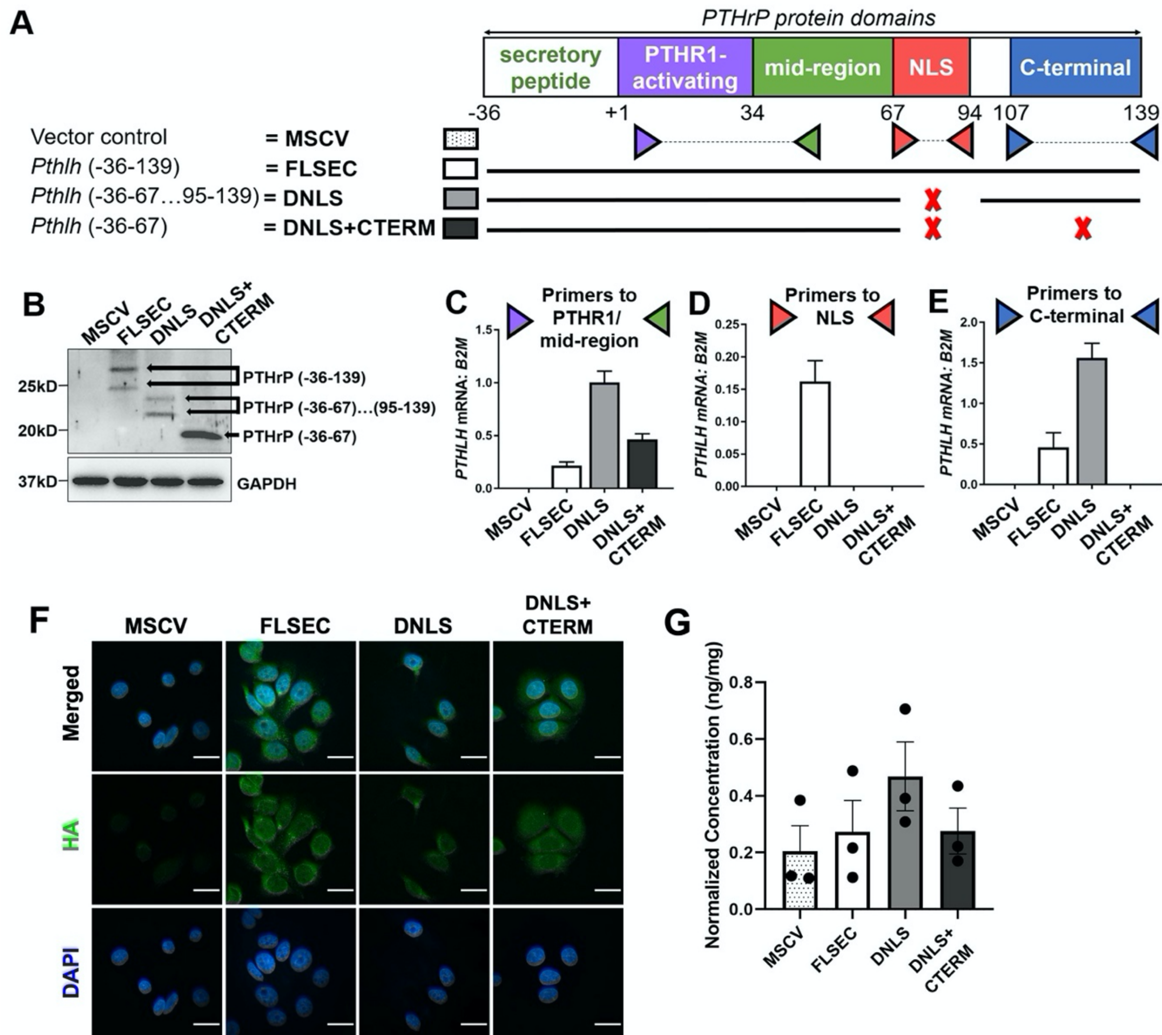


Figure 16. Validation of plasmids expressing specific PTHrP domains. (A) PTHLH overexpression construct design and validation in MCF7 cells by (B) western blot for the C-terminal HA-Tag and qPCR for the (C) mid-region, (D) nuclear localization sequence (NLS), and (E) C-terminal domain. MSCV=control, FLSEC=full-length secreted PTHrP, DNLS=NLS deleted PTHrP, DNLS+CTERM=NLS and C-terminal domain deleted PTHrP. Predicted molecular weights: FLSEC PTHrP (-36-139) = 21.2kD, DNLS PTHrP (-36-67)…(95-139) = 18kD, DNLS+CTERM PTHrP (-36-67) = 12.8kD. GAPDH=loading control. (F) Immunocytochemical staining for HA-Tag (green) and DAPI (blue). All panels = 100X and scale bars = 25μm. (G) Secreted PTHrP (1-34) levels measured by ELISA from conditioned media of cells described in (A). (B-E & G) n = 3 independent biological replicates. Graphs represent mean ± SEM.

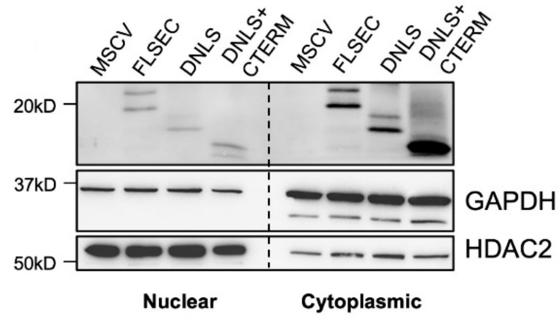


Figure 17. Subcellular localization of PTHrP peptides. (A) Western blot analysis for HA-Tag from nuclear and cytoplasmic fractions obtained from PTHrP mutant cells. GAPDH = cytoplasmic loading control, HDAC2 = nuclear loading control.

recognized NLS from amino acids 67-94. Lastly, we performed an enzyme-linked immunosorbent assay (ELISA) for PTHrP (1-34) and observed no statistically significant difference in full-length secreted PTHrP peptide levels secreted by the PTHrP mutant cell lines compared to controls (Figure 16G) indicating that altering expression of the NLS or the C-terminal domain does not affect PTHrP secretion by MCF7 human breast cancer cells.

The PTHrP NLS and C-terminal domain oppositely regulate breast tumor cell proliferation and tumor growth

To determine whether the PTHrP NLS and C-terminal domain regulate breast cancer cell proliferation *in vitro* we performed a trypan blue exclusion assay and monitored growth of the PTHrP mutant cells every 24 hours for four days. Deletion of the NLS alone significantly increased proliferation compared with MSCV control cells by the final day, while there were no significant changes in proliferation with overexpression of the full-length molecule or deletion of both the NLS and C-terminus (Figure 18A). To better understand the molecular mechanism underlying this *in vitro* phenotype, we performed RNA sequencing and Gene Set Enrichment Analysis (GSEA) on the PTHrP mutant cell lines and identified several hundred significantly altered genes ($\geq \log_2$ fold change 1 or $\leq \log_2$ fold change -1, $p<0.05$) and pathways that were differentially expressed across the mutants (Figure 18B). In the cells lacking the PTHrP NLS, there was a significant enrichment for genes that are upregulated in MCF7 cells overexpressing the oncoprotein and cell cycle promoter, cyclin D1 (Figure 18C). Ultimately, these data suggest that the NLS alters the expression of cell cycle regulators to regulate proliferation in MCF7 breast cancer cells.

Given these results, we sought to determine whether PTHrP and its biological domains regulate breast tumor growth *in vivo* by orthotopic inoculation of the PTHrP mutant cell lines. Overexpression of full-length PTHrP did not significantly alter time to tumor palpation (Figure 18D) or final tumor weight (Figure 18E) compared with controls. Strikingly, deletion of the NLS alone resulted in tumors that formed significantly earlier and grew larger than controls, while deletion of both the NLS and C-terminal domains completely reversed this phenotype such that the tumors grew significantly slower and smaller (Figure 18D & E). We performed immunofluorescence staining of the primary tumors for the C-terminal HA tag to verify that the PTHrP mutant plasmids were still expressed *in vivo* (Figure 19). Consistent with our *in vitro* findings, deletion of the PTHrP NLS alone significantly increased the percentage of Ki67+ positive tumor cells *in vivo* (Figure 18F), indicating these cells are more proliferative. There was no difference in apoptosis amongst the PTHrP mutant cell lines *in vivo* measured by cleaved PARP staining (Figure 18G), suggesting that the PTHrP NLS and C-terminal domain primarily regulate breast tumor growth by altering

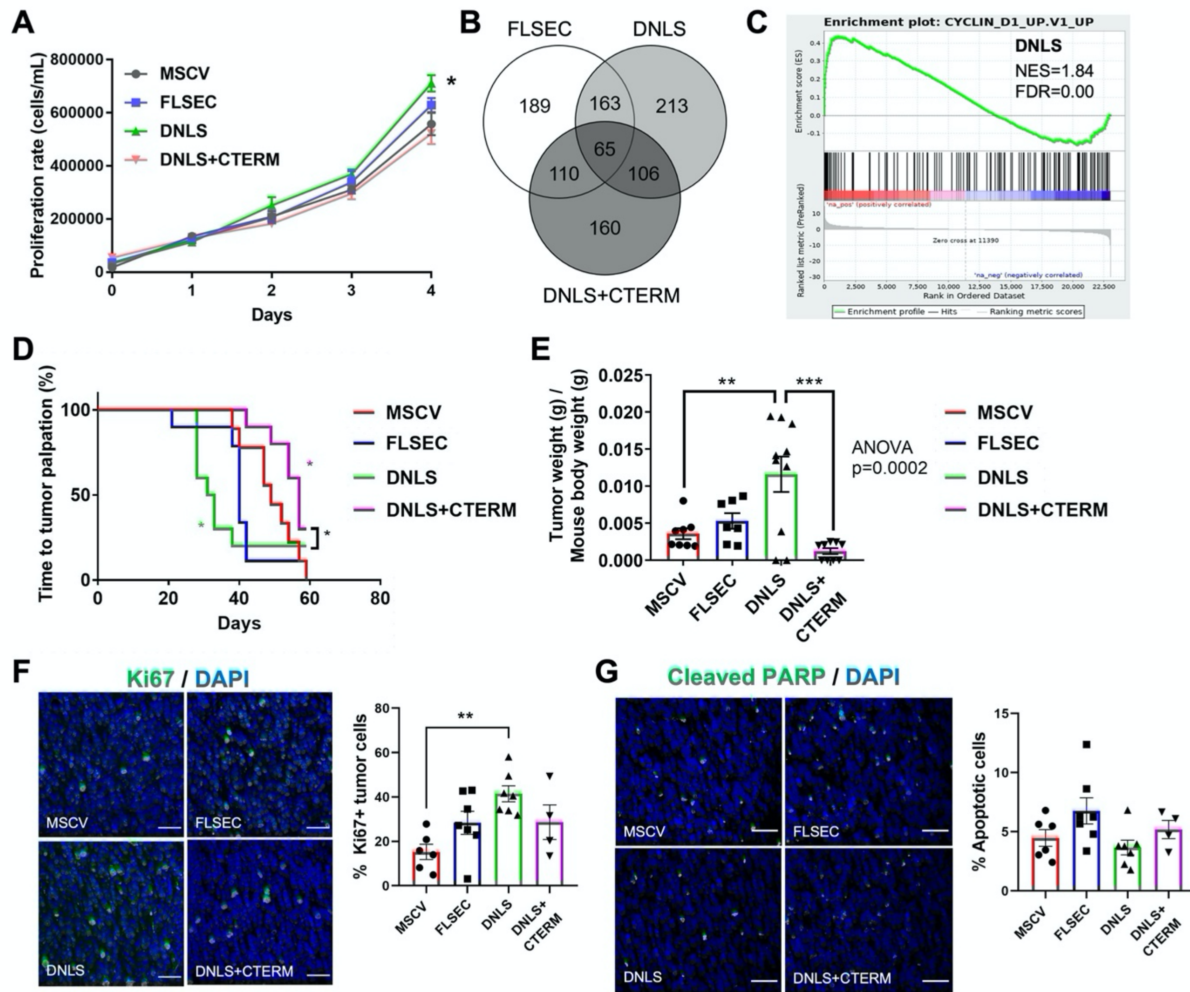
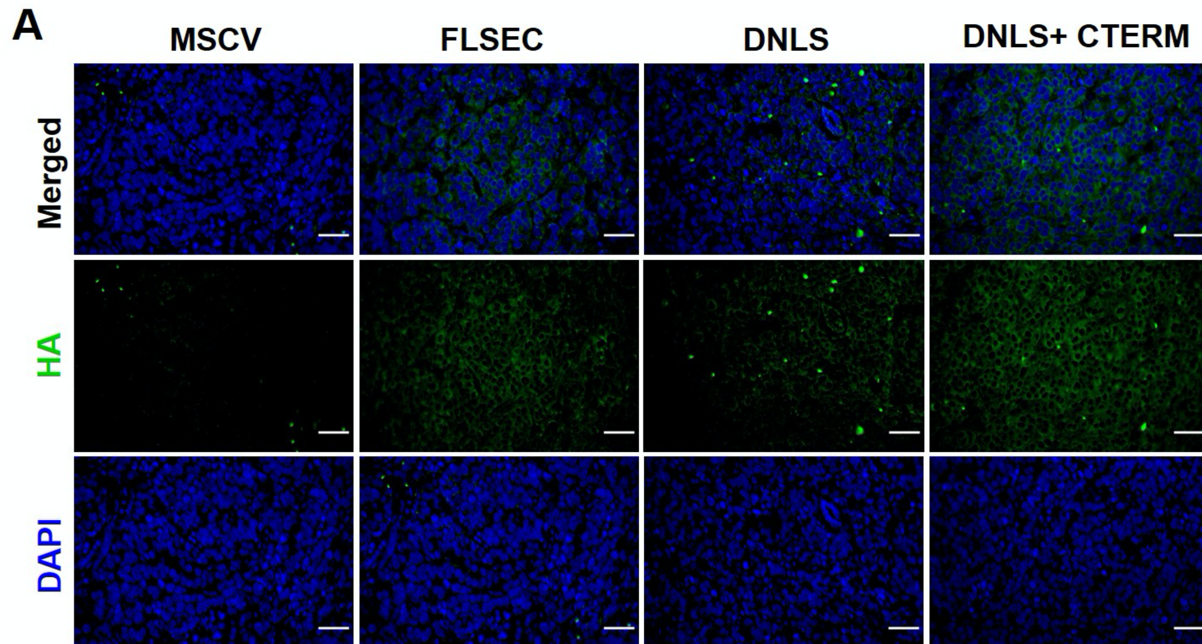


Figure 18. Deletion of the PTHrP NLS alters breast cancer cell proliferation and primary tumor growth. (A) Trypan blue exclusion assay to assess proliferation rate in MSCV, FLSEC, DNLS, or DNLS+CTERM cells. n=3 biological replicates from independent experiments. (B) Number of genes identified by RNAseq with $\log_2$fold change >1 and p<0.05. (C) GSEA plot from DNLS cells showing enrichment of genes upregulated in MCF7 cells overexpressing the cyclin D1 oncogene (*CCND1*). (D) Time to tumor palpation and (E) final tumor weight in mice inoculated with MSCV, FLSEC, DNLS, or DNLS+CTERM cells. n=7-10 mice/group. (F) Ki67 staining and quantification from tumors in (D & E). (G) Cleaved PARP staining and quantification from tumors in (D & E). All panels = 40X and scale bar = 50µm. (A) *p<0.05 vs MSCV by one-way ANOVA with multiple comparisons. (D) *p<0.05 vs MSCV by one-way ANOVA with multiple comparisons or *p<0.05 vs DNLS by unpaired t-test. (E) **p<0.01 vs MSCV by one-way ANOVA with multiple comparisons or ***p<0.001 vs DNLS by unpaired t-test. (F) **p<0.01 vs MSCV by one-way ANOVA with multiple comparisons. Graphs represent mean ± SEM.



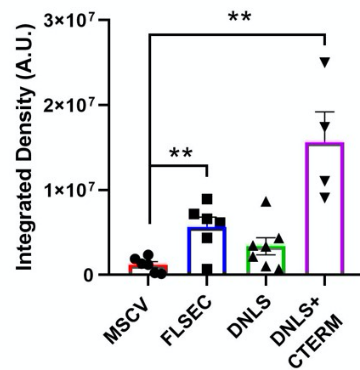


Figure 19. Plasmids expressing specific PTHrP peptides are retained in breast tumors *in vivo*. (A) Anti-HA immunofluorescence staining and quantification from primary tumors of mice inoculated with MSCV, FLSEC, DNLS, or DNLS+CTERM cells. All panels = 40X and scale bars = 50µm. *p<0.05 or **p<0.01 vs MSCV by unpaired t-test.

tumor cell proliferation. Together, these data suggest that the PTHrP NLS functions as a tumor suppressor to inhibit breast tumor growth while the PTHrP C-terminal domain may be oncogenic in breast cancer.

p27 is differentially regulated by the PTHrP NLS and C-terminal domains in breast cancer

To further understand the signaling mechanisms by which the PTHrP NLS and C-terminal domains regulate breast tumor cell proliferation, we analyzed expression of the cyclin-dependent kinase inhibitors p21 and p27, which are both regulated by PTHrP in other cell types [348-350]. Western blot analysis did not reveal any significant alterations in p21 or p27 with overexpression of the full-length PTHrP molecule or deletion of the NLS alone (Figures 20A-C), compared with MSCV controls. However, deletion of both the NLS and C-terminal domain significantly increased p27 expression, but not p21, compared with deletion of the NLS alone (Figure 20A-C). Immunocytochemical staining confirmed that overexpression of full-length PTHrP did not alter p27 levels (Figure 20D). Consistent with our biochemical analysis, immunocytochemical staining for p27 revealed significantly lower expression with deletion of the NLS alone compared to control cells. Furthermore, expression of p27 was significantly increased with deletion of both the NLS and C-terminal domain, exceeding levels in both MSCV controls and NLS-alone deleted cells (Figure 20D). Immunofluorescence staining of the primary breast tumors similarly revealed no change in p27 with overexpression of the full-length PTHrP molecule, but p27 protein levels were significantly decreased with deletion of the NLS alone compared to controls, and oppositely increased with deletion of both the NLS and C-terminal domain (Figure 20E). Interestingly, *in vivo* p27 protein levels still remained lower than controls with deletion of both domains. Overall, there was no consistent pattern of changes in p21 expression as measured by immunocytochemistry (Figure 21A) or immunofluorescence staining of the primary tumors (Figure 21B). Together, these *in vitro* and *in vivo* findings suggest that p27 is oppositely regulated by the PTHrP NLS and C-terminal domain in breast cancer, potentially contributing to the differential effects observed with cell proliferation and breast tumor growth.

PTHrP regulates downstream LIFR signaling to alter p27 expression in vitro

We previously demonstrated that PTHrP directly binds to the promoter [313] and downregulates breast cancer cell expression of leukemia inhibitory factor receptor (LIFR) [169], which is a known breast tumor dormancy regulator in bone [169, 314], breast tumor suppressor, and lung metastasis suppressor [329, 330]. The downstream signaling mechanisms by which LIFR regulates breast tumor cell proliferation remain incompletely understood. Here, we

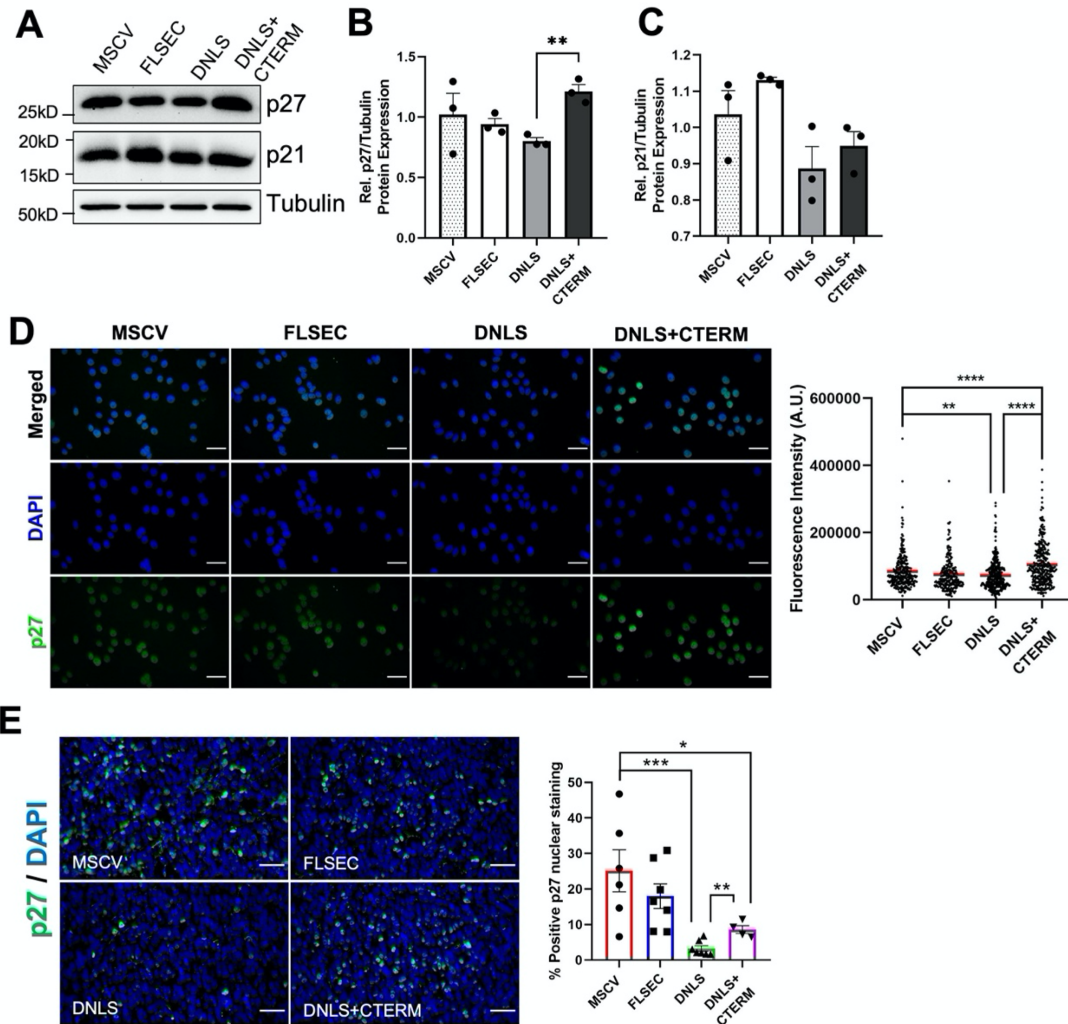


Figure 20. PTHrP lacking the NLS and C-terminal domain regulate proliferation by altering expression of the cyclin dependent kinase inhibitor p27. (A) Western blot analysis of p27 and p21 protein expression in MSCV, FLSEC, DNLS, or DNLS+CTERM cells. Tubulin = loading control. (B & C) Densitometry for western blots in (A). (D) Immunocytochemical staining and quantification for p27 in MSCV, FLSEC, DNLS, or DNLS+CTERM cells. N= 3 independent biological replicates. All panels = 40X, scale bar = 25µm. (E) Immunofluorescence staining and quantification for p27 in primary tumors from mice inoculated with MSCV, FLSEC, DNLS, or DNLS+CTERM cells. All panels = 40X, scale bar = 50µm. (B) **p<0.01 vs DNLS by unpaired t-test. (D) **p<0.01 or ****p<0.0001 vs MSCV by one-way ANOVA with multiple comparisons or ****p<0.0001 vs DNLS by unpaired t-test. (E) *p< 0.05 or ***p<0.001 vs MSCV by one-way ANOVA with multiple comparisons or **p<0.01 vs DNLS by unpaired t-test. Graphs represent mean ± SEM.

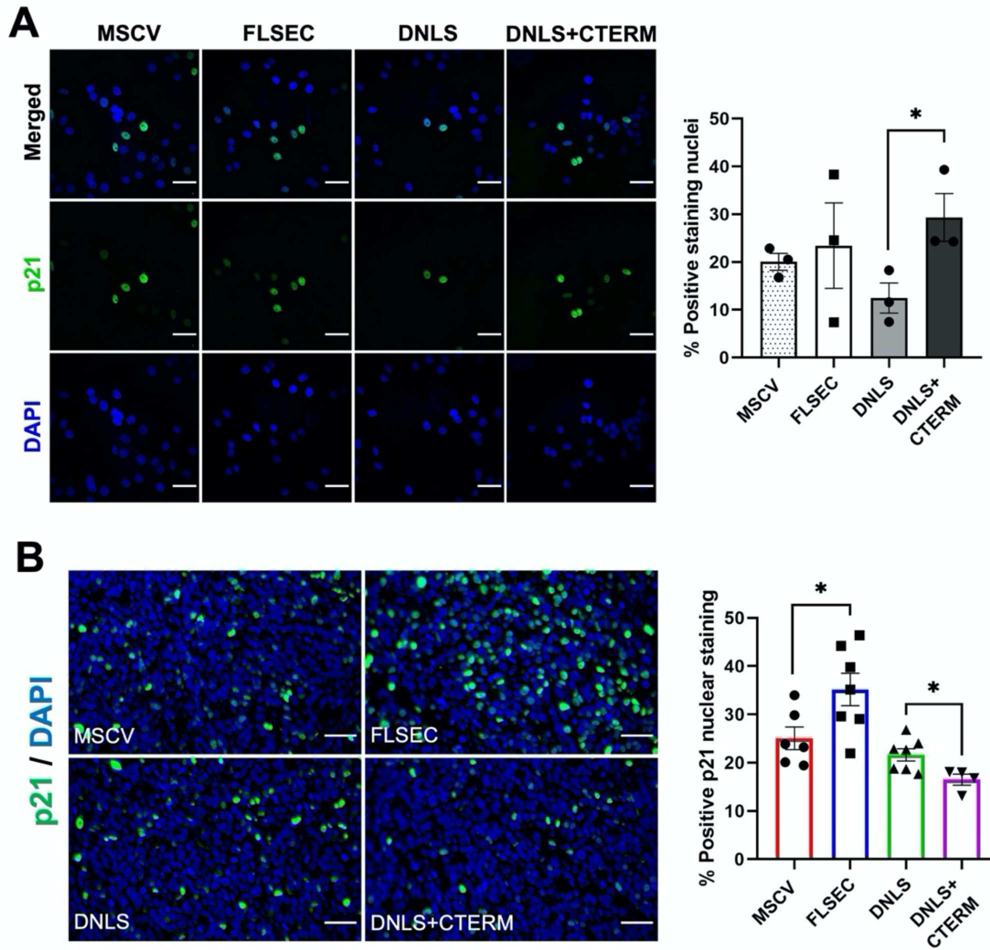


Figure 21. Expression of p21 in breast cancer cells expressing different domains of the PTHrP molecule. (A) Immunocytochemical staining and quantification for p21 in MSCV, FLSEC, DNLS, or DNLS+CTERM cells. N= 3 independent biological replicates. All panels = 40X and scale bars = 25µm (B) Immunofluorescence staining and quantification for p21 in primary tumors from mice inoculated with MSCV, FLSEC, DNLS, or DNLS+CTERM cells. All panels = 40X and scale bars = 50µm (A) *p<0.05 vs DNLS by unpaired t-test. (B) *p<0.05 vs MSCV by one-way ANOVA with multiple comparisons or *p<0.05 vs DNLS by unpaired t-test. Graphs represent mean ± SEM.

confirmed that *in vitro* levels of LIFR protein decreased in each of the PTHrP mutant cell lines compared with controls (Figure 22A & B). In the primary site, we surprisingly did not observe any difference in LIFR protein expression with overexpression of the full-length PTHrP molecule or deletion of the PTHrP NLS alone (Figure 23A). However, deletion of the NLS and C-terminus significantly increased expression of LIFR compared to tumors lacking the NLS alone, which restored levels close to that of the control tumors (Figure 23A). This pattern of increased LIFR expression with deletion of the PTHrP NLS and C-terminal domain (compared to NLS alone deletion) mirrors the previously observed trend in tumor p27 expression. Thus, we hypothesized that PTHrP may regulate downstream p27 signaling through LIFR, resulting in altered breast tumor cell proliferation. To investigate this, we treated the PTHrP mutant cells with a commercially available LIFR inhibitor (EC359) that blocks receptor/ligand interactions. After 24 hours of LIFR inhibitor treatment (100nM), cells containing PTHrP lacking the NLS and C-terminus were no longer able to induce p27 and even expressed significantly lower levels than control cells (Figure 23B & C), suggesting that these cells may induce p27 expression via a LIFR-dependent mechanism. Treatment of the PTHrP mutant cell lines with the LIFR inhibitor for 1 or 6 hours did not elicit the same effect on p27 as the 24-hour treatments, such that there was no change in the pattern of protein levels compared with vehicle treated cells (Figure 22C – F). This lack of effect with shorter treatments suggests that p27 is likely an indirect target of downstream LIFR signaling. Effective LIFR inhibition was confirmed by decreased phosphorylation of the downstream LIFR signaling factor, ERK1/2 (Figure 23B & D).

Since ERK activity is negatively regulated by p38 activation, and the ratio of p38 to ERK signaling in tumor cells is particularly important for determining whether the cells remain dormant (high p38/ERK signaling ratio promotes dormancy) [233, 351], we also analyzed phosphorylated p38 levels in the PTHrP mutant cells. While phosphorylated p38 and the p38/ERK ratio were unchanged in the untreated cells expressing full-length or NLS alone-deleted PTHrP, both parameters increased in cells expressing PTHrP lacking the NLS and C-terminal domain, compared to controls. This suggests that these cells preferentially activate p38 signaling to adopt a more dormant phenotype, which is consistent with the slowed tumor growth and reduced proliferation observed *in vivo* (Figure 18D & E, DNLS+CTERM group). Interestingly, there was a significant increase in phosphorylated p38 and the p38/ERK ratio in the LIFR inhibitor treated versus vehicle treated PTHrP mutant cells. This suggests that the LIFR inhibitor may preferentially decrease ERK signaling, which in turn increases p38 activity.

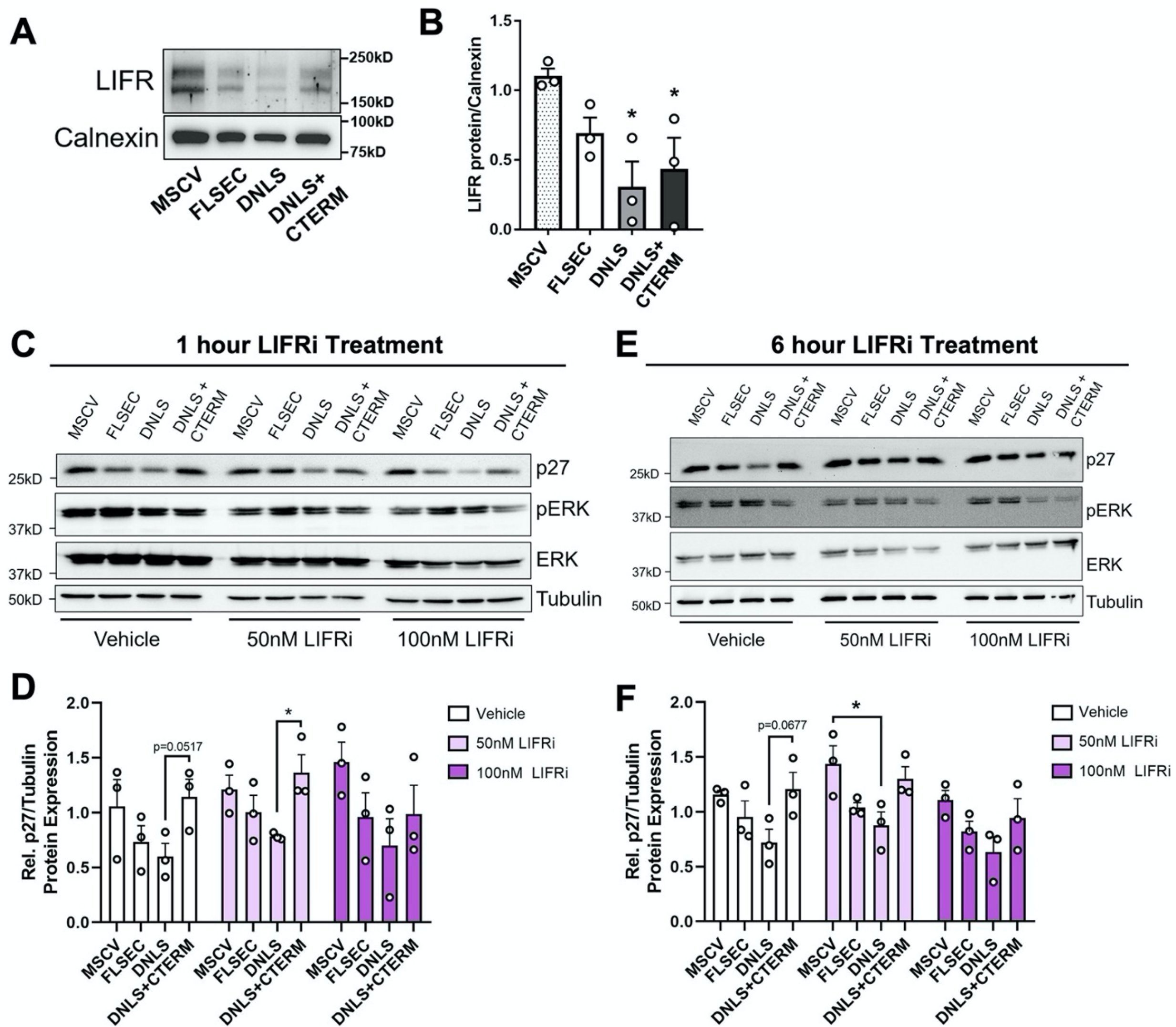


Figure 22. PTHrP regulates LIFR expression and signaling to alter p27 expression in breast cancer cells. (A) Western blot analysis and (B) densitometry of LIFR and calnexin (loading control) protein levels in MSCV, FLSEC, DNLS, or DNLS+CTERM cells. Western blot analysis and densitometry of p27, pERK, ERK and tubulin (loading control) protein levels in MSCV, FLSEC, DNLS, or DNLS+CTERM cells treated with LIFR inhibitor (EC359, 50nM or 100nM) for (C & D) 1 hour or (E & F) 6 hours. (B) *$p<0.05$ vs MSCV by one-way ANOVA with multiple comparisons. (D) *$p<0.05$ vs DNLS by unpaired t-test. (C) *$p<0.05$ vs MSCV by one-way ANOVA with multiple comparisons. Graphs represent mean ± SEM.

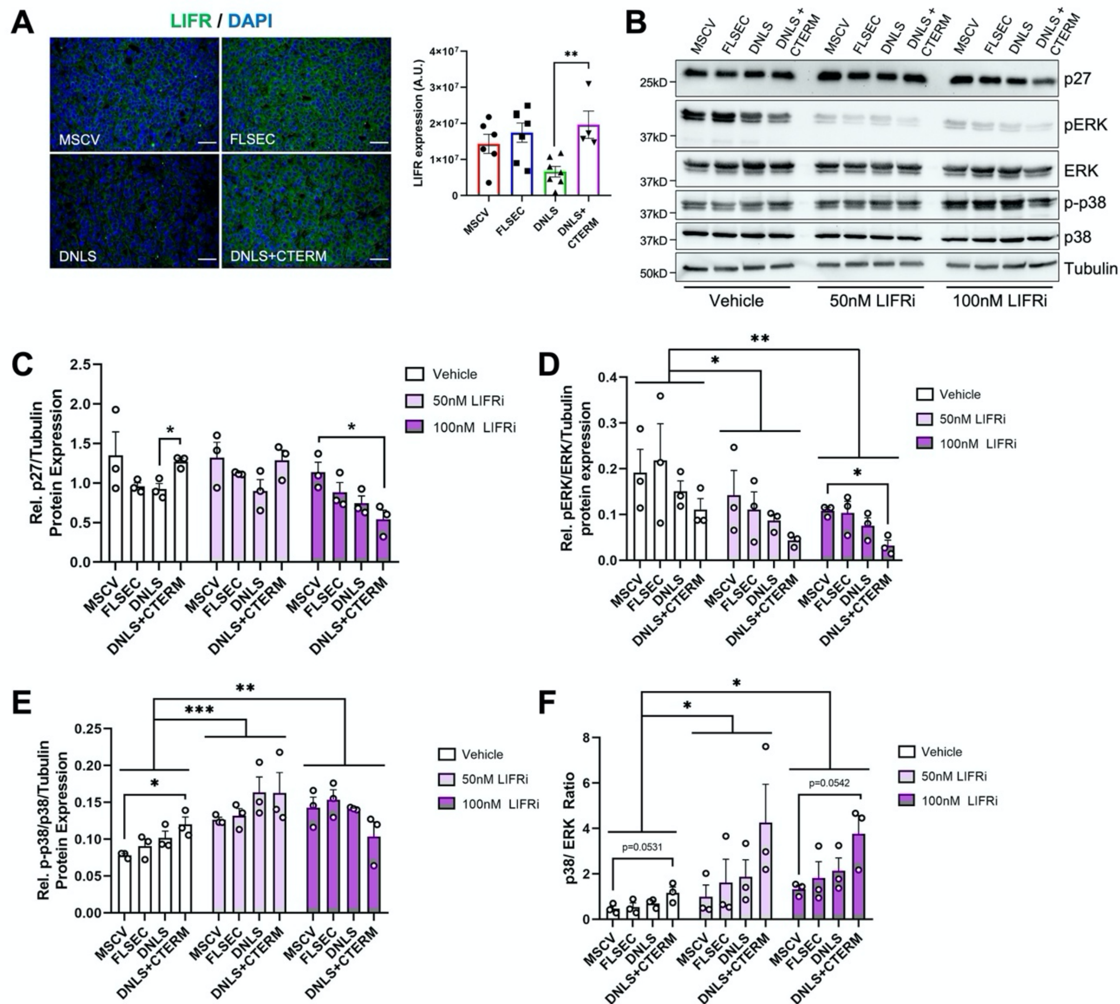


Figure 23. PTHrP regulates LIFR expression and signaling to alter p27 expression in breast cancer cells. (A) Immunofluorescence staining and quantification for LIFR in primary tumors from mice inoculated with MSCV, FLSEC, DNLS, or DNLS+CTERM cells. All panels = 40X and scale bars = 50µm. (B) Western blot analysis of p27, pERK, ERK, p-p38, p38 and tubulin (loading control) protein levels in MSCV, FLSEC, DNLS, or DNLS+CTERM cells treated with vehicle (DMSO) or LIFR inhibitor (EC359, 50nM or 100nM) for 24 hours. Densitometry for western blot analysis of (C) p27, (D) pERK, and (E) p-p38 described in (B). (F) p38/ERK signaling ratio calculated from densitometry in (D & E). (A) **$p<0.01$ vs DNLS by unpaired t-test. (C) *$p<0.05$ vs DNLS by unpaired t-test or *$p<0.05$ vs MSCV by one-way ANOVA with multiple comparisons. (D) *$p<0.05$ vs MSCV by one-way ANOVA with multiple comparisons, *$p<0.05$ or **$p<0.01$ vs vehicle by two -way ANOVA with multiple comparisons. (E) *$p<0.05$ vs MSCV by one-way ANOVA with multiple comparisons, **$p<0.01$ or ***$p<0.001$ vs vehicle by two -way ANOVA with multiple comparisons. (F) *$p<0.05$ vs vehicle by two -way ANOVA with multiple comparisons. Graphs represent mean ± SEM.

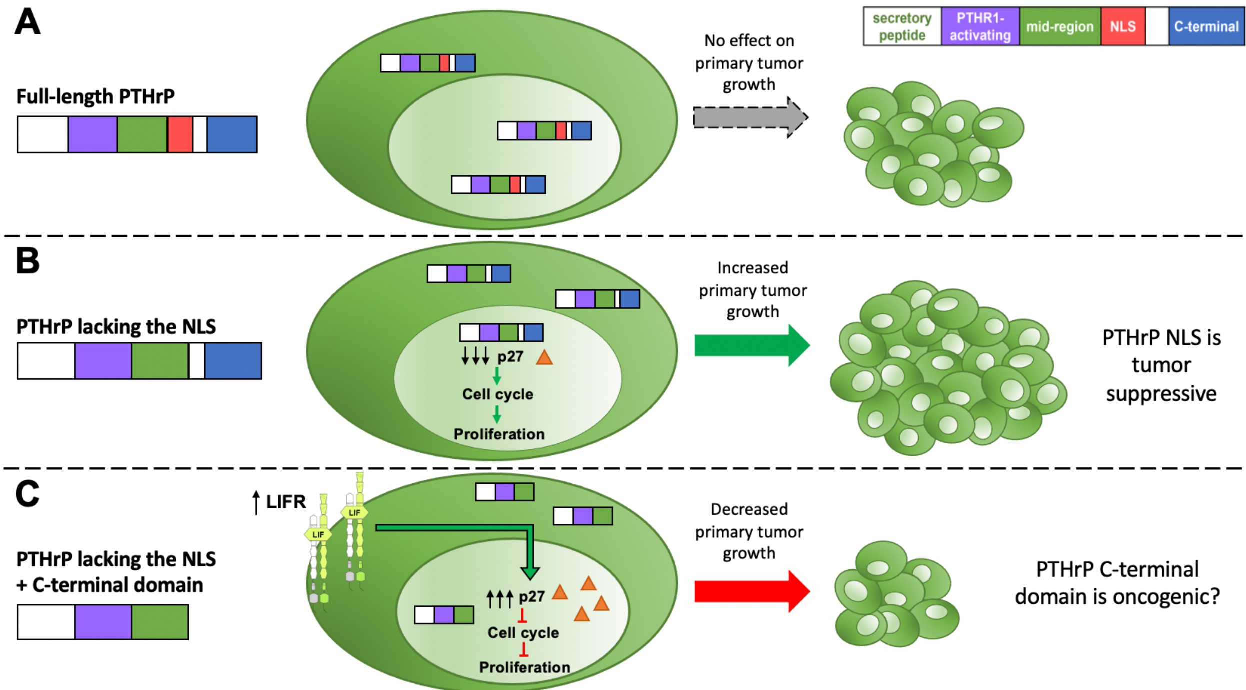


Figure 24. Summary of the effects of PTHrP and its truncated forms on breast tumor cell proliferation and primary tumor growth. (A) Full-length secreted PTHrP localizes in the nucleus and cytoplasm of human breast cancer cells but has no effect on primary tumor growth in our model. (B & C) Deletion of the PTHrP nuclear localization signal (NLS) does not preclude nuclear entry since truncated forms of PTHrP lacking the NLS alone or NLS and C-terminal domain were both able to localize within the cytoplasm and nucleus of human breast cancer cells. (B) In cells lacking the PTHrP NLS alone, p27 expression is decreased which may promote cell cycling and proliferation to increase primary tumor growth, suggesting that the PTHrP NLS may function as a tumor suppressor in breast cancer. (C) In cells lacking the PTHrP NLS and C-terminal domain, increased LIFR protein levels induce p27 expression which inhibits cell cycling and proliferation, resulting in decreased primary breast tumor growth. Thus, the C-terminal domain may be oncogenic in breast cancer.

PTHrP lacking the NLS dramatically induces osteolysis

Given the observed effects on cell proliferation and tumor growth in the primary site, and the known role for PTHrP in tumor-induced osteolysis, we sought to examine how the PTHrP NLS and C-terminal domain regulate breast cancer progression in the bone. Following intracardiac inoculation, tumor cells expressing PTHrP lacking the NLS alone or the NLS and the C-terminal domain dramatically increased the number and size of osteolytic lesions in the bone, even greater than overexpression of the full-length PTHrP molecule (Figure 25A – C). Furthermore, the increased osteolysis observed in this model is consistent with a dramatic increase in tumor burden in the bone with deletion of the NLS alone or NLS and C-terminal domain, as measured by flow cytometric analysis of CD298+ tumor cells in the bone marrow (Figure 25D). Together, these data suggest that the PTHrP NLS may play a particularly dominant role in regulating bone colonization, breast tumor cell proliferation, and osteolysis in the bone (Figure 26).

Discussion

The PTHrP NLS and C-terminal domains oppositely regulate breast tumor growth

PTHrP is a critical driver of tumor-induced bone disease and has more recently been recognized as an important regulator of breast tumorigenesis, cancer progression, and tumor dormancy [169, 213, 352, 353]. This multifaceted peptide has multiple domains with unique biological functions that mediate its secretion, activation of PTH receptor type I (PTH1R), nuclear localization (NLS), and cytoplasmic activity through the C-terminal region [342]. Here we investigated the role of the NLS and C-terminal domain in regulating breast cancer progression via these two domains that control many of the intracellular actions of PTHrP. We surprisingly found that deletion of the PTHrP NLS (amino acids 67-94) does not preclude entry into the nucleus. This suggests that our study outcomes are likely due to alterations in the ability of truncated forms of PTHrP to bind with other signaling proteins or interact in protein complexes, rather than solely the subcellular localization of the truncated peptides. Our studies revealed that breast cancer cells expressing PTHrP lacking the NLS were significantly more proliferative *in vitro.* In line with this phenotype, we similarly saw that expression of PTHrP lacking the NLS alone dramatically accelerated tumor growth and proliferation of tumor cells in the mammary fat pad. Surprisingly, PTHrP lacking both the NLS and C-terminal domain completely reversed this phenotype such that the primary tumors were significantly smaller and slower growing. These findings ultimately suggest that the PTHrP NLS functions as a tumor suppressor while the C-terminal domain may be oncogenic in breast cancer.

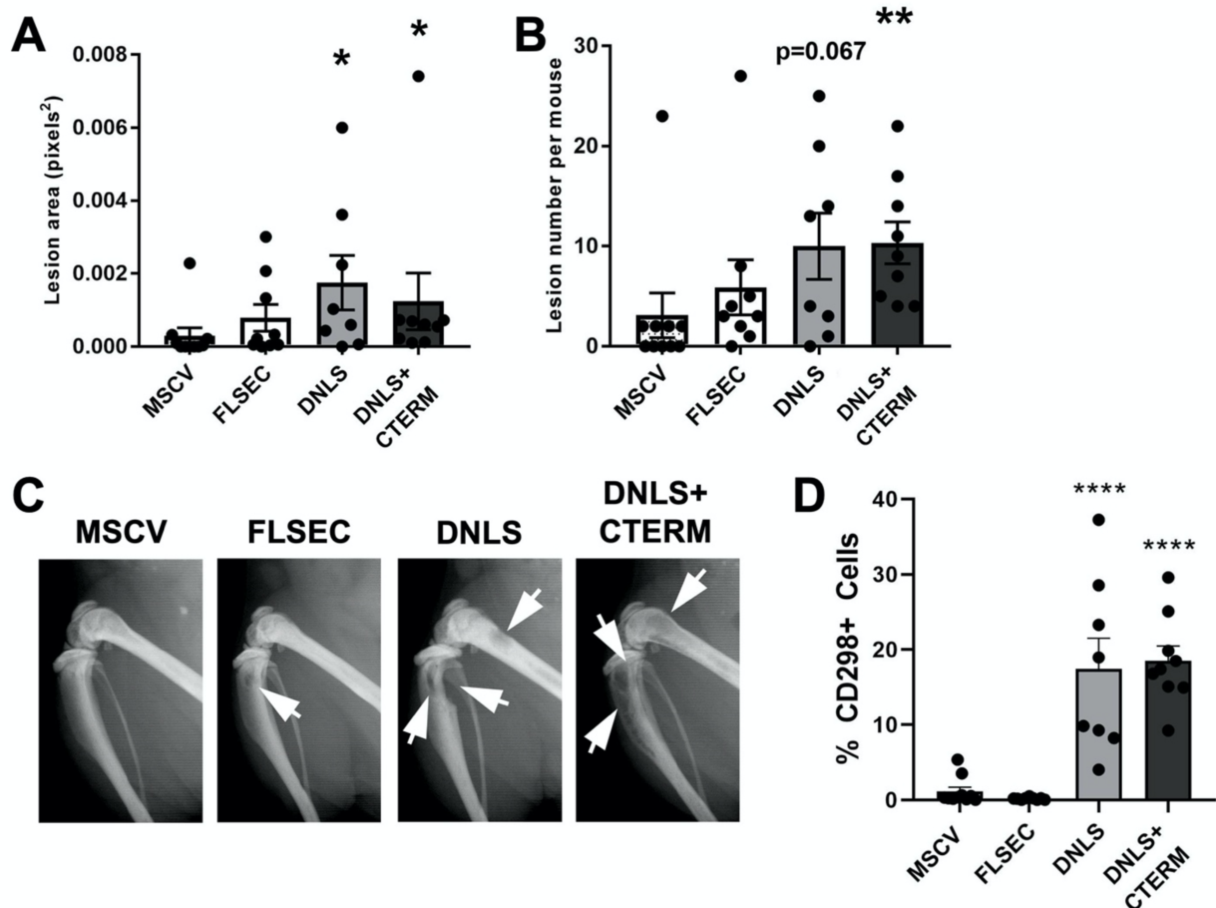


Figure 25. Deletion of the PTHrP NLS dramatically induces osteolysis and increases MCF7 tumor burden in the bone. (A-C) Total osteolytic lesion area and lesion number (per mouse) based on radiographic analyses for mice inoculated with MSCV, FLSEC, DNLS, or DNLS+CTERM cells via intracardiac injection. n=8-10 mice/group. (D) Flow cytometric quantitation of percent CD298+ tumor cells in the bone marrow of mice described in A-C. *p<0.05, **p<0.01 or ****p<0.0001 vs MSCV by one-way ANOVA with multiple comparisons. Graphs represent mean ± SEM.

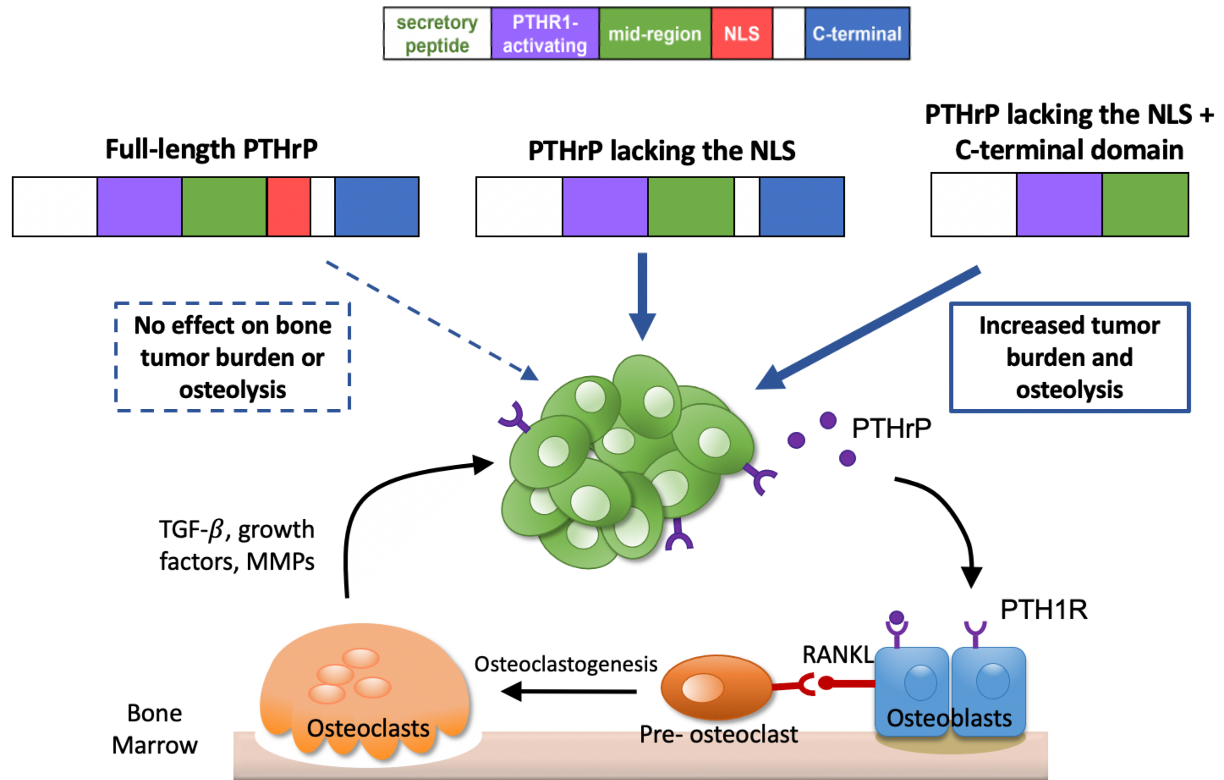


Figure 26. Summary of the effects of PTHrP and its truncated forms on breast tumor growth and osteolysis in the bone. PTHrP secreted by tumor cells binds to and signals through the PTH receptor (PTH1R) on osteoblast lineage cells to stimulate RANKL production and osteoclastogenesis. Osteoclast-mediated resorption releases pro-tumorigenic factors from the bone matrix such as TGF-β, matrix metalloproteinases (MMPs) and other growth factors that further fuel tumor cell colonization, proliferation, and PTHrP production. Breast tumor cell expression of full-length secreted PTHrP had no effect on bone tumor burden or osteolysis in our model. However, expression of PTHrP lacking the NLS alone or NLS and C-terminal domain increases bone tumor burden and osteolysis in the bone.

Our *in vivo* findings offer interesting insight into the complex role that PTHrP plays in breast tumor progression, which is likely driven by functions of its NLS and C-terminal domain that seem to have opposing effects on tumor cell behavior. While a large body of evidence clearly indicates that PTHrP has deleterious effects during late stages of breast cancer by promoting bone metastasis, tumor-induced osteolysis, and exit from dormancy [333, 354], PTHrP's role early in disease progression is highly controversial. Prior preclinical studies reported directly conflicting evidence suggesting that PTHrP inhibits primary breast tumorigenesis in some models [218], while promoting tumor growth in others [213]. It is plausible that the major discrepancies in these studies are explained by differences in expression of PTHrP fragments lacking either the NLS or C-terminal domain, which we have shown here to have divergent effects on breast tumor growth. It is also important to note that these studies, among numerous other preclinical and clinical investigations of PTHrP, frequently utilize commercially available antibodies targeted to the N-terminal region of the protein. Thus, truncated forms (like those with deletion of the NLS or C-terminus) are not fully taken into consideration. Importantly, fragments containing the amino terminal region (1-36), mid-regions (38-94), (38-95) and (38-101), as well as the C-terminal domain (107-139) have been detected in the plasma and urine of patients with solid tumors [288, 289]. Some limited investigation of the mid-region (38-94) fragment has been conducted in breast cancer [355], but deeper investigation of the function of these peptides in breast tumorigenesis is warranted. Future interpretation of PTHrP as a prognostic indicator in breast cancer should aim to more accurately detect truncated forms of PTHrP, in addition to the full-length molecule, since our data demonstrate they can have opposing effects on tumor cell behavior.

The PTHrP NLS and C-terminal domain differentially regulate p27 expression in breast cancer via a LIFR-dependent mechanism

Given the observed pleiotropic effects of the PTHrP NLS and C-terminus on breast tumor growth, we further explored the intracellular mechanisms by which PTHrP controls proliferation. Previous studies indicate that various cyclin dependent kinase inhibitors are regulated downstream of the PTHrP NLS and C-terminal domain in other non-breast cancer cell lineages [348-350]. Our studies show that p27 is oppositely regulated by the PTHrP NLS and C-terminal domain in breast cancer and may be an important downstream signaling factor mediating how these domains differentially alter breast tumor growth. Specifically, the PTHrP C-terminal domain appears to function as an oncogenic molecular switch able to induce proliferation and promote breast tumor formation via a mechanism that suppresses p27 expression. Future studies utilizing

breast cancer cells expressing PTHrP with deletion of the C-terminal domain only will be required to confirm this.

Since we previously determined that PTHrP downregulates LIFR [169, 313], a known breast tumor dormancy regulator in bone [169, 314], and that LIFR loss downregulates other pro-dormancy genes including p27 [169], we explored whether the PTHrP NLS and C-terminal domains regulate LIFR signaling to control downstream p27 expression. Indeed, LIFR expression was oppositely regulated by the PTHrP NLS and C-terminal domain such that breast tumors containing PTHrP lacking the NLS and C-terminal had significantly higher LIFR expression than tumors expressing PTHrP lacking the NLS alone. Importantly, this pattern of LIFR expression mirrors the pattern of p27 changes that we observed in the primary tumor, suggesting that the PTHrP NLS and C-terminal domain may alter p27 via a LIFR-dependent mechanism. Indeed, pharmacologic LIFR inhibition in cells overexpressing PTHrP lacking both the NLS and C-terminal domain were no longer able to induce p27 and in fact had significantly lower expression than control cells. This suggests that the PTHrP C-terminal domain regulates LIFR signaling to alter downstream p27 expression.

Activation of LIFR by the gp130 family of cytokines (LIF, OSM, and CNTF) is known to differentially induce STAT3 [175, 356-359], MAPK/ERK [356, 360-362], and AKT activity [358, 362, 363], as well as multiple other signaling pathways that were recently identified but have not been explored in-depth [364]. While LIFR:STAT3 signaling confers a dormant phenotype in bone-disseminated breast tumor cells [365-367], preferential activation of the other downstream signaling pathways can have either pro-tumorigenic or pro-dormancy effects in breast cancer cells residing in the primary site [364]. It remains unknown whether the PTHrP NLS and C-terminal domains differentially regulate LIFR signaling through the STAT3, MAPK, or AKT signaling pathways to alter p27 expression and downstream effects on breast tumor growth. In this study we investigated the *in vitro* effects of LIFR inhibition on p27 induction in the setting of basal levels of gp130 cytokine production. However, this does not take into account the *in vivo* behavior of the PTHrP mutant cells in the primary tumor or bone marrow niche where the cytokine levels may be elevated. It will be useful to study how the exogenous presence of the cytokines and their differentially activated downstream signaling pathways affect the influence of the PTHrP NLS and C-terminal domain on LIFR signaling and tumor cell behavior.

To further understand the mechanisms that regulate the effects of the PTHrP NLS and C-terminal domain on proliferation versus dormancy in breast cancer, we examined the balance between p38 and ERK signaling which is a well-established mechanism for regulating tumor cell dormancy [368]. Preferential p38 activation and a high p38/ERK signaling ratio induces dormancy

[233] since ERK signaling promotes cell cycle progression while p38 signaling can inhibit ERK-mediated proliferation and induce G0/G1 arrest [338-340]. In our studies of the breast cancer cells expressing PTHrP lacking the NLS and C-terminal domain, there was an increase in phosphorylated p38 and the p38/ERK ratio compared to controls, indicating that these cells have adopted a more dormant phenotype, which is consistent with their reduced tumor growth *in vivo* and the higher LIFR protein expression observed in these tumors *in vivo*. There was no change in the p38/ERK ratio when cells expressed PTHrP lacking the NLS only. Combined, these observations suggest that the C-terminal domain may be particularly important in controlling the function of PTHrP as a negative regulator of breast tumor dormancy through p38/ERK signaling.

Pharmacologic LIFR inhibition revealed an unexpected trend whereby the PTHrP mutant cells treated with the inhibitor had significantly elevated phosphorylated p38 and a p38/ERK signaling ratio compared to vehicle treated cells. This is an important finding as it suggests that the inhibitor may preferentially decrease LIFR activation of ERK signaling, shifting the balance towards p38 activity and potentially inducing a dormant phenotype *in vitro*. Recently, small molecule inhibitors and neutralizing antibodies targeting LIFR have been investigated as a strategy to inhibit breast tumor growth and metastasis in preclinical studies [369, 370]. Although anti-LIFR agents do show some evidence of effectively targeting primary breast tumors, caution should still be exercised in their use as a breast cancer therapy since inhibiting LIFR signaling could inadvertently increase metastatic outgrowth in bone where the LIFR:STAT3 pathway maintains disseminated tumor cells in a dormant state. Furthermore, it is still unclear how the PTHrP NLS and C-terminal domains may differentially regulate the downstream LIFR signaling pathways, and thus inhibiting PTHrP to prevent breast tumor progression should be approached with caution. Ultimately, more *in vivo* studies are needed to specifically examine the effects of LIFR-targeted therapies and PTHrP-targeted therapies on the growth of breast tumors expressing PTHrP or its truncated forms in the primary and bone-metastatic sites.

The PTHrP NLS regulates bone colonization and osteolysis

Finally, since breast cancer cells have a high predilection for metastasizing to the bone marrow [180] where PTHrP plays a critical role in driving osteolysis and metastatic outgrowth [221, 280, 291, 346], we investigated how the tumor cells home to and grow in the bone with overexpression of the full-length PTHrP molecule or truncated forms. Interestingly, we did not observe any significant difference in osteolysis and bone tumor burden with overexpression of our plasmid containing full-length PTHrP, although a previous study demonstrated that PTHrP (1-139) overexpression in MCF7 cells dramatically induces bone metastasis formation and

osteolysis [221]. Ultimately, further experiments will be necessary to confirm the role of the full-length PTHrP molecule in breast tumor cell exit from dormancy in the bone. Surprisingly, in our studies osteolysis and bone tumor burden were dramatically increased by bone-disseminated breast tumors cells expressing PTHrP lacking the NLS alone or NLS and C-terminal domain. It is intriguing that the effect of PTHrP on tumor growth and proliferation in the primary site, but not osteolysis, is completely reversed if the C-terminal portion of PTHrP is deleted along with the NLS. While the C-terminal domain seems to be a unique molecular switch controlling breast tumorigenesis in the primary site, that effect does not seem to exist in the bone. Thus, alternative signaling mechanisms other than LIFR-mediated changes in p27 likely control how the PTHrP NLS and C-terminal domain regulate bone colonization and osteolysis. Further studies are needed to understand how the PTHrP NLS and C-terminal domain affect the signaling and behavior of bone-disseminated tumor cells. Since PTHrP regulates tumor dormancy in the bone [221], investigation of these processes may offer mechanistic insight into why/how breast cancer cells lie dormant for years and then re-emerge and how PTHrP could be targeted to reduce or prevent bone metastasis.

Concluding Remarks

In summary, these data reveal how the PTHrP NLS and C-terminal domain differentially regulate breast tumor cell signaling and tumor growth in the primary site versus the bone. Due to its multifactorial roles in tumorigenesis and breast cancer metastasis, PTHrP has the potential to be leveraged as a therapeutic target for the treatment of breast cancer at multiple stages of disease progression and possibly for the prevention of bone metastasis formation. However, much work is still required to further understand how PTHrP and its truncated forms affect breast tumor growth in different microenvironments. This knowledge, coupled with a better understanding of the abundance of PTHrP fragments in tumors, will be necessary to determine the appropriate patient population and timing of administration of any future PTHrP targeted therapies.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Despite remarkable improvements in the detection and treatment of primary breast tumors, metastatic breast cancer continues to cause considerable morbidity and mortality in patients. Unfortunately, there is still no cure or prevention for metastasis, leaving a significant unmet clinical need for identifying effective targeted therapies. In recent decades, substantial progress has been made towards understanding the mechanisms that regulate dissemination of tumor cells to distant sites and their eventual metastatic outgrowth or existence in a dormant state for prolonged periods of time. One of these key factors, leukemia inhibitory factor receptor (LIFR), is a recognized breast tumor suppressor and dormancy regulator in bone [169, 314]. However, the specific mechanisms that regulate its expression and downstream signaling in breast cancer cells localized in various microenvironments are not completely understood. Studies presented in this dissertation demonstrate one mechanism whereby LIFR is downregulated in hypoxia (low oxygen tensions) by increased repressive histone modifications (methylation) and reduced transcriptional activation marks (acetylation) within the promoter region. This is a key finding as tumor cells experience hypoxic conditions when solid tumors grow beyond several millimeters or take up residence in hypoxic regions of the bone marrow. Given these findings, we ultimately sought to determine whether targeting histone acetylation could effectively stimulate LIFR expression in low oxygen conditions, such as what would be encountered by tumor cells residing in hypoxic regions of the primary tumor or bone. We previously demonstrated that HDAC inhibitors epigenetically increase *LIFR* expression and induce a pro-dormancy phenotype under normal oxygen conditions [314], and our findings here indicate that the HDAC inhibitors entinostat and panobinostat retain their ability to robustly stimulate LIFR in hypoxia.

Along with hypoxic repression, we have also previously determined that LIFR is downregulated by PTHrP overexpression, which promotes exit from dormancy and metastatic outgrowth in the bone [169, 221]. Our studies herein demonstrate for the first time that PTHrP localizes to the *LIFR* promoter and downregulates its protein expression in breast cancer cells. HDAC inhibition with entinostat or panobinstat stimulated LIFR even in the setting of repression by PTHrP overexpression by enriching for H3K9ac, a marker of active transcription. These results indicate that HDAC inhibitors can overcome LIFR repression driven by either hypoxia or PTHrP overexpression, which are two characteristics of the breast tumor and microenvironments that will

influence whether tumor cells proliferate or remain in a dormant state. Moreover, these findings are clinically relevant because while HDAC inhibitors are FDA approved for the treatment of hematologic malignancies, entinostat failed in a recent phase III clinical trial as combination for metastatic breast cancer [328]. This leaves many questions as to why the HDAC inhibitors have shown promising efficacy in preclinical and early clinical trials, but have been unsuccessful in large, later phase patient studies. While the answer is likely multifactorial, our findings here rule out an inability for the inhibitors to overcome the effects of hypoxia or PTHrP-induced LIFR repression. Thus, there is still potential for HDAC inhibitors to be utilized clinically to induce dormancy and reduce breast cancer recurrence.

In addition to its roles in promoting exit from dormancy and metastatic outgrowth in the bone [169, 221], emerging studies have also established an important role for PTHrP in regulating primary breast tumorigenesis. Overall, clinical studies indicate that PTHrP expression in the primary tumor is correlated with improved patient survival and decreased bone metastasis. However, some preclinical studies have produced directly conflicting results suggesting that PTHrP can inhibit [218], or promote breast tumorigenesis [213]. While these major discrepancies have not been fully resolved, the work presented herein likely explains some of the differences in these study outcomes by offering useful insight into the intracellular actions of PTHrP driven by its nuclear localization signal (NLS) and C-terminal domain, which we have found to have divergent effects on breast tumor growth. Specifically, our *in vivo* models demonstrate that expression of PTHrP lacking the NLS dramatically accelerates primary breast tumor growth and proliferation, while this phenotype is completely reversed by expression of PTHrP lacking the NLS and C-terminal domain. These findings indicate that the PTHrP NLS functions as a tumor suppressor whereas the C-terminal domain may be an oncogenic switch in primary breast cancer. Surprisingly, tumor-induced bone destruction and bone tumor burden were dramatically increased by bone-disseminated breast tumors cells expressing PTHrP lacking the NLS alone or NLS and C-terminal domain. It is interesting that the same phenotypic switch did not occur in the bone as in the primary tumor, highlighting how the function of these domains can differentially alter the behavior of tumor cells in different microenvironments. To fully understand the prognostic role for PTHrP in breast cancer in all stages of disease progression, it will be necessary to take into account the abundance and function of these truncated forms of PTHrP since differences in their expression could drive or inhibit tumor progression in multiple sites.

Further investigation of the signaling mechanisms that underlie our *in vivo* phenotype revealed that the PTHrP NLS and C-terminal domain oppositely regulate expression of the cyclin dependent kinase inhibitor, p27. Specifically, the PTHrP C-terminal domain appears to function

as an oncogenic molecular switch able to induce proliferation and promote breast tumor formation via a mechanism that suppresses p27 expression. Since we previously determined that PTHrP overexpression downregulates LIFR [169, 313], and LIFR downregulates numerous other pro-dormancy genes (e.g. p27) [175], we investigated whether the PTHrP NLS or C-terminal differentially regulate p27 expression via a LIFR-dependent mechanism. Indeed, LIFR expression was oppositely regulated by the PTHrP NLS and C-terminal domain such that breast tumors containing PTHrP lacking the NLS and C-terminal had significantly higher expression than tumors expressing PTHrP lacking the NLS alone. Furthermore, pharmacologic LIFR inhibition blunted the ability of the cells expressing PTHrP lacking both the NLS and C-terminal domain to induce p27. Beyond examining changes in LIFR expression that alter p27, deeper work is still needed to understand specifically how the PTHrP NLS and C-terminal domain differentially alter downstream signaling through the STAT3, MAPK, or AKT signaling pathways. These pathways are all activated downstream of LIFR but can have pro-tumorigenic or pro-dormancy effects in breast cancer in certain contexts.

PTHrP is an incredibly complex peptide with multiple distinct domains that can each influence its endocrine, paracrine, autocrine and intracrine signaling activity. This coupled with the fact that its different isoforms and fragments can elicit diverse cellular responses could result in PTHrP-targeting therapies that inadvertently induce tumor development and recurrence if used in the wrong patient population or stage of disease progression. If PTHrP-targeted therapies are to be effectively utilized for the treatment of breast cancer, much more work is required to gain a deeper understanding of the signaling mechanisms by which PTHrP and its truncated forms alter breast tumor cell behavior in the primary tumor or metastatic sites.

Future Directions

What is the mechanism by which PTHrP acts as a transcriptional regulator of LIFR?

We previously published that overexpression of PTHrP (-36-139) downregulates *LIFR* and several other pro-dormancy factors in MCF7 breast cancer cells [169] independent of autocrine/ paracrine activation of the PTH1R domain and downstream cAMP signaling [226]. Thus, the effects of PTHrP on dormancy gene expression must be regulated by intracellular actions of domains other than the PTH1R activating region. In Chapter III, our chromatin immunoprecipitation (ChIP) analyses provide evidence for the first time that PTHrP localizes to the *LIFR* promoter. However, it remains unknown how this occurs and whether PTHrP functions as a transcription factor capable of directly binding DNA. Although PTHrP does have a known

RNA binding motif within its mid-region [343], it has no identified DNA binding motif. Additional studies would be useful in determining whether any of the PTHrP biological domains also possess DNA-binding capabilities. Our ChIP studies utilized a model system where MCF7 human breast cancer cells overexpress HA-tagged PTHrP, since commercially available antibodies are unreliable for the detection of the endogenous protein. There are inherent limitations to this approach since the overexpressed recombinant protein may not fully mimic the behavior of the endogenous protein expressed at basal levels. To confirm whether endogenous PTHrP functions as a transcription factor that directly binds DNA within the *LIFR* promoter, an electrophoretic mobility shift assay (EMSA) should be performed to detect protein- nucleic acid interactions [371, 372]. To more definitively conclude from the EMSA that any binding activity associated with the nucleotides of interest in the promoter region is indeed due to PTHrP (rather than the presence of another binding partner within the protein complex), use of a highly purified extract of endogenous PTHrP would be most useful. Unfortunately, without an antibody that reliably detects endogenous PTHrP, antibody-based purification methods will not be possible and alternative purification methods should be optimized. Alternatively, an unpurified cell extract can be utilized by coupling the EMSA with mass spectrometry to identify PTHrP or other unknown transcription factors and proteins that bind the target nucleotide sequences in the *LIFR* promoter [373]. Lastly, in the setting of PTHrP overexpression, as is the case for the studies presented in this dissertation, an EMSA should also be performed to confirm whether the recombinant PTHrP directly binds the *LIFR* promoter in MCF7 cells expressing our plasmid of interest. In this case, the PTHrP is HA-tagged and can be purified with an anti-HA antibody prior to performing the EMSA.

Alternatively, if PTHrP does not directly bind DNA, it likely exists in a complex with other DNA-binding proteins, though to our knowledge, none have been identified yet. To identify additional unknown proteins that bind the *LIFR* promoter, a DNA pulldown assay followed by mass spectrometry could be performed [374]. These analyses will more broadly shed light on the factors that control the transcriptional regulation of *LIFR* and possibly elucidate how PTHrP directly or indirectly regulates gene expression in breast cancer.

How does the PTHrP C-terminal domain independently influence breast tumor cell behavior?

The studies presented in chapter IV utilized a model where MCF7 cells were stably transfected with plasmids containing the full-length PTHrP molecule or truncated forms with deletion of the NLS alone or NLS and C-terminal domain. These plasmids were utilized since we originally hypothesized that the NLS would play the key role in regulating breast tumor cell

behavior based on previous data in other non-breast cancer cell lineages investigating the effects of the NLS on proliferation. However, our studies here indicate that both the NLS and C-terminal domain are important for regulating breast tumor cell proliferation. While our *in vivo* and *in vitro* experiments examining the effects of deletion of both the NLS and C-terminal domain suggest that the C-terminus may be oncogenic, it is still difficult to interpret the independent effects of this domain. Perhaps the C-terminal domain decreases LIFR and p27 expression to drive proliferation in breast cancer, but this oncogenic activity is kept in check by the presence and activity of the NLS. However, when the tumor suppressive activity of the NLS is also absent, the C-terminal domain is uninhibited and able to drive breast tumor growth.

Future studies should be conducted utilizing MCF7 cells that stably express PTHrP lacking the C-terminal domain only (in comparison to the other existing PTHrP mutant cells) to examine effects on (I) *in vitro* cell proliferation assessed by trypan blue exclusion assay, (II) primary tumor growth assessed following tumor cell implantation into the mammary fat pad, (III) osteolysis and bone tumor growth assessed following intracardiac injection of tumor cells and (IV) exit from dormancy assessed by the p38/ERK signaling ratio, Ki67 positivity and LIFR, p21 and p27 expression. To confirm the *in vitro* findings proposed here as well observations from Chapter IV suggesting that PTHrP lacking the C-terminal domain upregulates LIFR to induce p27 and decrease primary tumor growth, an *in vivo* study with LIFR inhibitor administration or LIFR knockdown should also be conducted. If our hypothesized mechanism is correct, pharmacologic LIFR inhibition in mice inoculated by mammary fat pad injection with cells expressing PTHrP lacking the NLS and C-terminal domain or C-terminal domain only, or LIFR knockdown in these cells may result in increased primary tumor growth and decreased p27 expression compared to the control group. Primary tumors from this study should be stained by immunofluorescence for LIFR, pERK, pSTAT3, p21, p27, p38 and Ki67 to confirm effective LIFR inhibition and to assess readouts of dormancy and the other *in vitro* signaling mechanisms outlined in Chapter IV of this dissertation.

How does exogenous presence of the gp130 cytokines and their differentially activated downstream signaling pathways affect the influence of the PTHrP NLS and C-terminal domain on LIFR signaling and tumor cell behavior?

Our findings in chapter IV suggest that the PTHrP NLS and C-terminal domain oppositely regulate p27 via a LIFR-dependent mechanism. Activation of LIFR by the gp130 family of cytokines (LIF, OSM, and CNTF) induces signaling through the STAT3 [175, 356-359], MAPK/ERK [356, 360-362], and AKT pathways [358, 362, 363], among others [364]. While

activation of the LIFR:STAT3 signaling axis maintains bone-disseminated breast tumor cells in a dormant state [365-367], preferential activation of the other downstream pathways can have either pro-tumorigenic or pro-dormancy effects in the primary tumor or other metastatic sites [364]. It is unknown whether the PTHrP NLS and C-terminal domains preferentially activate the STAT3, MAPK, or AKT signaling pathways downstream of LIFR to alter p27 expression and how this changes in cells that remain in the primary tumor or disseminate to the bone where the cytokines are also produced by bone marrow niche cells [375-379]. To investigate this further, the PTHrP mutant cells should be treated with the LIFR inhibitor for 24 hours and stimulated with recombinant LIF, OSM, or CNTF (alone, or in combination to mimic the bone microenvironment where all three are produced by bone marrow niche cells). Cytokine concentrations of 1ng/ml can be utilized since we have previously tested a range of concentrations from 0-100ng/ml and seen that this low concentration is able to robustly activate LIFR signaling [169]. Optimization of a higher LIFR inhibitor concentration may be needed to induce substantial alterations in downstream LIFR signaling factors since this compound functions as a competitive inhibitor that disrupts the receptor/ligand binding site. In the present studies, a maximum inhibitor concentration of 100nM was sufficient to reduce LIFR activation/ pERK levels in the setting of basal cytokine expression, but this may need to be increased with recombinant cytokine treatment. Following treatment, western blotting and immunocytochemistry should be performed to examine STAT3, ERK, and Akt activation (assessed by pSTAT3, pERK, and pAkt levels, respectively) as well as p38 signaling to elucidate how these cytokines differentially influence pro-tumorigenic versus pro-dormancy signaling in cells expressing PTHrP or its truncated forms. In the setting of exogenous cytokine stimulation, these studies will also shed light on whether the LIFR inhibitor predominantly targets LIFR activation of ERK signaling and induces p38 as we saw in the *in vitro* studies in Chapter IV, or whether STAT3 or Akt signaling is preferentially inhibited. This knowledge will be important to understand since it may determine whether LIFR inhibition is a viable therapy to induce dormancy in metastatic sites like the bone where LIFR:STAT3 signaling is necessary to maintain quiescence.

What is the mechanism by which the PTHrP NLS and C-terminal domain regulate exit from dormancy in the bone?

In chapter IV we observed an interesting phenotype whereby the effect of PTHrP on primary tumor growth and proliferation, but not osteolysis and bone tumor burden, is completely reversed if the C-terminal portion is deleted along with the NLS. Why does the PTHrP C-terminal

domain seem to function as a phenotypic switch in the primary tumor, but not the bone? We currently know very little about the signaling changes induced when PTHrP versus its truncated forms are expressed in bone-disseminated tumor cells. It will be useful to further examine our RNA sequencing and Gene Set Enrichment Analysis (GSEA) data to identify genes that may be implicated in PTHrP-induced exit from dormancy in the bone. For instance, our GSEA data showed a significant enrichment for genes involved in the epithelial-to-mesenchymal transition (EMT) in cells expressing PTHrP lacking the NLS alone or NLS and C-terminal domain (data not shown). While EMT is implicated in dormancy, it is not completely understood whether this process maintains or drives bone-disseminated tumor cells out of dormancy, or how the PTHrP domains influence this effect. However, in our studies we did observe a significant increase in p38 signaling and the p38/ERK ratio in cells expressing PTHrP lacking the NLS and C-terminal domain. TGFβ2, a main mediator of EMT, increases p38 and reduces ERK signaling, thus increasing the p38/ERK signaling ratio [380]. Perhaps in the primary tumor, breast cancer cells lacking the PTHrP NLS and C-terminal domain have elevated TGFβ2 which induces p38 signaling and a dormant phenotype (consistent with the observed reduction in tumor growth *in vivo*). Cells expressing PTHrP lacking the NLS alone may be more proliferative in the primary tumor, in part, because they do not induce TGFβ2/ p38-mediated dormancy. However, once the cells disseminate to the bone, there is a molecular switch where cells expressing PTHrP lacking the NLS and C-terminal domain are no longer able to induce TGFβ2, pushing the cells out of dormancy into a more proliferative and osteolytic state. Consequently, expression of either truncated form of PTHrP results in the observed increase in osteolysis and tumor burden in bone.

To investigate whether truncated PTHrP lacking the NLS and C-terminal domain reduces proliferation and tumor growth by increasing TGFβ2/ p38 signaling, the genes encoding both of these factors should be individually deleted via CRISPR/Cas9 (or lentiviral shRNA silencing as an alternative). This should be performed in the MSCV control cells and MCF7 cells expressing PTHrP lacking the NLS and C-terminal domain, which have elevated TGFβ2 (data not shown). If TGFβ2 (gene name, *TGFB2*) does alter dormancy in these cells, p38 (gene name, *MAPK14*) knockout should phenocopy *TGFB2* deletion. The cells lacking the PTHrP NLS and C-terminus with and without *TGFB2* or *MAPK14* ablation should be analyzed for p38/ ERK signaling activation as well as p21 and p27 expression by qPCR, western blot and immunocytochemistry. *In vitro* proliferation as a readout of dormancy should be analyzed by a trypan blue exclusion assay or a CellTrace Violet dye assay.

In vivo analysis with these cells +/- *TGFB2* and *MAPK14* deletion should also be performed following mammary fat pad and intracardiac injection to analyze changes in

proliferation and dormancy pathways in the primary site versus bone. Mammary fat pad tumors should be stained for TGFβ2, p38, pERK/ERK, p21, p27, and Ki67 by immunofluorescence. Bone-disseminated cells from mouse femur can be prepared for DNA staining by Hoechst to analyze cell cycling by flow cytometry. These cells should also be flow sorted out by CD298 selection to examine expression of TGFβ2, p38, ERK, p21, and p27 by Western blot. From the intracardiac study, other portions of the mouse hindlimb can be utilized for microCT analysis of bone volume (to assess bone destruction) as well as immunofluorescence staining for cytokeratin (to measure tumor burden) and p38, p21, p27 and Ki67 (as readouts of tumor dormancy). To more broadly assess signaling in the bone, even without TGFβ2 or p38 knockout, CD298+ cells from the bones of mice inocluated with each of the PTHrP mutant cells should be flow sorted and analyzed by RNA sequencing to assess which signaling factors are changed in the bone disseminated tumor cells *in vivo.* Investigation of these processes will enhance our understanding of tumor dormancy and disseminated tumor cell interactions with the bone metastatic niche.

What is the incidence of PTHrP mutations or abundance of its truncated peptides in breast cancer patients?

Numerous preclinical and clinical studies have investigated the expression of PTHrP in primary and metastatic breast tumors, although the studies primarily utilize antibodies targeted to the N-terminal region of the protein that cannot differentiate between truncated forms such as those with deletion of the NLS or C-terminus. Additionally, some studies have isolated fragments encompassing portions of the N-terminal, mid-region and C-terminal domains from plasma [288] and urine of patients with HHM [289]. However, to gain a more complete understanding of the prognostic role of PTHrP in breast cancer tumorigenesis and progression, these truncated peptides must be taken into deeper consideration. Does the abundance of PTHrP or its truncated forms change throughout disease progression or in different tumor sites? To analyze this, future studies should analyze patient primary tumors and any available matched metastatic lesions (bone, lung, liver, or brain), which can be obtained through the Cooperative Human Tissue Network (CHTN) at Vanderbilt University Medical Center. These analyses should utilize the PTHrP domain-specific primers we have generated in these studies to perform qPCR analysis on RNA extracted from the tissue specimens. Additionally, RNAscope (RNA *in situ* hybridization) can be performed on embedded tissue sections. These two analyses would allow detection of changes in expression of *PTHLH* at the gene level if alternative splicing gives rise to any truncated PTHrP isoforms.

Since a commercially available antibody to the mid-region or C-terminal domain does not exist, truncated forms of PTHrP generated by proteolysis could be detected in patient tumor sections by Matrix-Assisted Laser Desorption/ Ionization Imaging Mass Spectrometry (MALDI MS) as previously described [381]. This mass spectrometry technique allows for determination of the molecular composition, relative abundance, and spatial distribution of proteins directly from thin tissue sections [382]. However, there are still limitations to this approach as the process is performed on proteolytic fragments generated by enzyme digestion and may not definitely distinguish between peptides generated from intact or truncated PTHrP isoforms. Ultimately, much more work is needed to develop tools that can accurately detect truncated PTHrP peptides in human tissue samples. In particular, development of a polyclonal antibody suitable for western blotting would prove most useful. Finally, from patient samples obtained from the Vanderbilt CHTN, tumor sections should also be fixed and stained for p38, ERK, p21, p27 and Ki67 to examine changes in molecular markers of dormancy and downstream PTHrP signaling factors that we have identified in the present studies. Lastly, additional genetic studies are needed to investigate specific mutations in PTHrP in breast cancer. One previous analysis including multiple genome-wide association studies in breast cancer identified *PTHLH* (the gene name for PTHrP) as a susceptibility locus in both ER+ and ER- breast cancer [278]. However, it would be interesting to know if specific mutations are associated with better or worse outcomes in breast cancer patients.

Concluding Remarks

The work presented in this dissertation provides substantial insight into the nuanced factors that influence breast cancer cell behavior in the primary site and bone. These studies provide a strong foundation for future efforts to better understand the signaling mechanisms that influence the response of breast cancer cells to existing therapies such as HDAC inhibitors. While many questions remain unanswered, our work also offers a better understanding of the prognostic role for PTHrP in breast cancer and highlights additional targets to explore as anticancer therapeutics. Ultimately this knowledge will help identify the stage of disease progression and patient population where novel targeted therapies will be most efficacious in treating breast tumors and possibly preventing recurrence to improve patient outcomes.

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