Sympathetic Signaling Mediator, Norepinephrine, Re-Activates Prostate Cancer Bone Metastases through Direct and Indirect Mechanisms

by

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Dedication

For Joe

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Ach	acetylcholine
ATF4	Activating Transcription Factor 4
ADR	adrenergic receptor
BMP	bone morphogenetic protein
cAMP	cyclic adenosine monophosphate
CREB	cyclic adenosine monophosphate response element binding protein
CCL5	chemokine ligand 5
CXCL12	cysteine-x-cysteine motif chemokine ligand 12
DTC	disseminated tumor cell
FLT3L	fms-like tyrosine kinase 3 ligand
GAS6	growth arrest specific 6
GKO	gas6 knockout mouse
HSC	hematopoietic stem cel
IL	interleukin
M-CSF	macrophage colony stimulating factor
MERTK	mer tyrosine kinase
NE	norepinephrine
OB	osteoblast
PCa	prostate cancer

RAR	retinoic acid receptor
TAM	tyro3 axl mer
TRAP	tartrate-resistant acid phosphatase
VEGF	vascular endothelial growth factor
TRACER	transcriptional activity cell array
WT	wild type mouse

Abstract

Metastatic prostate cancer can recur months or years after clearance of the primary disease and is associated with poor clinical outcomes. The bone marrow represents the preferred sire for metastasis, with disseminated tumor cells (DTCs) able to establish residency and remain dormant until some outside stimulus leads to their reactivation. A number of signaling factors in the bone marrow such as growth arrest specific 6 (GAS6) have been identified to elicit dormancy (reviewed in Chapter 2), however less is known about signals that might trigger reactivation. This dissertation explores the hypothesis that signaling from the sympathetic nervous system may lead to reactivation of dormant prostate cancer cells. This hypothesis is motivated by clinical observations of a correlation between stressful life events and recurrence as well as mechanistic observations linking adrenergic signaling to the mobilization of bone marrow resident stem cells. Chapter 3 establishes a causal link between norepinephrine (NE) and reactivation of dormant prostate cancer cells in a variety of models for prostate cancer dormancy. These experiments establish a direct effect of NE on prostate cancer cells that can lead to cell cycle re-entry. Chapter 4 examines the effects of NE on the bone marrow microenvironment and identifies downregulation of GAS6 as the primary indirect factor mediating re-entry. Chapter 5 establishes a mechanism through which adrenergic signaling downregulates GAS6 expression in order to suggest clinical strategies to target this interaction. Finally, Chapter 6 suggests future directions for this project, focused on the role of GAS6 in long-term

bone homeostasis, another key factor leading to recurrence. Overall, this dissertation provides a new mechanism for DTC reactivation and establishes a potential new direction for clinicians to treat patients at risk for recurrence.

Chapter 1

Introduction

Disseminated tumor cells (DTCs) give rise to metastatic disease, which remains the cause of death for the vast majority of patients. In prostate cancer (PCa), DTCs primarily traffic to the bone, where they either proliferate into a secondary tumor or undergo cell cycle arrest. This second fate, DTCs that have acquired a quiescent phenotype, is the main topic of this dissertation. These dormant cells can become reactivated months or years after the patient has been cured, resulting in painful tumors in the bone and a poor prognosis. Technologies to detect, or clear these cells, are actively sought after, however much more knowledge of the biology of these DTCs is needed before dormant DTC-targeted therapy becomes a reality. Chapter 2 provides an in-depth review of possible signaling factors related to dormancy and how these biological mediators relate to the issue of reactivation.

Systemic factors leading to reactivation of DTCs are often hypothesized, however few causal mechanisms have been proposed in the literature. This dissertation addresses one of those hypotheses, specifically the relationship between the sympathetic nervous system and cell cycle re-entry for DTCs. Several sources have suggested a correlative relationship between psychosocial stress and poor cancer outcomes (1,2). Adrenergic signaling, in particular, has been previously demonstrated to activate numerous pathways associated with oncogenesis, and has also been tied to stem cell

egress from the bone marrow (3). This fact suggests a potential connection between adrenergic signaling, quiescent DTCs, and cell cycle re-entry, however no mechanisms connecting these processes has been proposed previously.

Three specific aims were established for this dissertation in order to address the hypothesis that adrenergic signaling may reactivate dormant PCa cells. These aims were as follows:

Aim 1: Establish a link between sympathetic signaling and cell cycle re-entry

The first set of experiments described in Chapter 3 are designed to establish a causal relationship between adrenergic signaling and cell cycle re-entry in dormant PCa cells. The existing literature on this topic suggested a correlative relationship when specifically examining cancer in the bone marrow, and as such these experiments were a logical place to start examining our hypothesis. A number of different models, each with a different biochemical milieu and different potential routes to dormancy and reactivation were investigated. The results of these studies provided a basis to continue probing for a mechanism that may connect dormancy to adrenergic signaling in the bone marrow.

Aim 2: Determine an indirect mechanism for NE leading to cell cycle re-entry

This aim is explored in Chapter 4 of this dissertation. These series of investigations sought to connect indirect changes in the microenvironment activated NE, and an awakening phenotype from dormant cells. These experiments were performed in a non-biased manor to facilitate single or combinations of secreted factors produced by osteoblasts that could connect adrenergic signaling to dormancy. Our rational for

choosing osteoblasts (OBs) as a niche regulating cell was derived from prior work demonstrating that once DTCs engage an osteoblastic-niche they become quiescent (4). Bioinformatic tools were used to identify a single factors, growth arrest specific 6 (GAS6) as essential to reactivation of PCa cells by NE.

Aim 3: Determine a mechanism for NE-mediated downregulation of GAS6

Finally, a third set of experiments described in Chapter 5 establish a clear mechanistic link between adrenergic signaling and the expression of GAS6. These experiments focused on the receptors and transcription factors mediating adrenergic signaling to GAS6. These experiments were able to identify a mechanism and link the phenotypes of the PCa/OB cultures and the molecular players mediating this response.

The work presented in this dissertation used a variety of model systems and techniques to establish a mechanistic relationship between GAS6, NE, and PCa dormancy. These data provide evidence that this relationship can be targeted pharmacologically to the potential benefit of PCa patients.

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Chapter 2

Background

Introduction

While localized prostate cancer (PCa) presents a generally positive prognosis, metastatic disease remains the primary cause of PCa-related death (1). Importantly, the incidence of metastatic prostate cancer increased 72% between 2004 and 2013, according to a recent study, possibly due to increased detection of metastatic disease(2,3). To improve these statistics, a deeper understanding is needed as to the events which surround metastatic disease, the effect of the marrow microenvironment on metastatic cells and disease progress, and the factors instigating recurrence. The aim of this work is to discuss the cues within the bone microenvironment that support metastatic PCa cell growth including systemic signaling molecules, local signaling molecules, local adhesion molecules, local extracellular matrix molecules, and current therapeutic targeting modalities regarding metastatic disseminated tumor cells (DTCs).

PCa metastasizes to the bone marrow

The development of clinical metastatic disease is described in a series of biological steps. Cancer cells disseminate from a primary tumor and enter the circulation (4). Hematogenous circulation and lymphatic routes appear to be major routes through which disseminating tumor cells (DTCs) navigate. In this regard, there are many challenges that tumor cells must overcome during the metastatic process including dissociation from

neighboring cells of the primary tumor, extravasation, survival, and establishment in distant sites. DTCs have a number of different fates including death, dormancy, or proliferation (5). The role of the microenvironment in tumor cell fate regulation has been reported as early as Paget's "seed and soil hypothesis" (6). This hypothesis was expanded upon by (5), who suggested that tumor cells (i.e. seed) extravasate into circulation, survive, and establish in a distant site (i.e. soil), and their fate (death, dormancy, or growth) is directly influenced by the microenvironment of the distant site.

The 'seed and soil' hypothesis has been used to describe many different tumor-related diseases, including prostate cancer, which has a particular predilection for metastasis to bone which also houses the hematopoietic stem cell (HSC). 80% of advanced prostate cancer cases exhibit distant site metastasis in bone accompanied by a median survival of approximately 40 months (7). Furthermore, many men ostensibly cured of their local disease may develop clinically detectable bone metastases many years following resection or radiation of the primary tumor, suggesting that cancer cells likely escape early in the disease process and are able to maintain a dormant phenotype within the bone marrow prior to conversion to a proliferative phenotype years later (8,9).

Microenvironment signaling factors and ECM components play a significant role in the progression of PCa from a primary lesion to metastasis. The prostate gland itself is comprised of many defined regions surrounded by a smooth muscular stroma that is perforated by the cavernous nerve and neurovascular bundles of the pelvic plexus serving autonomic innervation to the prostate(10). The greatest innervation has been observed in the prostate's peripheral zone and perineural invasion may provide a means of cancer cell escape from the PCa capsule(11). Interestingly, though normal prostate tissue

expresses several combinations of integrin units, PCa cells predominantly express the laminin binding integrins α6β1 and α3β1 (12). Further, post-translational modification of α6β1 increases PCa cell migration and invasion as well as metastasis to laminin-rich bone (11,13,14). Many cell-cell and cell-extracellular matrix (ECM) interactions occur in the migration of cancer cells from the primary tumor to a metastatic site and these data suggest that biomechanical cues can be involved in cancer cell progression.

Metastasis of PCa to the bone marrow microenvironment is directed through several known mediators, including the CXCL12/CXCR4 signaling axis. CXCL12 (previously described as stromal-derived factor-1 (SDF-1)) is a homeostatic chemokine that functions in health to regulate hematopoietic stem cell (HSC) and lymphocyte localization to the bone marrow. Expression of CXCL12 increases with cardiac infarctions, peripheral ischemia, excessive blood volume loss, and tissue damage related to chemotherapy (15). CXCR4 is also widely expressed on CD34+ HSCs, T-lymphocytes, B-lymphocytes, monocytes, macrophages, neutrophils, neuronal cells, endothelial cells, and smooth muscle progenitors, allowing these cells to migrate along the CXCL12 gradients(15). Expression of CXCR4 by PCa cells also provides a mechanism for their migration to bone marrow sites, including the HSC niche (16-19). In addition to homing, CXCL12 can transiently regulate the expression of the αvβ3 integrin, which may also play a role in PCa metastatic localization to the bone marrow niche(20). Further, annexin II receptor (ANXA2r) located on HSCs and PCa cells bind directly with annexin II (ANXA2), expressed by osteoblasts, and facilitates the anchorage of HSCs in health and PCa cells in disease conditions(21,22). Thus, many niche factors involved in PCa metastasis are

increasingly relevant to the support of PCa disease progression and localization to the bone marrow metastatic site.

Regulation of PCa cells within the bone marrow niche

Niche cells are known to include mesenchymal stem cells, progenitor osteoblasts, osteoblasts, progenitor osteoclasts, osteoclasts that are primarily involved in the formation and maintenance of this microenvironment as demonstrated in a recently published model of the niche and its microenvironment (23). Each of these cell types, and likely others within the bone marrow environment, actively contribute to the cytokine gradients which dictate quiescence, survival, and effect of proliferative status on the newly engaged PCa DTCs through cytokine/chemokine signaling, adhesion, and ECM remodeling.

PCa DTCs can target and engage the HSC niche following dissemination to the bone marrow (16). Similar to HSCs, when DTCs are engaged with osteoblasts within the marrow niche, PCa cells can attach to the cell surface of adjacent osteoblasts via many cell-cell interactions that regulate cell quiescence, survival, and lower proliferative capacity. Specifically, it was shown that binding of PCa cells to osteoblasts in the bone marrow induces TANK binding kinase 1 (TBK1) expression, which subsequently inhibits mTOR signaling, induces cell cycle arrest, and increases chemotherapeutic resistance (24).

Effects of cytokine/chemokine signaling within the bone marrow niche on pca cells

GAS6/TAM Receptors

GAS6 is a growth factor expressed by osteoblasts within the bone marrow microenvironment that regulates the cell cycling of HSCs. GAS6 is a ligand for the TYRO3 (Dtk/SKY/Rse/Brt/ETK2/Tif), AXL (Ufo/Ark), and MERTK (Eyk), commonly referred to as the TAM family of tyrosine kinase receptors. GAS6 binds to the TAM receptors via tandem G domains at its C terminus(25). For HSCs, GAS6 inhibits HSC proliferation (26). Similarly, GAS6 inhibits PCa proliferation and appears to participate in the induction of tumor cell dormancy, such that they can remain quiescent for prolonged periods in the marrow (27). GAS6, expressed by osteoblasts regulates PCa cell cycle in the bone marrow, through induction of G_1 cell cycle arrest and S cell cycle phase delay (28). Further GAS6 appears to also ensure cell survival by protecting PCa cell apoptosis signals through inhibition of cleavage of caspase-3 and PARP (28). Thus, PCa engagement with the endosteal niche exposes DTCs to osteoblast-secreted GAS6, causing PCa cell cycle arrest, survival, and resistance to chemotherapeutic advances.

Interestingly, the TAM receptors may also have an effect on PCa cell phenotype within the bone marrow. Here, the phenotype of dormant PCa DTCs includes a decrease in the p-ERK/p-p38 ratio, upregulation of the transcription factors NR2F1, SOX2, SOX9, NANOG, and RARB (29). Recently, we reported that MERTK knockdown alone induced PCa cell cycle arrest via decreased p-ERK1/2 to p-p38 and increased cell cycle inhibitors/dormancy associated transcription factors p27, NR2F1, SOX2, and NANOG(30). Furthermore, GAS6 overexpression activated phosphorylation of MERTK in PCa cells, leading to an increase in the number of cancer stem cells (CSCs) among DTCs recovered from the bone marrow, suggesting that activation of Mer receptor signaling by

endogenous GAS6 can contribute to the establishment of PCa CSCs (CD133+/CD44+) in the bone marrow(31).

In addition, the TAM receptor ratio of AXL/TYRO3 has also been associated with PCa cell cycling (32). Specifically, *in vivo* studies demonstrated that when Axl receptor levels were more highly expressed compared to other TAM receptors, PCa cells became growth-arrested compared to PCa cells that expressed lower Axl expression(33). Cells that had a lower Axl/Tyro3 receptor ratio were able to escape from dormancy, suggesting that in addition to the presence of GAS6 there may be an association of the receptor ratios and the ability to enter or exit dormant or proliferative states(33,34).

TGF-ß/TGFBR Family Molecules

TGF-ß is a growth regulatory factor that is produced by most replicating cells and has a wide range of effects on the cells within the PCa/Bone marrow niche cells. At the site of the primary tumor, TGF-β promotes transition from an epithelial to mesenchymal phenotype and subsequent escape of the tumor cell from the primary site (35) Similar morphogenetic and phenotypic changes occur in bone metastatic sites, particularly in the context of the native osteoblasts and osteoclasts. Osteoblasts have been shown to synthesize and respond to TGF-ß (36)**.** In general, TGF-B signaling tends to have a suppressive effect on the cells of the bone marrow; for example, forced overexpression of TGF-β 2 in osteoblasts leads to bone loss (37), which indicated the homeostasis between osteoblasts and osteoclasts may be at least partly regulated by TGF- β. Cancer cells have been found to promote metastasis in the bone through secretion of TGF-β and subsequent control of osteoblast/osteoclast differentiation (38). The promotion of osteoclast bone resorption by TGF-ß aids in the bioavailability of cell-survival markers in

the bone marrow, which in turn enhances proliferation and growth of DTCs. Other regulatory targets can have an effect on TGF-β signaling in the osteoclasts of the metastatic site. For example, posttranslational regulation of TGF-ß-induced factor 2 by miR-34a has been shown to suppress osteoclastogenesis and the formation of the bone metastatic niche (39).

TGF-β has a wide range of effects on the cells within the PCa/marrow niche. Recently it was reported that GAS6 binding to the TAM receptor Axl on PCa cells induces TGF-β1 and TGF-β2 expression and increases expression of TGFβ R2 and TGF-β R3(40). Further, expression of paracrine TGF-β (from local osteoblasts) and autocrine TGF-β (from PCa cells) in turn can induce PCa dormancy(40). TGF-β 2 signaling initiates a dormant state in DTCs through up-regulation of p27, a ubiquitous cell cycle inhibitor through phosphorylation of p38 and downstream activation of Smad2 and Smad1/5 with a resultant phenotype of TGF-$\beta 2^{high}$, $(ERK/p38)^{low}$, $DEC2^{high}$, $p53^{high}$, $p27^{high}$ and $P\text{-}H3^{low}$ (41).

BMP7 is a TGF-ß family member, secreted by stromal cells within the bone marrow. BMP7 signaling through BMPR2 on PCa cells induces senescence in PCa CSCs through activation of p38 MAPK and increasing cell cycle inhibitor p21(42). Moreover, continued growth of PCa cells following withdrawal of BMP7 both *in vitro* and *in vivo* was also observed (42).

EGF

Epithelial growth factor (EGF) has a well-characterized role in primary tumor growth and eventual patient outcomes. EGF signaling proceeds through a number of

receptors (EGFR, HER2, ErbB2) that have been linked to oncogenesis and metastasis. These receptors are often upregulated in the primary tumor, which can lead to uncontrolled proliferation and ultimately metastatic disease.

EGF is present in the bone marrow and contributes to tumor metastasis and growth in the niche microenvironment, where the EGF signaling cascades are important for the expansion of stem cells (43)**.** ErbB2 overexpression of metastatic breast cancer cells in the bone marrow has been linked to poor clinical outcome, supporting the role of EGF signaling in promoting growth (44). In the case of PCa, EGF has been shown to promote proliferation (45). EGF and similar ligand signaling from metastatic cells have been shown to suppress osteoprotegerin (OPG) expression by osteoblasts, which promotes osteoclast differentiation and subsequent osteolytic events (46). EGF has been shown to significantly alter the effects of bone marrow macrophages on the bone marrow metastatic niche.

Macrophages have been demonstrated to support PCa growth in bone (47), and milk fat globule-EGF factor 8 has been demonstrated to initiate efferocytosis (the clearance of dead and dying cells) by macrophages which induces the expression of a gene repertoire promoting the tumor-associated macrophages that promote PCa growth (48). EGFR inhibition has also been shown to decrease macrophage promoted invasion in osteosarcoma (49).

IGF

Insulin-like growth factor (IGF) promotes tumor growth through signaling of the AKT pathway through IRS1/PI3K and activation of the RAS/RAF pathway through SHC.

IGF promotes osteoblastic niche expansion and HSC cell engraftment (50). IGF has been shown to select for metastatic clones that have a predisposition to colonize and form recurrent tumors in the bone marrow (51). These cells are selected for high Src activity (an enhancer of PI3-K-Akt activation), which confers a predisposition to colonize bone. Interestingly, the loss of the IGF receptor has been associated with advancement of PCa to bone metastasis clinically (52)**,** however numerous others report IGF levels as an enhanced risk factor for PCa (53,54)**.** IGF1 release from resorbing bone enhances breast cancer metastatic growth (55)**.** Osteoclastogenesis is partly regulated by IGF1 through regulation of osteoprotegerin and RANKL (56)**.** Additionally, IGF signaling regulates osteoblast differentiation as well (57), highlighting the complex role of the this signaling pathway in the native bone environment. The complex dynamics of IGF1 on osteoblasts, osteoclasts and tumor cells within the bone marrow environment remains a topic of active research.

VEGF

Vascular endothelial growth factor (*VEGF*) is a mediator of angiogenesis in healthy and cancerous tissues. VEGF mobilizes bone-marrow derived endothelial progenitor cells to promote a number of repair/remodeling functions (angiogenesis(58), bone resorption(59)). Tumor cells likely upregulate VEGF production for this reason and have been shown to exploit this pathway as both a mechanism for establishing a blood supply at the primary site as well as creating a permissive environment for metastasis. $VEGFR1^{+}$ bone marrow progenitor cells have been implicated in the establishment of the premetastatic niche in cancers(60). While not marrow specific, these findings indicate the

role of VEGF in establishing a permissive environment to disseminated tumor cells and the formation of a secondary tumor.

IL-6 and RANK/RANK

Interleukin 6 (*IL-6*) is an interleukin that binds to the IL-6R and activate three major signaling pathways: the Janus-tyrosine family kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, the ERK1/2 and MAPK pathway and the PI3-K pathway. Through these pathways, IL-6 regulates apoptosis/cell survival, and proliferation(61). While, IL-6 has been implicated in many stages of PCa progression and metastasis, it appears to play a key role in bone metastases specifically. IL-6 secreted from PCa cells can mediate osteoblastic differentiation and enhance osteoclastogenesis, thus inducing bone turnover and a key event in establishment of osteoblastic bone metastases(62-64). In return, osteoblastic production of IL-6 stimulates PCa cell proliferation, initiating a “vicious cycle” whereby PCa cells stimulate osteoblastic activity, which in turn stimulates tumor growth in a paracrine fashion (64-66). Analysis of human PCa soft tissue and bone metastatic samples indicates that IL-6 is more highly expressed in bone metastases compared to soft-tissue counterparts (67). Thus, IL-6 remains a key signaling mediator in the growth of PCa metastases through action on both PCa cells as well as the bone microenvironment.

Receptor activator of nuclear factor kappa-B ligand (*RANKL*), expressed by osteoblasts and other cells within the bone microenvironment, is one of the primary factors leading to the activation of osteoclastogenesis and accelerated bone resorption. Proposed as a “vicious cycle,” osteoclastogenesis is necessary to create space for the tumor, but also releases PCa growth stimulating factors embedded in the demineralizing

matrix (66). More recently, the relationship of IL-6 and RANKL has been explored. RANKL, released from local osteoblasts, can stimulate the expression of IL-6 in PCa cells and also increase RANK expression, increasing PCa sensitivity to RANKL (68). Conversely, in a murine model, inhibition of IL-6 signaling with tocilizumab, inhibits skeletal tumor growth and decreased RANKL serum levels, as well as RANK expression in PC3-derived bone tumors (68).

Effects of adhesion molecules/ECM components within the bone marrow niche on PCa cells

Integrins/RGDs

Integrins are transmembrane adhesion molecules that are comprised of noncovalently linked α and β subunits, whereby each heterodimer binds to different ECM proteins, such as collagen, laminin, vitronectin, and fibronectin. In the bone the most abundant protein is type I collagen. Integrin binding is dependent on divalent cations and specific binding sequences such as Arg-Gly-Asp or Asp-Gly-Glu-Ala in the ECM protein (69). The β unit of the integrin binding pair can initiate a signal transduction pathway that is facilitated with intracellular molecules such as focal adhesion kinase (FAK), which in turn lead to ligand-mediated activation of ras/mitogen activated protein kinase (Ras/MAPK) and phosphatidylinositol 3-kinase (PI-3kinase) signal transduction pathways (69). In normal prostate, FAK expression is low or non-detectable; however, in metastatic PCa it is significantly elevated compared to both healthy, benign PCa, or low-grade adenocarcinoma(70). FAK association with Src is critical for prostate cell migration; however, FAK association with PI3K activation affects proliferation, survival, differentiation, and migration through the intermediator, serine/threonine protein kinase B

(AKT) (71). In addition, FAK can activate the Ras proteins, a large family of GTPases that function to stimulate many signaling cascades, such as ERK, that affect cell cycle and proliferation (71). In fact, these pathways are monitored continuously when evaluating PCa cellular dormancy in the bone marrow through evaluation of the p-ERK1/2 to p-p38 ratio (29).

Integrin αvβ3 is another integrin involved in PCa cellular binding to fibronectin, vitronectin, thrombospondin (TSP), among other ECM matrix proteins. Interestingly osteoblast secreted CXCL12 binds to CXCR4 on the resident PCa cells, upregulating αvβ3 and CD164, both adhesion molecules that bind PCa cells to osteoblasts and ECM components (27). Further, ANXA2r on PCa cells binds to osteoblastic ligand ANXA2, resulting in transcription of TAM receptor, Axl, decreasing proliferative cell cycle signaling and subsequent quiescent phenotypes (27).

Integrin pairs (α1β1, α2β1, and α6β1) for collagen appear to be important mediators in PCa metastasis to bone. Interestingly, it was recently reported that bone metastatic PCa cells bound collagen I, whereas cells that only formed visceral metastases failed to bind collagen (72). Since Ras mutations are uncommon in PCa, it was previously reported that chronic stimulation of Ras/MAPK pathway is most likely stimulated through alterations in upsteam regulars such as integrins, growth factors, and growth factor receptors during PCa progression (71). One group reported that PCa-Collagen I attachment was mediated by α2β1 to initiate motility programs through Rho-family of small GTPases, RhoC(72).

TSP1

Thrombospondin 1(TSP1) is a potent angiogenesis inhibitor and down-regulation TSP1 has been suggested to alter tumor growth. In wound healing, TSP1 delays neoangiogenesis via activation of the caspase death pathway in endothelial cells (73). In tumor progression, TSP1 is upregulated by p53 and down-regulated by oncogenes, Myc and Ras (74). Further, TSP1 activates TGF-β1, suggesting a critical role in the regulation of tumor progression (74,75). Interestingly, androgen is reported to increase VEGF-A and decrease TSP1 expression in PCa, suggesting that androgen may play an important role in the angiogenic process of cancer (76). Together the pro-angiogenic factors, such as VEGF, and anti-angiogenic factors, such as thrombospondin 1(TSP1), remain important mediators of the angiogenesis balance, ECM remodeling, and cellular recruitment. TSP1 is an ECM glycoprotein is produced by many different cell types and has important roles in cell attachment, angiogenesis, inflammation, and fibrosis.

Therapeutic implications

Therapeutics designed to target the abnormal microenvironment induced by PCa have been proposed. As discussed above, the vicious cycle model proposes that PCa cells stimulate increased bone remodeling, which subsequently liberates IL-6, TGF-β and other factors that further increase proliferation of tumor cells. Thus, the use of drugs which inhibit osteoclast function was proposed to halt the abnormal osteoclast activation component of the vicious cycle and thereby slow PCa progression (77). Both the bisphosphonate, zoledronate, and the anti-RANKL antibody, denosumab, are proven to be effective in prevention of skeletal events such as pathologic fracture, spinal cord compression, and bone pain (78). The majority of positive studies have been in patients with bone metastases from castration resistant PCa (i.e. progressing despite medical or

surgical castration). Either denosumab or zoledronate is standard of care in this patient population (79). Furthermore, denosumab is effective and indicated for prevention of osteoporotic fracture in all prostate cancer patients treated with androgen deprivation therapy, regardless of disease stage or castration resistant status (80).

Yet despite their promise, it remains unclear as to whether osteoclast targeted drugs ultimately change outcomes in PCa. For despite, their useful benefits in prevention of skeletal complications, denosumab and zoldronate have not shown improvements in overall survival in any PCa patient population – as would be expected if the drugs were targeting cancer cells (77). Additionally, clinical data on osteoclast targeted drugs has not supported their use to prevent formation of bone metastases. Specifically, zoledronate did not increase the time to first skeletal related event in men with castration sensitive metastatic prostate cancer (81). Also, while denosumab did increase metastasis free survival in men with non-metastatic castration resistant PCa (PSA rising after castration, but no gross metastases on imaging), it did not increase overall survival and is not FDA approved in this setting (82). Similarly, in the adjuvant setting, zoledronate did not prevent PCa progression or mortality in patients with high risk localized disease (83). Therefore, although they have prevented much morbidity from bone complications in PCa patients, osteoclast targeted drugs have not yielded all the desired beneficial effects in clinical trials. However, because of the research avenues discussed above, we are confident that targeting the bone microenvironment will continue to yield effective therapeutics in the future.

Summary

There are many was to address the problems arising from metastatic disease. One approach is to target the microenvironment in which metastatic cells colonize, survive, and proliferate. There are many molecular signals that direct the homing of PCa cells to the bone marrow and regulate DTC proliferative activity, as summarized in Table 2.1 and Figure 2.1. Identification of these players has been increasingly a point of interest to the research setting, however, translation of these findings to the clinic remains limited. Future efforts need to be made to identify how these molecular players distinctly regulate PCa cell survival, dormancy, and re-activation to determine more effective clinically relevant therapeutic targets that can not only increase the life-span of these patients but also improve the quality of a cancer patient's life.

Table 2.1**:** Description of important biochemical mediators of PCa entry and survival in the bone marrow.

Biochemical Components	**Description**	**Effect on PCa Bone Metastasis**	**Citation**
Growth Factors			
CXCL12	Homing molecule secreted by osteoblasts	Induces HSC mobilization from the HSC niche and recruits PCa cells.	(16-19)
GAS6	Growth factor expressed by osteoblasts	Ligand for PCa TAM receptor reducing cell cycling and induction of PCa dormancy.	(25) (27) (33,34)
TGF-β	Growth regulatory factor expressed and produced by a wide-variety of cells including osteoblasts and PCa cells	Autocrine (from PCa cells) and Paracrine (from osteoblasts) signaling reduces cell cycling, inducing a dormant state.	(35) (36) (38) (39)
BMP7	TGF-β family member, secreted by stromal cells in the bone marrow	Induces cellular senescence in PCa CSCs.	(42)
EGF	Endogenous growth factor that is linked with cell growth.	Present in the bone marrow, increasing PCa cell proliferation and osteoclast differentiation/promoting osteolytic events.	(43) (45) (46) (44)
IGF	Growth factor affecting the growth and differentiation of a variety of tissues.	Promotes osteoclastogenesis and expansion of the osteoblastic niche. May promote colonization of the bone marrow in PCa cells.	(57) (56) (53,54) (51)

VEGF	Mediates angiogenesis in various tissues both in homeostasis and cancer.	Mobilizes bone-marrow derived endothelial precursors to mobilize angiogenesis and bone resorption creating a permissive environment for disseminated tumor cells.	(60)
IL-6	Interleukin that regulates apoptosis/cell survival and proliferation.	PCa IL-6 secretion mediates osteoblastic differentiation and osteoclastogenesis. Osteoblast secretion of IL-6 results in PCa proliferation.	(61) (67)
RANKL/ RANK	RANKL expressed by osteoblasts can bine RANK on osteoclast precursor cells and induce osteoclastogenesis during the normal bone remodeling process.	RANKL released from osteoblasts can increase PCa IL-6 secretion and RANK expression.	(66) (68)
Adhesion and ECM Components			
ANXA2	Protein expressed by OBs	ANXA2r located on PCa cells bind directly ANXA2 on osteoblasts to facilitate PCa anchorage within the bone marrow.	(21,22)
αVβ3	Binds fibronectin, vitronectin, TSP, and other ECM proteins	Osteoblastic CXCL12 causes upregulation of PCa αVβ3 promoting PCa adhesion to osteoblasts in the bone marrow.	(27)
α1β1, α2β1, α6β1	Binds collagen	Engages PCa cells with bone and may initiate bone metastatic motility programs.	(71) (72)
TSP1	Anti-angiogenic ECM glycoprotein produced by various cell types including	Downregulated in progression of PCa to promote angiogenesis in the area.	(74,75)
Therapeutics			
Denosumab	Anti-RANKL antibody	Indicated for prevention of osteoporotic fracture in all prostate cancer patients treated with androgen deprivation therapy. Increased metastasis	(82)

		free survival in men with non-metastatic castration resistant PCa.	
Zoledronate	Bisphosphonate	Did not prevent PCa progression or mortality in patients with high risk localized disease.	(83)

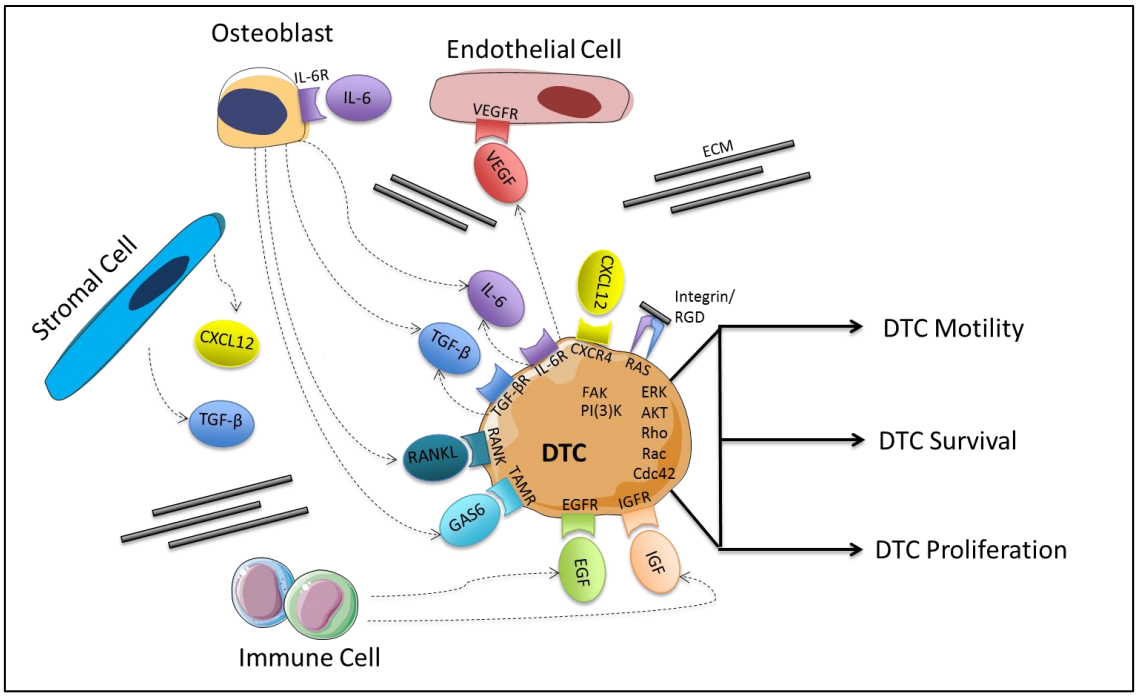

Figure 2.1**:** Summary of the molecular interactions of PCa cells in the bone marrow microenvironment. IL-6 = Interleukin 6; VEGF = Vascular endothelial growth factor; CXCL12 = SDF-1 = Stromal derived factor 1; CXCR4= CXC chemokine receptor 4; TGF-β = Transforming growth factor β; RANK = Receptor activator of nuclear factor kappa-B; TAMR = TYRO3, AXL, MERTK receptor; GAS6 = Growth arrest specific 6; EGF = Epithelial growth factor; IGF = Insulin growth factor; ECM = Extracellular matrix.

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Chapter 3

Sympathetic Signaling Reactivates Quiescent Disseminated PCa Cells in the Bone Marrow

Introduction

Prostate cancer (PCa) remains the second leading cause of cancer-related deaths in the male population within the United States (1). Distant site bone metastasis is present in 80% of cases of advanced PCa with a mean survival time of 40 months (2). Disseminated tumor cells (DTCs) that metastasize to the bone marrow can undergo apoptosis, proliferate or become dormant, and their fate/mitotic rate is directly influenced by the surrounding metastatic microenvironment (3). Dormant DTCs cannot be targeted with cell-cycle dependent chemotherapies and thus can remain latent in the bone marrow for years following an initial course of treatment. As a result, dormant DTCs may become activated at a future time leading to recurrent disease with a poor prognosis (4).

Previously, it was demonstrated that PCa DTCs home to a CXCL12-rich osteoblastic area (niche) of the bone marrow that is typically supportive of hematopoietic stem cell (HSCs) homeostasis (5,6). One mechanism suggested to be involved in PCa dormancy includes osteoblastic secretion of BMP7, which activates p38/MAPK signaling pathway signaling of PCa cells (7). It has also been demonstrated that expression of paracrine TGF-β (from local osteoblasts) and autocrine expression of TGF-β (from PCa cells) in turn can induce PCa dormancy (8). TGF-β2 signaling initiates a dormant state in DTCs through up-regulation of p27, a ubiquitous cell cycle inhibitor through

phosphorylation of p38 and downstream activation of Smad2 and Smad 1/5 with a resultant phenotype of TGF-β2^{high}, (ERK/p38)^{low}, DEC2^{high}, p53^{high}, p27^{high} and P-H3^{low} (9). Another mechanism potentially regulating DTC dormancy is osteoblastic secretion of growth arrest specific-6 (GAS6), which increases PCa exit from the cell cycle or dormancy, and PCa survival in the presence of chemotherapeutics (10). These fundamental observations are critical to understanding how dormancy is induced or maintained but fail to establish how reactivation or escape from the dormant state occurs which ultimately leads to recurrent disease.

The fight-or-flight (i.e. stress) response is a major physiological reaction to potentially harmful events. Stress induces hypothalamic signaling which activates the sympathetic nervous system, ultimately resulting in the systemic release of norepinephrine/epinephrine. Stress may also be an important factor that influences activation of dormant DTCs after long periods of time in marrow through immune system suppression (11), activating release of pro-inflammatory mediators (12,13), and activation of neurotransmitters from adrenergic neurons (14,15). Growing evidence supports the observation that β-adrenergic receptor antagonists, which interfere with norepinephrine (NE) signaling, may reduce cancer relapse or slow disease progression (16-19). Part of the mechanism by which NE blockade may interfere with disease progression is through the regulation of the PCa-bone homing factor, CXCL12 (20) and altering the expression of RANKL, which regulates bone-turnover (21). However, the effects of autonomic nervous system (ANS) signaling through NE on dormant tumor cell reactivation in the HSC niche is currently unknown.

In this study we demonstrate that NE induces quiescent PCa cell re-entry into the cell cycle both *in vitro, ex vivo,* and *in vivo*. We show that following NE stimulation, PCa transition into the proliferative, G_2/M cell cycle phases in multiple models of quiescence. This study demonstrates that PCa DTCs undergo cell cycle re-entry in the presence of NE, and NE may be one of the factors causing metastatic disease relapse following a cancer free diagnosis.

Methods

Cell Culture

Human PCa cell lines (PC3, DU145) were obtained from American Type Culture Collection (Rockville, MD). Murine osteoblasts were established from C57BL/6J mice as previously reported (5). All prostate cancer cell lines were cultured with RPMI 1640 (Life Technologies, Carlsbad, CA), and murine or human osteoblasts were grown in α-MEM or DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, GEMINI Bio-Products, Sacramento, CA, 1% penicillin-streptomycin (P/S, Life Technologies) and maintained at 37ºC, 5% CO2, and 100% humidity.

PC3-FUCCI Cells

To monitor the cell cycle in PCa cells, PC3 cells were transduced with lentivirus containing fluorescent ubiquitination-based cell cycle indicator (FUCCI) vectors (Clontech, Mountain View, CA). Cells contain chromatin licensing and DNA replication factor 1 (CDT1)-Cherry reporter and Geminin-Cyan reporter. Early S phase cells are double-positive for CDT1 and Geminin and fluoresce yellow. A pRetroX-G_1-Red vector (cat. 631463, Clontech) and pRetroX-SG2M-Cyan vector (cat. 631462, Clontech) were packaged into lentivirus by the University of Michigan Vector Core Facility. Lentiviral

pRetroX-G_1-Red vector and lentiviral pRetroX-SG_2M-Cyan vector were co-infected into PC3 cells. Cells were selected for 7 days in media containing 1μg/ml Puromycin and analyzed by FACS analysis. Cell cycle monitoring was performed in PC3-FUCCI cell culture with direct NE (2.5μg/ml) treatment alone or in co-culture with osteoblasts (OBs) (MC3T3-E1, or primary murine OBs). PC3-FUCCI cells were cultured for 24 hours in RPMI with 10% FBS, 1% P/S, followed by culture for 48h in RPMI with no added FBS, 1% P/S and treated with Vehicle or NE (2.5μg/ml). In PC3-FUCCI/OB co-culture experiments cells were cultured in OB culture conditions of α-MEM with 5% FBS and 1% P/S.

Quantitative PCR

Total RNA was extracted from cells using the RNeasy mini kit (cat. 74104, Qiagen, Valencia, CA) and converted into cDNA using a First-Strand Synthesis Kit (Invitrogen). Quantitative PCR (real-time PCR) was performed on an ABI 7700 sequence detector using TaqMan Universal PCR Master Mix according to the directions of manufacturer (Applied Biosystems, Foster City, CA). TaqMan MGB probes (Applied Biosystems) were as follows: ADRα1 (Hs00169124_m1), ADRα2 (Hs01099503_s1), ADRβ1 (Hs02330048_s1), ADRβ2 (Hs00240532_s1), ADRβ3 (Hs00609046_m1), GAS6 (Mm00490378_m1), TGFβ2 (Mm00436955_m1), SPARC (Mm00486332_m1), BMP7 (Mm00432102_m1), Jagged 1 (Mm00496902_m1), Annexin 2 (Mm00500307_m1), CXCL12 (Hs03676656_mH, Mm00445553_m1). β-Actin (Hs01060665_g1; Mm02619580_g1) was used as internal controls for the normalization of target gene expression.

Western Blots

Lysates were prepared in cOmplete lysis M (Roche #04 719 956 001) supplemented with proteinase inhibitor Mini cOmplete Tablets (Roche #04705378) and phosphatase inhibitor PhosSTOP *EASY*pack Tablets (Roche #04 906 837 001). Protein concentration was calculated using the BCA protocol. 20 µg of total protein was added per lane of 4-20% reducing SDS polyacrylamide Tris-Glycine gels after sample preparation in Laemmli sample buffer. The samples were transferred to PVDF membranes and blocked for 1 hour in 5% dry milk in TBS with 0.1% Tween-20 (TBST). Antibodies for phosphorylated proteins were applied at 4˚C overnight in 5% BSA TBST, washed and visualized with a horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (Cell Signaling #7074S) and SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific #34075). Images were acquired with a ChemiDoc Touch imager (BioRad). The following antibodies were procured from cell signaling (catalog number, dilution) were used: Phosphorylated-Erk 1/2 (P-Erk) Y204 (#4377S, diluted 1:500), total Erk (#4695, 1:500), Phospho-p38 (P-p38) T190/Y182 (#4511, 1:500), total p38, (#9212, 1:500), p21 Waf1/Cip1 12D1 (#2947, 1:500), p27 Kip1 (#2552, 1:500), β-actin (#4970, 1:1000). β2-AR (SC-81577; 1:500) antibody was procured from Santa Cruz Biotechnologies. Images representative of biological replicates are shown and cropped for presentation. For P-Erk, P-p38, and p27 quantification, images from four independent experiments were quantified relative to each vehicle treated condition with BioRad ImageLab software and then normalized to housekeeping gene expression. The P-Erk to P-p38 ratio was obtained by dividing the normalized P-Erk and P-p38 values for each independent experiment. All data are shown as fold change from the corresponding vehicle control.

Chemotaxis Assays

Cell invasion into a reconstituted extracellular matrix coating of Matrigel™ overlaid on 8μM pore-size in polyethylene terephthalate membranes was performed in a dual chambered 12-well plate (BD Biosciences, San Jose, CA), as previously described (22). Spontaneous invasion was compared to invasion supported by NE (2.5μM) and propranolol (10μM) conditions.

Immunostaining

Cells (PC3, DU145, MC3T3-E1, and primary murine OBs) were used for immunostaining. Cells were fixed and permeabilized with Perm/Wash Buffer (cat. 554723, BD Biosciences). Cells were incubated for 2 hours at room temperature with primary antibodies combined with reagents of Zenon Alexa Fluor 488 (green) or 555 (red) labeling kit (Invitrogen). HLA (BioLegend), and Ki67 (BioLegend) were used as primary antibodies. After washing with PBS, the slides were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Images were taken with Olympus FV-500 confocal microscope. Results were quantified by counting the positive cells confirmed by two different independent investigators.

Flow Cytometry

OBs and PCa cells (PC3 and DU145) were removed from co-culture experiments with trypsin and suspended in flow buffer (1X PBS + 2%FBS). Cells were then labeled with a Fixable Viability Kit according to package instructions (Biolegend, Cat# 423111), HLA-A,B,C (Biolegend, Cat# 311426), and in the case of parental PCa cells anti-human Ki67 (Biolegend, Cat# 350514) for 30 min at room temperature. Cells were then washed

2x with flow buffer and re-suspended in flow buffer for flow analysis. Live cells were gated for HLA-A,B,C prior to analysis of Ki67 or FUCCI cell cycle expression.

Ex Vivo Experiments

Femur explants were dissected from 5-week old C57BL6/J mice. Femur dissection was completed as previously described (23). Sorted G_0/G_1 FUCCI cells were injected into the marrow space of the femur explants and cultured for 24 hours in α-MEM (Life Technologies, Carlsbad, CA) supplemented with 5% FBS. 24 hours after dissection, media was changed to α-MEM supplemented with 10% FBS and 2.5μM NE or vehicle control. After 48h of culture in experimental conditions, the bone marrows were flushed from the femur explants with FACS buffer (PBS +2% FBS). Mouse cells were depleted using a MACs Mouse Cell Depletion Kit (Miltenyl Biotec, Cat# 130-021-694) using a MACs automated Pro Cell Separator (Miltenyl Biotec). Remaining cells were labeled with a Live/Dead stain (Zombie Green, BioLegend, Cat# 423111), murine IgG2b b haplotype (mH-2Db) (BioLegend, Cat# 111516, PE/Cy7), mCD45 (BioLegend, Cat# 103112, APC), HLA-A,B,C (Biolegend, Cat# 311426, APC/Cy7). Live cells were negatively gated for mH-2Db and mCD45, and positively gated for HLA-A,B,C prior to cell cycle analysis using the FUCCI spectrum.

In Vivo Experiments

PC3 cells were stained with DiD (ThermoFisher) and allowed to rest for 24 hours. DiD staining was confirmed through flow cytometry. Following confirmation, 2e6 cells were introduced into the bone marrow of NSG mice through intratibial injection. NE (1mg/kg) was subsequently administered through intraperitoneal (IP) injection, beginning 24 hours after intratibial injection and continuing once per day for three days. After three

days, mice were euthanized and tibias extracted for analysis. Bone marrow was flushed to remove prostate cancer cells and marrow stroma. These cells were digested with trypsin, stained in a similar manner to the *ex vivo* assay, and analyzed through flow cytometry. Cells were analyzed as the fraction of cells gated as human that were positive for DiD stain.

Statistical Analyses

Results are presented as mean ± standard deviation and fold change ± standard deviation normalized to standard vehicle control conditions. Significance of a difference between values was determined by use of an ANOVA with correction of multiple comparisons using the Sidak's multiple comparison test through GraphPad Prism version 7.0. If the comparison of two values were assessed, an unpaired Student's t-test was used. Error bars reported in all figures represent standard deviation.

Results

Adrenergic signals in the bone marrow can reactivate dormant PCa cells

To first explore the impact of neuronal regulation of PCa dormancy in the bone marrow, we evaluated marrow for the presence of nerve elements in the femurs of mice. Immunohistochemistry of C57BL/6J femur sections revealed innervation of adrenergic nerves at the proximal end of a longitudinal bone marrow section. We observed tyrosine hydroxylase (TH) expressing nerves in endosteal region in bone marrow (Figure 3.1A).

As previous reports suggest that the β2-adrenergic receptor is present on PCa cells (24,25) and OB cells (26-28), we sought to verify this data and to identify the expression panel of adrenergic receptors on PCa and OB cell lines. Expression of the β2-adrenergic receptor was confirmed using qPCR and by Western blot (Figure 3.1B-E).

Messenger RNA for β2 was expressed to a higher degree than other adrenergic receptors and was expressed at a protein level on both PCa and OB cell types. Given the presence of adrenergic nerves in the marrow and receptors for adrenergic signaling on both bone marrow-resident cells and PCa cells we explored further the impact of sympathetic signaling on disseminated PCa cells in the bone marrow niche.

NE induces reactivation of dormant PCa cells.

Growing evidence supports the observation that β-adrenergic receptor antagonists, which interfere with norepinephrine (NE) signaling, may reduce cancer relapse or slow disease progression (16-19). Given the presence of sympathetic nerves in the marrow and β2-adrenergic receptors expressed by PCa and niche cells, we explored the role of NE on DTC proliferation by first evaluating the impact of NE on PCa cells. Here, PCa cell lines were treated *in vitro* with NE and cell cycle activity was assessed by Ki67 expression using flow cytometry. Following culture with 2.5μM NE, PCa cells demonstrated a 2.6 fold increase in Ki67-positive cells, indicating cell cycle entry in PC3s and a 1.7 fold increase in DU145 cells compared to vehicle controls under serum free (SF) conditions (Figures 3.3A and 3.3B). Treatment with NE had no effect on AR+ cell lines (Figure 3.2A) or invasiveness of PC3 or DU145 cells (Figure 3.2B).

Treatment with the β-adrenergic receptor antagonist propranolol (PPL),) decreased the proportion of Ki67 positive cells (Figure 3.3C). Using immunocytochemistry to confirm the flow cytometry results, a 2.5 fold increase in Ki67 positive PC3 cells were observed when cultured with NE for 48 hours compared to vehicle controls (Figure 3.3D and 3.3E).

Previously we determined that when PCa cells become proliferatively quiescent when co-cultured with osteoblasts (8,29,30). To determine whether NE is able to alter the quiescence of PCa cells induced by osteoblasts, co-culture experiments were performed where in the human PCa cells were selectively analyzed using antibodies specific for human HLA. Under the co-culture conditions, a 4.6 fold increase in Ki67 positive HLA-expressing PC3 cells was observed in the presence of NE for 48 hours compared to vehicle controls (Figure 3.3D and 3.3E).

Previous investigations have demonstrated that a PCa G_1 cell cycle arrest is induced through PCa-OB co-culture, and serves as a model for PCa dormancy within the bone marrow (30). To further explore how NE impacts PCa in the bone marrow, the use of cell cycle specific FUCCI-vectors were employed to isolate cells at different stages of the cell cycle. Here, PC3-FUCCI cells were cultured under serum free conditions with 2.5uM NE or a vehicle control to assess the effect of NE on cell cycle status. A 1.8 fold increase in the proportion of cells in the G_2/M cell cycle phase compared to dormant cells in the G_1 cell cycle phase was observed (Figures 3.3F and 3.3G). These data suggest that NE has the capacity to drive G_0/G_1 cell cycle arrested cells into a proliferative state.

Western blots were employed to evaluate the impact of NE on the expression or phosphorylation of proteins that regulate dormancy. Culture of PCa cells with NE demonstrated a decrease in expression of both p27 and p21 (Figure 3.4A-C). Previous reports have demonstrated that an increased ratio of p-ERK 1/2 to p-p38 MAPK is associated with re-activation from dormancy in prostate and other cancers (7,9,31). Thus, we also evaluated p-ERK and p-p38 levels following exposure to NE (Figure 3.4D-E). NE treatment did not alter the level of phosphorylation of ERK relative to treatment control

groups. In contrast, NE exposure decreased the level of p-p38 under the culture conditions (Figure 3.4D-E). Together these results suggest that an increase in the p-ERK/p-p38 levels observed in PC3 cells, with a modest albeit not significant impact on DU145 levels. Together these observations suggest NE is able to alter a quiescent phenotype of PCa cells towards a proliferative signature (Figure 3.4F). These data suggest that re-activation of PCa cells by NE could be mediated through the inhibition of cell cycle regulators.

NE alters the dormancy – inducing signals expressed by cells of the HSC niche.

Our evaluation of β2-adrenergic receptor expression suggested that both PCa cells and osteoblasts may be targets of NE signaling in the marrow (Figure 3.1E). Previously, we and others have demonstrated that PCa cells metastasize to the HSC niche and interact with osteoblasts. To determine the extent to which NE alters niche-localization signals expressed by osteoblasts, we examined mRNA expression by PCR for the following adhesion molecules: Annexin II (ANXA2/Annexin IIR (ANXA2R)), Notch/Jagged, and homing molecule CXCL12 (5,6,32). As shown in Figure 3.4A, NE treatment of OBs decreased mRNA expression of *Anxa2* but failed to induce significant changes in the expression of *Jagged 1*, *Anxa2R* or *CXCL12*.

We next considered that NE may alter the expression of molecules known to be associated with the induction of dormancy of PCa cell. For these investigations, we evaluated the impact of NE on the expression of mRNA for GAS6, TGF β2, Secreted Protein Acidic and Rich in Cysteine (SPARC), and Bone Morphogenetic Protein 7 (BMP7). NE increased the expression of *BMP7* mRNA by osteoblasts but significantly decreased expression of *GAS6* osteoblastic mRNA (Figure 3.5A).

We also assessed the effects of NE on proliferation of PCa cells in co-culture with primary murine OBs. NE did not have a statistically significant effect on OB proliferation (Figure 3.5B). This result indicated that NE stimulation primarily affected cytokine output through signaling pathways and not alterations in cell proliferation.

Adrenergic signaling reactivates PCa cells in the native marrow environment

We next developed a novel ex vivo model system to explore the impact of NE on dormant PCa cells in the bone marrow without the systemic effects of altered NE signaling (Figure 3.6A). For these studies explant femurs were injected with PCa cells in the presence or absence of NE. First, G_0/G_1 phase PC3-FUCCI cells were injected into *ex vivo* bone explants. After 48-hour incubation with NE in murine femurs, PCa cells were isolated by flow cytometry using positive selection for the Human cells and negative population selection for the murine cells, and subsequently the cell cycle state determined. A 26.6% increase in the number of cells in the G_2/M phase was observed following NE treatment compared to controls demonstrating that addition of NE to *ex vivo* culture conditions can induce cell-cycling in PCa cells within the context of an induced DTC model system (Figure 3.6B,C,D).

Endogenous NE activates adrenergic signaling *in vivo*, which can lead to reactivation of dormant PCa cells residing in the bone marrow [10]. As such, we also tested the effects of NE signaling *in vivo*, to complement the results of the *ex vivo* experiment. An intratibial injection of cycling PC3 cells followed by NE injection provided further evidence for this point (Figure 3.7A). Intraperitoneal injection of NE for three days decreased the dormant fraction of PC3 cells in the bone marrow (Figure 3.7B), indicating

the effects that adrenergic signaling can have an impact on the marrow microenvironment.

Discussion

Bone marrow relapse remains a central problem in the care and maintenance of men with PCa disease. Despite the importance of this issue, little is known about how dormant tumor cells become reactivated. Contributory factors are still under investigation, however one suggestion is that stress may influence relapse (34). In this study, we demonstrated that NE can induce re-entry of dormant tumor cells into the cell cycle in three separate models of dormancy; serum starvation, co-culture with OBs, and in a novel *ex vivo* culture. NE can exert a direct effect on PCa cells to cause them to re-enter the cell cycle and can also have an effect on the microenvironment by affecting secretion of *GAS6*. The sum of these activities suggests that NE can induce quiescent PCa cells to reenter proliferative phases of the cell cycle.

The experiments in this manuscript specifically examined the effects of NE on dormant, androgen receptor (AR) negative PCa cell lines. AR+ lines were found to be less responsive to NE stimulation (Figure 3.2A), possibly due to their reliance on androgen signaling for growth. Further, the effect of NE was strongest on proliferation in the serum starvation, co-culture and *ex vivo* models we used in this study. Examination of invasiveness in serum starved PCa cells showed a decrease in invasiveness when treated with NE (Figure 3.2B). These data suggest that other confounding factors may be necessary to induce EMT or migration of previously dormant cells in the bone marrow, beyond proliferation.

Adrenergic signaling has recently gained prominence as a factor leading to progression in cancer. Adrenergic signaling and chronic stress have been shown to accelerate metastasis in breast, prostate, leukemia and pancreatic cancers (35-38). Innervation of the primary tumor has been shown to promote invasiveness, and examination of adrenergic signaling on primary tumor cells and cell lines has demonstrated increased invasiveness and metastatic potential of these cells (39,40). Further, therapy which suppresses beta-adrenergic receptor signaling has been shown to improve relapse free survival, supporting the role of adrenergic signaling in reactivation of dormant tumor cells (41,42). Moreover, Magnon and co-authors (40) completed a series of studies using mice bearing PC3 prostate tumor xenografts that developed tumors and were monitored for metastases. The authors also determined adrenergic and cholinergic nerve densities in radical prostatectomies from 43 patients with prostate cancer. The authors reported that nerve fibers at the primary tumor site can alter tumor behavior and that the autonomic nervous system can 1) promote early stages of tumorigenesis (sympathetic) and 2) cancer dissemination (parasympathetic). These data together suggest that NE increases the metastatic potential of primary tumor cells and could potentially lead to reactivation of dormant cells through direct action on the cells.

In this study, we demonstrated that NE can exert a direct effect on prostate cancer cells, and this direct effect can overcome stimuli leading to dormancy. The prostate gland itself is comprised of many defined regions surrounded by a smooth muscular stroma that is perforated by the cavernous nerve and neurovascular bundles of the pelvic plexus, serving autonomic innervation to the prostate (43). Interestingly the greatest innervation has been observed in the prostate's peripheral zone and perineural invasion may provide

a means of cancer cell escape from the PCa capsule (44). Our study suggests a similar mechanism may apply to micrometastases in the bone marrow.

PCa most often disseminates to the bone marrow where PCa cells are able to engage the HSC niche and dormancy is induced (6). Adrenergic signaling from stress in a healthy HSC niche microenvironment can promote HSC mobilization into the blood stream (20). Recently, our group demonstrated that DTCs assume a stem cell-like phenotype in the bone marrow, which suggests that signals involved in HSC retention and mobilization may play a role in reactivation of these dormant cells (45). The effect of stress-mediated cytokines, and NE in particular, on the niche environment is an active area of investigation; however, other studies support the conclusion that adrenergic signaling within the niche environment could lead to mobilization of cancer cells from that environment (20,21).

A number of other dormancy and adhesion associated genes were profiled through qPCR (Figure 3.5A). Annexin2, an adhesion molecule, and BMP7, a member of the TGFβ family, were both changed significantly in response to NE, increasing 0.75 fold and decreasing 1.4 fold, respectively. Annexin2 promotes cell localization to the HSC niche (5,32). The decrease in Annexin 2 expression aligns with the other data presented here in which NE promoted activation from dormancy in PCa cells. BMP7 has been previously shown to promote dormancy in cancer cells by predominantly direct actions on tumor cells. (7). Yet BMP7 promotes differentiation of osteoblast precursors into osteoblasts (47). It is possible that maturity of osteoblasts is related to their secretion of dormancy inducing molecules and expression of adhesion proteins, and that the effect of BMP7 on differentiation of osteoblasts and related gene expression is stronger than its direct effects

on the PCa cells in the co-cultures used in this study. Further investigation on the specific cytokines involved in NE-mediated reactivation of quiescent PCa cells was performed in Chapter 4.

In conclusion, we employed *in vitro* assays to demonstrate reactivation of dormant PCa cells with direct application of NE to culture conditions. Together, these data demonstrate that NE may be one of the critical factors causing metastatic disease relapse following a cancer free diagnosis. These experiments additionally form the foundation for further studies to isolate indirect mechanisms through which NE may reactivate disseminated PCa cells.

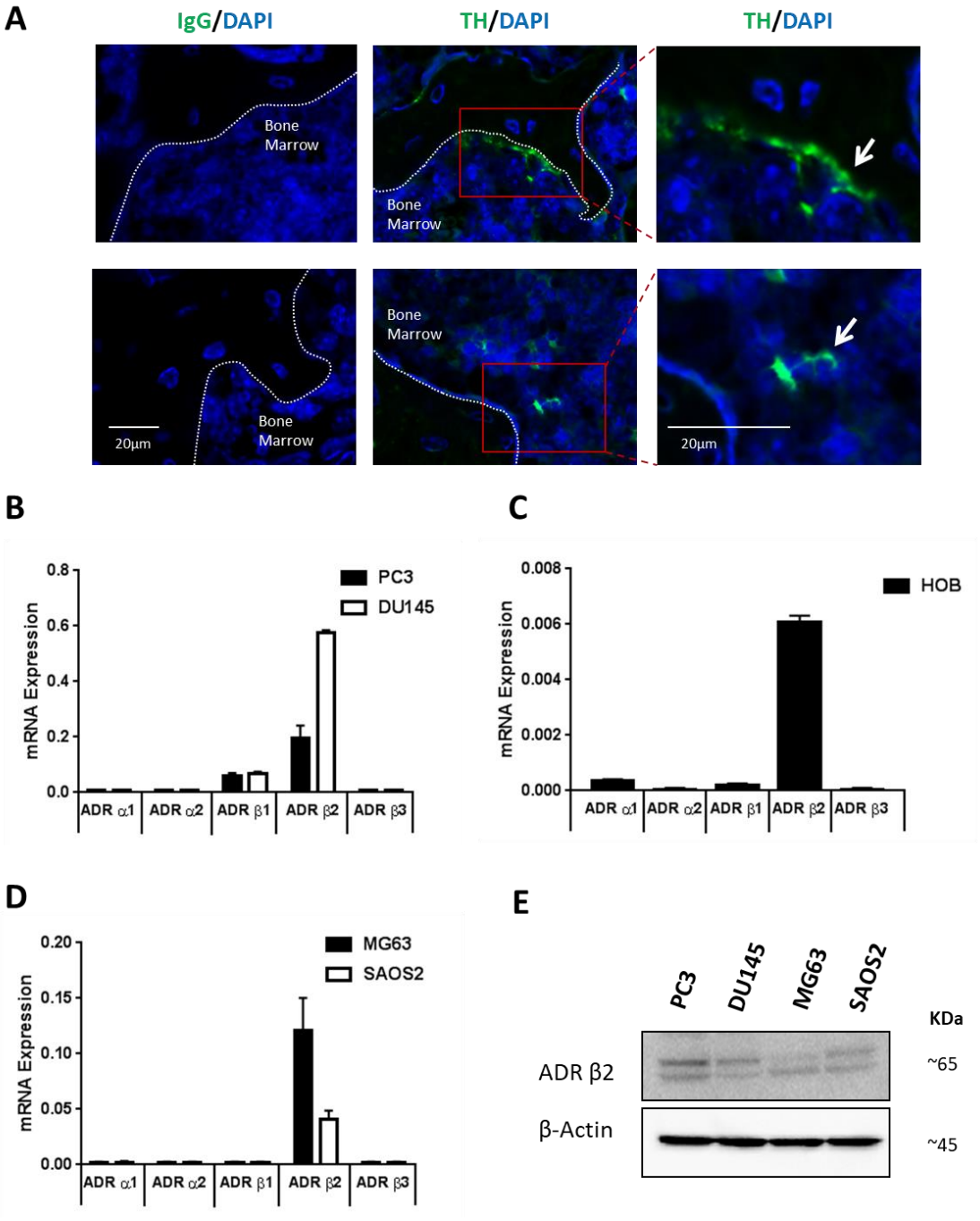


Figure 3.1. Adrenergic neurons in the bone marrow.
(A) C57BL/6J mouse femur sections were stained with tyrosine hydroxylase (TH) to identify adrenergic nerves or non-specific IgG control. qPCR was completed to identify adrenergic receptor isotypes on (B) PCa cells (PC3, DU145), (C-D) Human osteoblasts (HOB, MG63, SAOS2) (N=3). (E) Western blots depict protein expression of adrenergic receptor isotype β2 (ADRβ2) in PCa and OB cell types.

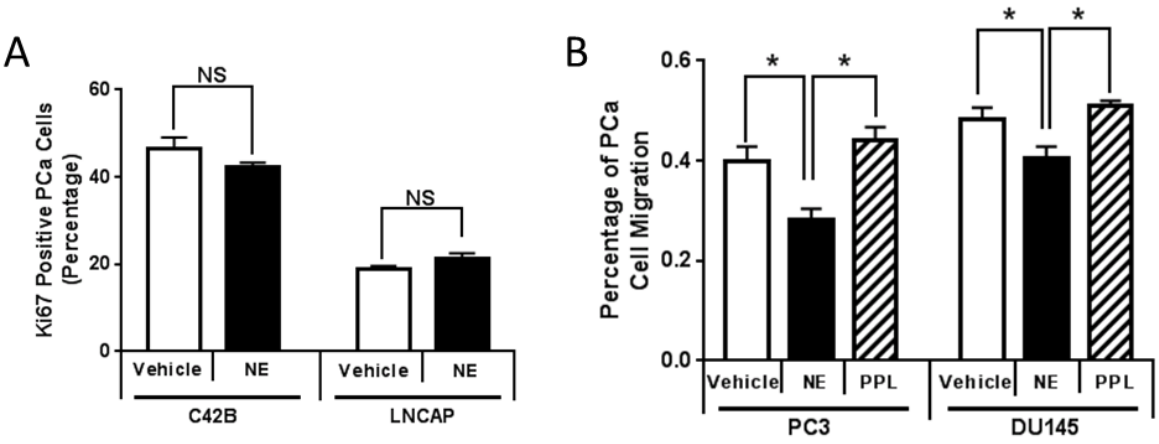


Figure 3.2: AR+ cell line proliferation and migration assays. A) NE does not have an effect on Ki67 expression in AR+ C42B and LNCAP PCa Cells. B) NE-induced migration suppression of PCa cells is negated in the presence of PPL

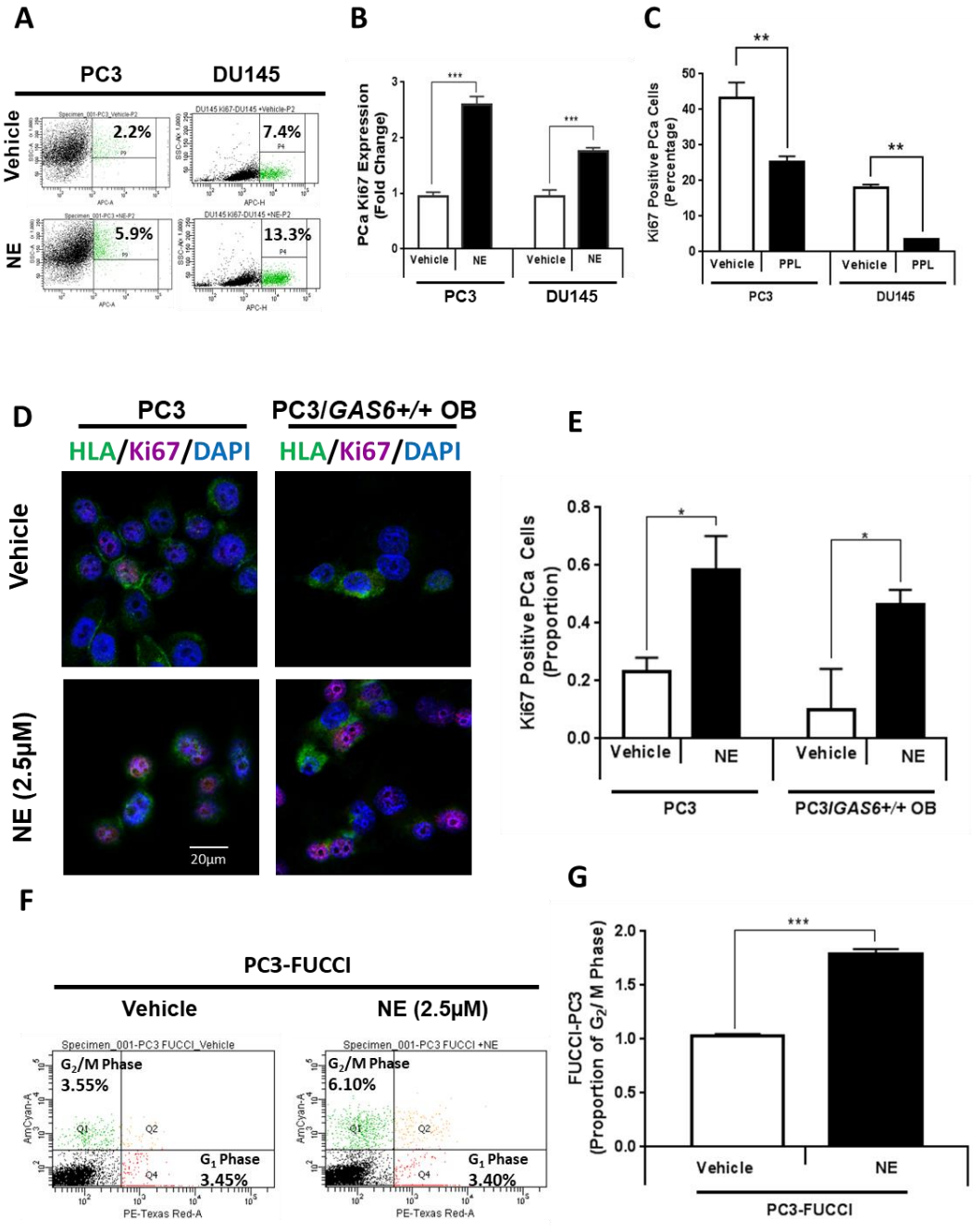


Figure 3.3. NE induces reactivation of dormant PCa cells.
(A-C) PCa cells (PC3 and DU145) cultured with NE, Propranolol, or vehicle control and evaluated for Ki67 expression using flow cytometry (N=3; Student's t-test). (D-E) Immunocytochemistry for Ki67 expression by PCa cells following *in vitro* culture of PC3 cells and *GAS6*+/+ OB co-culture in the presence of NE or vehicle control (N=3; Student's t-test). (F-G) PC3-FUCCI cells cultured *in vitro* with NE or vehicle control and evaluated for cell cycle phase using flow cytometry (N=3; Student's t-test).

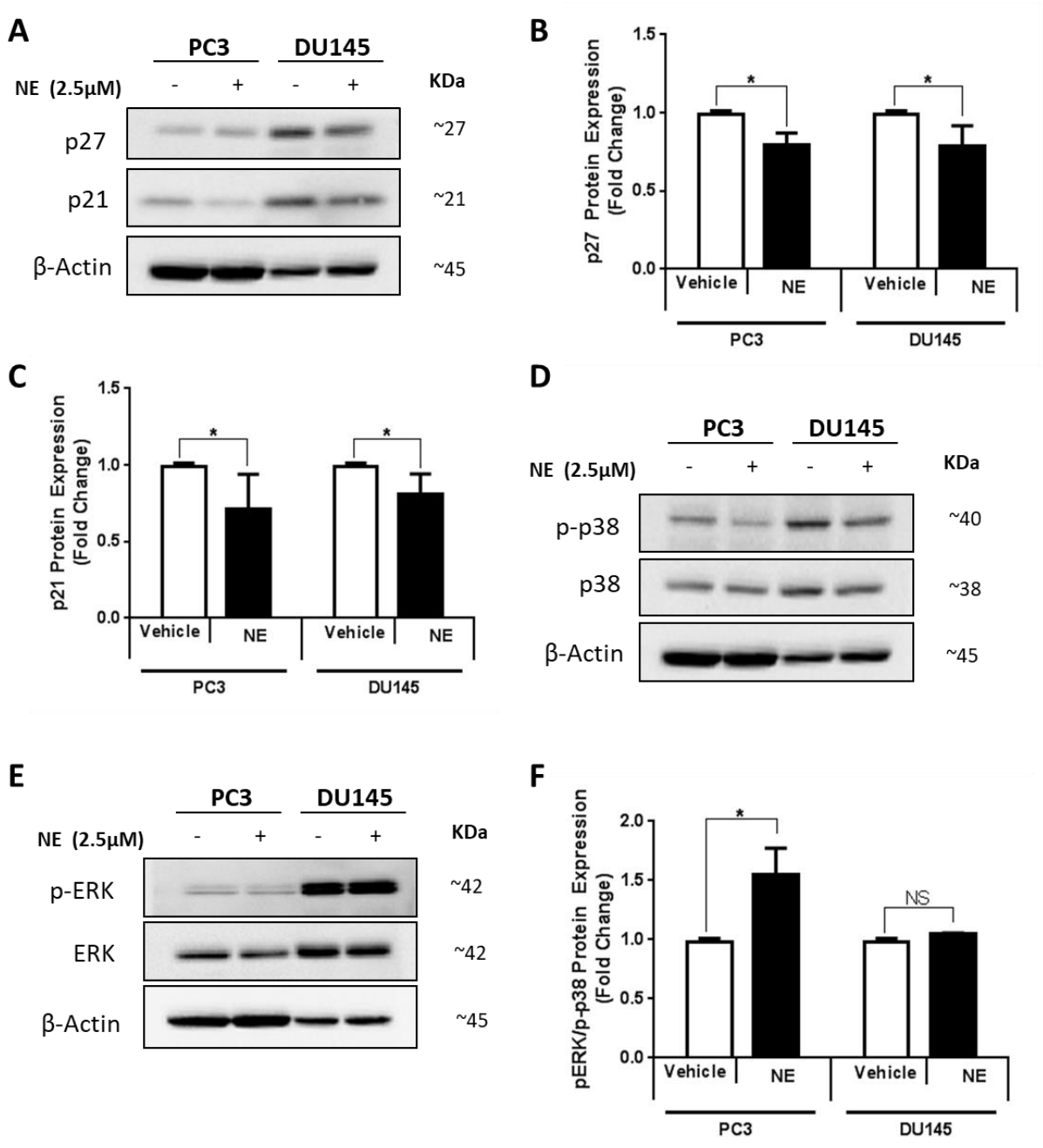


Figure 3.4. NE induces reactivation of dormant PCa cells.
(A-C) PCa (PC3 and DU145) expression of cell cycle inhibitors p21 and p27 was determined in the presence of NE or vehicle control by Western blot and quantified using Image J. Band values were normalized to β-Actin (N=4; Student's t-test). (D-E) PCa (PC3 and DU145) expression of the cell cycle inhibitor p-p38 and growth promoting factor p-ERK were determined in the presence of NE or vehicle control by Western blot. (F) The ratio of p-p38 to p-ERK was quantified (N=4; Student's t-test).

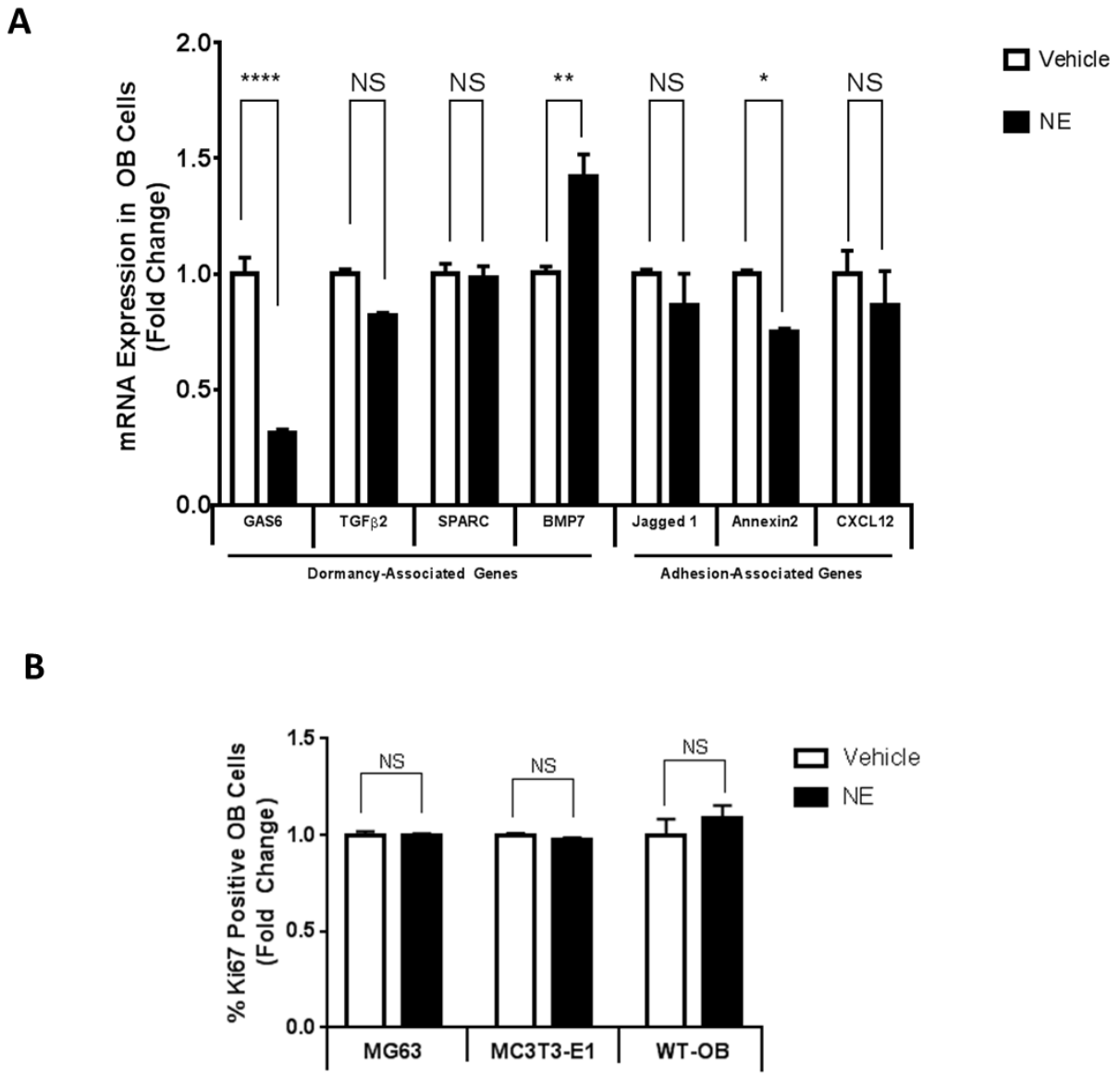


Figure 3.5. NE disturbs niche microenvironment signaling.
(A) Expression of mRNA dormancy-associated genes and adhesion/homing-associated genes expressed by OBs were determined in MC3T3-E1 OB cultures treated with NE or vehicle control using qPCR (N=2; Student's t-test). Values were normalized to GAPDH levels. (B) Ki67 expression of OBs (MG63, MC3T3-E1, and wild type (WT) OBs) cultured with NE or vehicle control was determined by flow cytometry (N=3; Student's t-test).

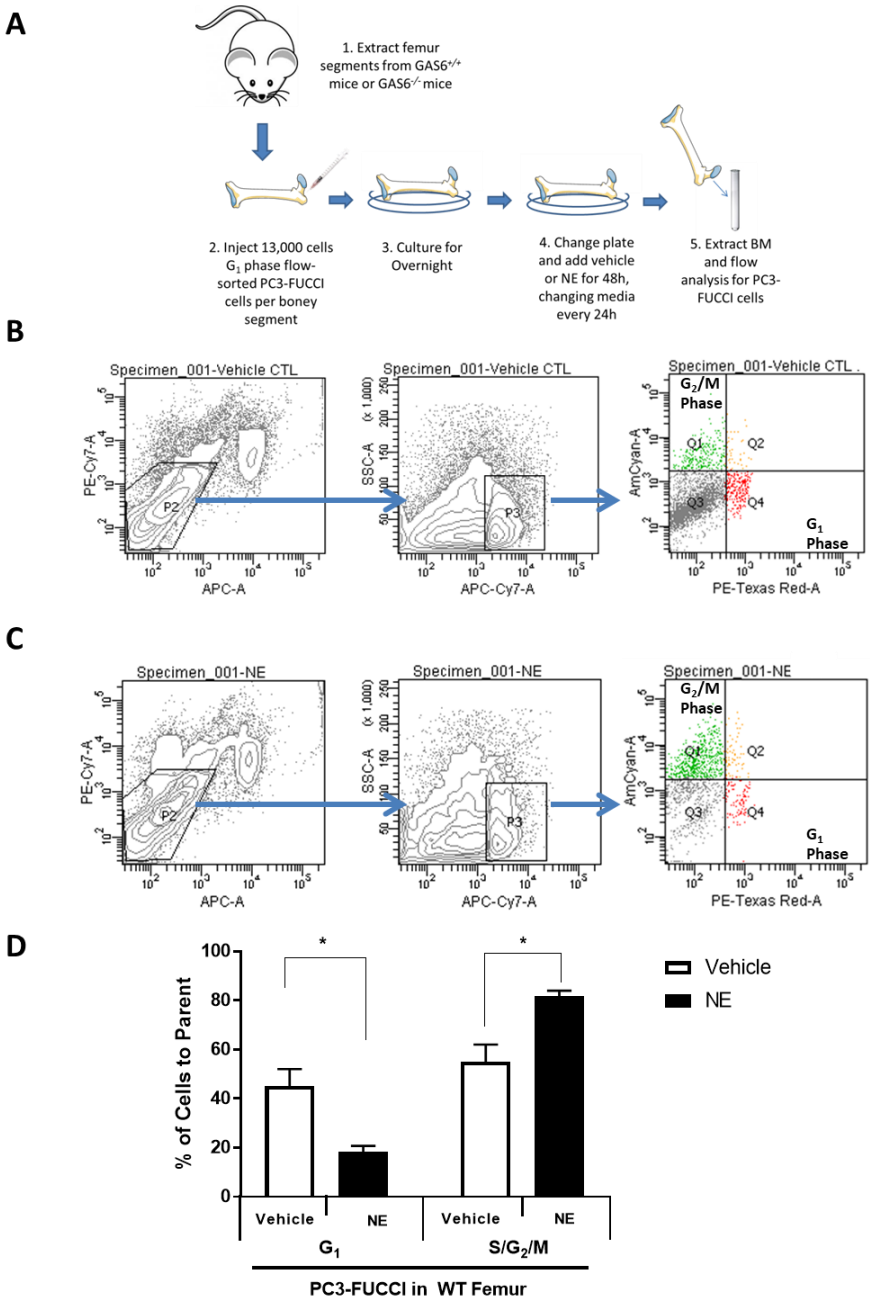
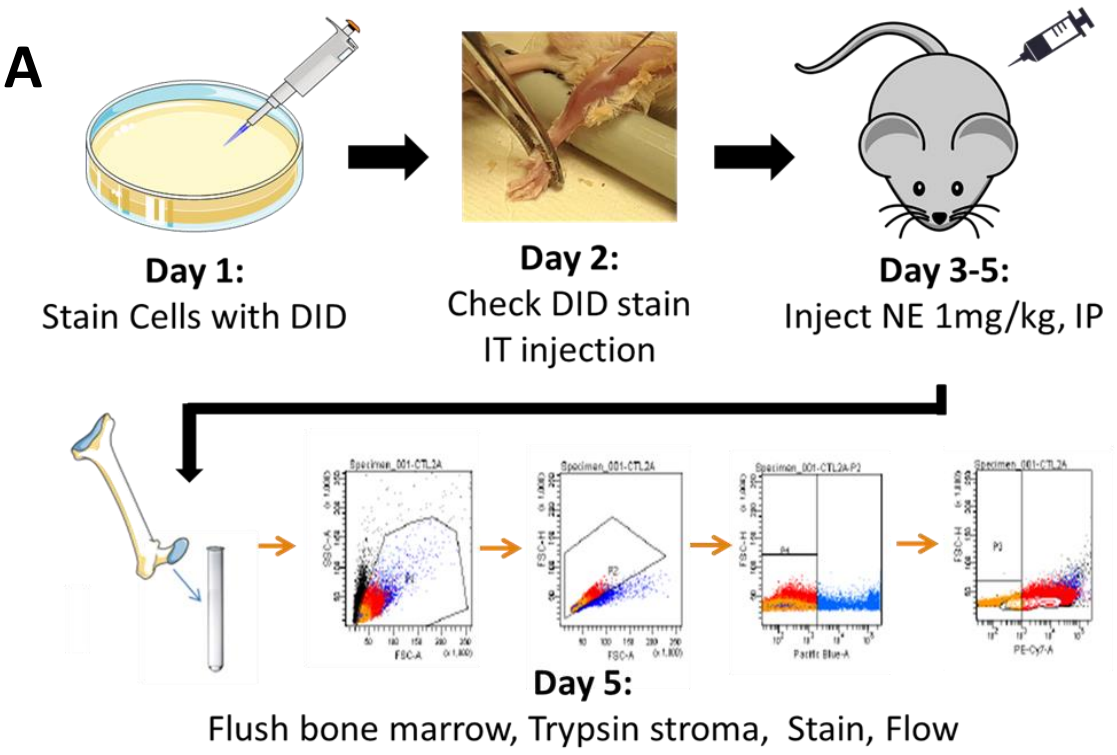


Figure 3.6. NE induces cell cycle entry by PC3 in the bone marrow.
(A) Pictorial diagram demonstrating the experimental protocol for the *ex vivo* culture model. (B,C) Representative sample of the flow plot gates found from PC3-FUCCI cells injected into *ex vivo* femurs treated with vehicle (B) or NE (C) added to the culture conditions. Live cells were negatively gated for murine IgG2b b haplotype (mH-2Db) (BioLegend, Cat# 111516, PE/Cy7) and mCD45 (BioLegend, Cat# 103112, APC), which were then positively gated for HLA-A,B,C (Biolegend, Cat# 311426, APC/Cy7). After these gates were applied, cells were plotted on the FUCCI spectrum. (D) PC3-FUCCI cells were isolated following injection into *ex vivo* femur explants and cell cycle was determined using flow cytometry (N=3; Student's t-test).



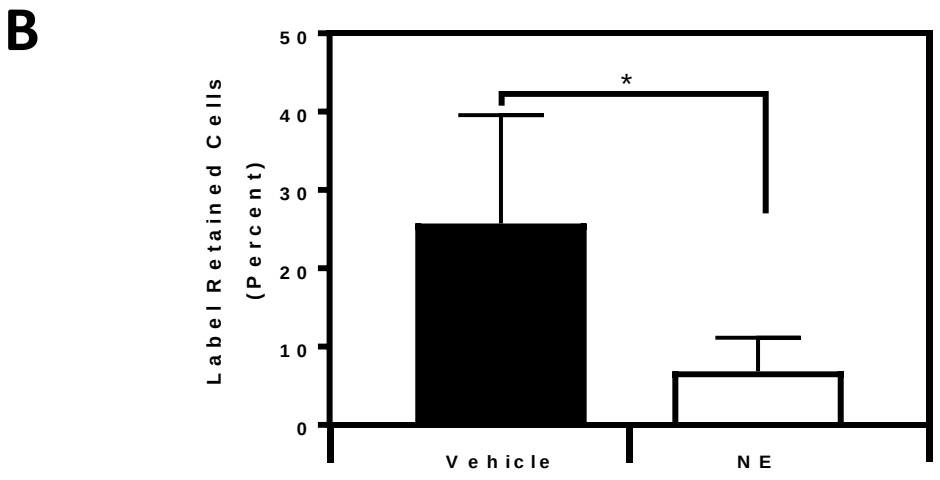


Figure 3.7: NE significantly decreases the proportion of DID positive cells in marrow after IP injection. A) Schematic of experimental protocol. B) Percent cells isolated on Day 5 that were positive for DiD in both conditions. Error bars are standard deviation of multiple explanted tibias.

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Chapter 4

GAS6 Mediates Indirect Effects of Adrenergic Signaling on Dormant Pca Cells

Introduction

Prostate cancer (PCa) remains one of the most common cancers in men, with high mortality related to metastasis and the formation of secondary tumors. PCa has been previously shown to metastasize to bone, resulting in painful secondary tumors and eventually mortality. Upon dissemination, PCa cells can undergo one of three fates: 1) the cells can die due to incompatibility with the microenvironment, 2) cells may colonize and proliferate, resulting in secondary tumors, or 3) the cells may undergo cell-cycle arrest and remain dormant for months or years before reentering the cell cycle (1). The mechanisms regulating dormancy of disseminated tumor cells (DTCs), and when DTCs enter the marrow has been a considerable source of scientific debate (2). Late recurrence (more than five years after curative therapy) has been shown to account for 20% of all recurrences in PCa, which strongly suggests that the presence of DTCs in marrow is a predictor of poor clinical outcome (3). However, the signaling mechanisms within the bone marrow controlling proliferation of these cells is poorly understood.

We have previously demonstrated that PCa DTCs replace resident stem cells in marrow (4)**,** and are subject to similar signaling within the bone marrow microenvironment. Extracellular signaling from soluble factors such as GAS6 (5),TGFβ (6), or BMP7 (7) have all been shown to induce DTC dormancy through a variety of

intracellular signaling mechanisms**.** Internal factors, such as signaling from ERK or NR2F1 (8) also play an essential role in regulating dormancy**.** Other intrinsic factors, such as VEGF, may affect the initial entry into dormancy and could potentially lead to egress of DTCs (9)**.** However, despite the body of work on what signaling factors can lead to cell-cycle arrest, less is known regarding how these signals are reversed resulting in cell-cycle reentry.

Our recent work has shown that adrenergic signaling through norepinephrine (NE) may drive dormant DTCs to reenter the cell cycle (10)**.** Adrenergic signaling can act directly on a primary tumor to promote proliferation and metastasis (11), and circadian fluctuations in NE within the bone marrow have been shown to mediate hematopoietic stem cell activation and entry into circulation (12). For dormant PCa cells, several intrinsic and extrinsic models for dormancy all suggested adrenergic signaling both directly on dormant cells and indirectly on the microenvironment can alter the proliferative phenotype of these cells. NE was found to directly influence expression of key cell-cycle regulators p21, p27, p38 and ERK, further supporting the connection between adrenergic signaling and cell-cycle reentry. However, the indirect mechanisms through which adrenergic signaling influences the dormant microenvironment are largely unknown.

The goals of this study were to identify the indirect mechanisms through which adrenergic signaling leads to proliferation in quiescent tumor cells in marrow. Our hope is that by identifying how NE alters the production of niche-derived factors which regulate DTC dormancy, we will gain a greater understanding of how dormancy is induced and maintained as well as be in a far better position to target the process for therapeutic gain.

Methods

Cell Culture

Human PCa cell lines (PC3) were obtained from American Type Culture Collection (Rockville, MD). Primary murine osteoblasts were established from C57BL/6J (WT or $GAS6^{+/+}$) and GAS6 knockout mice (GKO-C57BL/6J ($GAS6^{-/-}$)) mice as previously reported. The murine osteoblastic cell line MC3T3-E1 Subclone 4, was obtained from ATCC (CRL-2593). All prostate cancer cell lines were cultured with RPMI 1640 (Life Technologies, Carlsbad, CA), and murine or human osteoblasts were grown in αMEM or DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, GEMINI Bio-Products, Sacramento, CA, 1% penicillin-streptomycin (P/S, Life Technologies) and maintained at 37ºC, 5% CO2, and 100% humidity.

Lentivirus

Lentivirus was produced by co-transfecting lentiviral packaging vectors (pMDL-GagPol, pRSV-Rev, pIVS-VSV-G) and lentiviral vectors using JetPrime (Polyplus) into HEK-293T cells, as previously described(13). Viral supernatant was collected after 48 hours in culture and concentrated using PEG-it (Systems Biosciences). Virus was re-suspended in phosphate buffered saline (PBS) and stored at -80^{o}C until use.

Reporter arrays

Transcriptional activity cell arrays (TRACER) were used to identify transcription factors (TFs) leading to adrenergic signaling-mediated reentry into the cell cycle(14-16). PC3 cells were infected with a library of reporter viruses, cultured for at least two days, and subsequently plated at a low density onto a confluent monolayer of MC3T3 cells in a black 384 well plate. Three days later, 2.5μM NE was added to the culture and transcription factor activity measured after 2, 4, 6, 8, 24, 48, and 72 hours using an IVIS

Spectrum (Perkin Elmer). TRACER data were processed as previously described (16). Briefly, activity measurements were background subtracted, normalized to the empty control reporter and log2 transformed prior to analysis(17). Only reporters measured above background were included in the final analysis. Data were taken from a minimum of six replicates and presented as the mean ± standard error. Statistical analysis was performed using the *limma* R package(18). P values were adjusted using the false-discovery rate correction(19).

Network analysis

NTRACER (networks for TRACER) was used to analyze connections between dynamic TF activity measurements, as previously described (16,20). Briefly, this method uses a combination of inference methods (PLSR(21), similarity index(22), linear ordinary differential equations based on TIGRESS(23), random forest(24), ARACNE(25), CLR(26), MRNET(27)) to infer high-confidence connections (inferred by >1 inference method) between factors based on their dynamic activity. Eigenvector centrality is then used to identify nodes that are central to the network. Networks were visualized and analyzed using the R package *iGraph*(28)**.**

Dot Blot

Lysates were prepared in cOmplete lysis M (Roche #04 719 956 001) supplemented with proteinase inhibitor Mini cOmplete Tablets (Roche #04705378) and phosphatase inhibitor PhosSTOP EASYpack Tablets (Roche #04 906 837 001). Protein concentration was calculated using the BCA protocol (Sigma). 20 µg of total protein was added to dot blot nitrocellulose sheets and subsequent incubation and wash steps were followed according to manufacture guidelines (R&D Systems). SuperSignal West Dura

Chemiluminescent Substrate (Thermo Scientific #34075) was added for 1 minute on shaker in the dark and images were acquired with a ChemiDoc Touch imager (BioRad). Expression of protein was analyzed relative to the control spot on the blot and reported as the per-blot average.

ELISA

An ELISA assay was performed following the manufacturer's instructions (R&D Systems, Dy986) to evaluate GAS6 expression in OBs (MC3T3-E1s and primary murine OBs) in the presence of vehicle or NE.

Ex Vivo Experiments

Ex vivo experiments were performed as described in Chapter 3. Femur explants were dissected from 5-week old *GAS6*$^{+/+}$ or *GAS6*$^{-/-}$ mice. Briefly, sorted G_0/G_1 FUCCI cells were injected into the marrow space of the femur explants and cultured for 24 hours in α-MEM (Life Technologies, Carlsbad, CA) supplemented with 5% FBS. 24 hours after dissection, media was changed to α-MEM supplemented with 10% FBS and 2.5μM NE or vehicle control. After 48h of culture in experimental conditions, the bone marrows were flushed from the femur explants with FACS buffer (PBS +2% FBS).

Statistical Analyses

Results are presented as mean ± standard deviation and fold change ± standard deviation normalized to standard vehicle control conditions. Significance of a difference between values was determined by use of an ANOVA with correction of multiple comparisons using the Sidak's multiple comparison test through GraphPad Prism version 7.0. If the comparison of two values were assessed, an unpaired Student's t-test was used. Error bars reported in all figures represent standard deviation.

Results

Adrenergic signaling can reactivate dormant cells through RAR and ATF1 signaling

We used a transcriptional activity cell array (TRACER) to investigate the mechanisms through which adrenergic signaling reactivates dormant PCa cells. We used a previously defined co-culture system in which a monolayer of osteoblasts (MC3T3-E1 cells) act as a surrogate for the osteoblasts in the bone marrow, while sparsely plated PC3 cells are used as a model of PCA dormancy (Figure 4.1A). TRACER can be easily used to separate signal from one cell type in co-culture and provide intracellular activity information in living cells, making it an ideal tool for this type of co-culture assay. Activity was monitored for three days after administration of NE, during which significant alterations in TF activity were observed. Specifically, 16/66 TFs had their activity altered over three days of NE stimulation leading to cell-cycle reentry (Figure 4.1B). In order to determine which of these factors were most essential to reactivation, we used a network analysis to determine controlling factors. This network analysis identified five factors as central to reactivation: E2F, RAR, ATF1, CMYC and STAT4 (Figure 4.1C). Of these factors, only ATF1, RAR, and E2F were significantly altered during the experiment, leading to the conclusion that these three factors were primarily responsible for the observed effects.

NE causes downregulation of dormancy-inducing cytokine GAS6

Adrenergic signaling may reactivate dormant PCa cells through both direct effects on the cells themselves or indirectly on the osteoblasts that reside in the bone marrow. Direct effects of NE on the PC3 cells used for the co-culture are mediated through ATF1 activity (Figure 4.1D). However, despite ATF1 being central to reactivation (Figure 4.1C),

co-culture was found to significantly limit the immediate effects of NE stimulation (Figure 4.1D).

Therefore, we focused on identifying an indirect mechanism for reactivation of these cells in co-culture. We explored the impact of adrenergic signaling on cytokine secretion and the expression of membrane bound factors by osteoblasts using a dot blot comprised of 111 different mouse cytokines. This panel showed significant alterations in MC3T3 cytokine expression in response to adrenergic signaling (Figure 4.2A and 2B). A total of 12 cytokines were differentially expressed in the NE treated group compared with vehicle control ($p<0.05$), with one upregulated cytokine (CCL5) and 11 downregulated cytokines. Downregulated cytokines included GAS6, M-CSF, and osteopontin (Figure 4.2B).

We next employed TRACER to identify transcriptional regulators of NE signaling in MC3T3 cells. We screened activity of 50 different TFs over 24 hours of stimulation with NE. A total of 26 different factors (52%) were found to have their activity significantly altered during the first 24 hours of stimulation with NE. Of these, YY1, EGR1, MEF2 and ATF4 were the most significantly altered. A network analysis was subsequently employed to discover connections between dynamic TF activity during adrenergic stimulation in an effort to identify the predominant factors regulating the impact of NE treatments on osteoblasts. This analysis identified the CRE binding protein family as mediating the response to NE in MC3T3-E1 cells (Figure 4.2C). We connected the TRACER results to the dot blot measurements through searching the ENCODE database for experimental binding of CREB1 to the promoter regions of the 12 significantly altered cytokines. The ENCODE project database had experimental evidence of binding of CREB1 to 5 of the

12 factors (GAS6, FLT3L, IGFBP6, PTX3 and VCAM1) identified in the dot blot. Of these 5 factors, only 2 (GAS6 and FLT3L) had binding at a canonical CREB binding site, which was used as the criteria for regulatory binding of the CREB1 factor to the promoter (Figure 4.2D and 2E).

We next sought to confirm a connection between GAS6, FLT3L, and reentry into the cell cycle in the co-culture model. We screened the transcription factor reporter library against signaling from soluble GAS6 or FLT3L and compared these results to significantly altered factors from the reactivation experiment (Figure 4.3A). GAS6 signaling significantly altered activity of reporters in the CRE binding protein family (CREB, ATF1, ATF4), along with others, for a total of 12 factors of the total 43 screened. FLT3L signaling significantly altered 8 of 43 factors. Of these factors, GAS6 and FLT3L had two in common (NANOG and SRF) (Figure 4.3B). FLT3L had 0 of a possible 16 factors in common with the reactivation experiment, while GAS6 had 25% overlap (4/16) with the reactivation experiment. Importantly, all three factors identified as both significant and central to reactivation (ATF1, E2F, and RAR) from the co-culture experiment were significantly altered by soluble GAS6 signaling, indicating GAS6 was mediating the indirect effects of NE on dormant PCa cells in co-culture (Figure 4.3B).

In order to confirm the connection between adrenergic signaling and GAS6, several cell lines were screened for expression following NE stimulation (Figure 4.3A). These cells showed consistent, adrenergic mediated downregulation of GAS6 protein, as measured by ELISA (Figure 4.4A). Additionally, qPCR of bone marrow extracted from C57BL6/J mice treated with norepinephrine confirmed downregulation of GAS6 by

adrenergic signaling *in vivo* (Figure 4.4B), confirming the connection between adrenergic signaling and GAS6 regulation in the native bone marrow environment.

Further confirmation of these data was sought using immunohistochemistry. NE stimulation reduced the expression of GAS6 by wild-type (*GAS6*$^{+/+}$) osteoblasts, but as expected had no impact on expression of GAS6 by osteoblasts isolated from *GAS6* deficient mice (*GAS6*$^{-/-}$ OBs) (Figures 4.5A and 4.5B).

To directly test whether alterations in GAS6 signaling by osteoblasts in response to NE is responsible for the alteration of proliferation of PCa cells in co-culture, experiments were performed in which PCa cells were cultured on *GAS6*$^{+/+}$ or *GAS6*$^{-/-}$ OBs. Co-culture of PCa cells with *GAS6*$^{+/+}$ OBs reduced the proportion of proliferating PCa cells compared to PCa co-cultured with *GAS6*$^{-/-}$ OBs (Figure 4.5C), which is consistent with previous reports and induction of PCa dormant states (29). Co-culture of PCa cells with *GAS6*$^{+/+}$ OBs also revealed a 1.9 fold increase in Ki67 positive PCa cells when cultured with NE compared to vehicle controls as determined by flow cytometry (Figure 4.5D). Critically, when co-cultured with *GAS6*$^{-/-}$ OBs, there were no statistical differences in the number of Ki67 expressing PCa cells suggesting that GAS6 expression is a critical parameter regulating PCa proliferation on OBs (Figure 4.5C). These data suggest that PCa cell re-activation may be the result of diminished osteoblast-mediated *GAS6* signaling.

These experiments were repeated using a second human PCa line, DU145 cells (Figure 4.5D). Increased Ki67 expression was observed under conditions where NE was added to DU145 and *GAS6*$^{+/+}$ OB co-cultures compared to vehicle controls. However, no differences were observed between NE and vehicle control conditions, when DU145 cells

were co-cultured with *GAS6*$^{-/-}$ OBs, suggesting that culture with NE induced DU145 cell cycle re-activation. Interestingly, Ki67 expression in DU145 cells did not change significantly when cultured with *GAS6*$^{+/+}$ or *GAS6*$^{-/-}$ OBs in the presence of vehicle controls, as previously reported (29). Thus, the effects of NE on DU145 PCa cell cycling may instead be mediated more directly through NE signaling than indirectly via GAS6 suppression, while PC3 PCa cells appear to be influenced through both direct NE application and the presence of dormancy molecule, GAS6.

Next, PC3 cells expressing the FUCCI vectors were co-cultured with osteoblasts to observe the effects of NE on cell-cycle induction. PC3 cells exhibited a 7.2 fold increase in the proportion of cells in G_2/M phase when cultured on *GAS6*$^{-/-}$ OBs compared to *GAS6*$^{+/+}$ OBs suggesting that GAS6 is responsible for the maintenance of PCa cells in a dormant state in co-culture (Figure 4.5E). Moreover, co-culture of PC3-FUCCI cells with *GAS6*$^{+/+}$ OBs in the presence of NE doubled the proportion of G_2/M phase PCa cells compared to vehicle controls (Figure 4.5E). Co-culture of PC3-FUCCI cells with *GAS6*$^{-/-}$ OBs in the presence of NE did not alter the proportion of cells in the G_2/M cell cycle phases. These data suggest that NE changes the proportion of cells in an active cell cycling phases compared to vehicle controls in a *GAS6* competent system. The proportional increase in cycling cells was negated upon deletion of *GAS6*, suggesting that NE may target the *GAS6*-dormancy inducing axis, resulting in the observed results of PCa cell re-activation.

Finally, we applied the *ex vivo* model system to explore the impact of NE on dormant PCa cells in the bone marrow without the systemic effects of altered NE signaling (Figure 4.6). G_0/G_1 phase PC3-FUCCI cells were injected into *ex vivo* bone *GAS6*$^{+/+}$ or

GAS6$^{-/-}$ explants. After 48-hour incubation with NE in *GAS6*$^{+/+}$ femurs, PCa cells isolated from the *GAS6*$^{-/-}$ femur did not have the proportion of cells in G_2/M phase significantly altered, which differed significantly from the *GAS6*$^{+/+}$ cultures.

Discussion

Transcription factor analysis of quiescent PCa cells reactivated through adrenergic signaling suggested three factors (ATF1, RAR, and E2F) were primarily responsible for the change in phenotype. The E2F family of transcription factors are responsible for cell cycle regulate on and DNA synthesis (30). We observed an increase in activity of this reporter during reactivation, which is consistent with reentry into the cell cycle. RAR was previously shown to interact with SOX9 and NR2F1 to cause dormancy in a model of head and neck squamous cell carcinoma (8). Further, RAR has been demonstrated to be involved in dormancy of hematopoietic stem cells (31), and the Taichman Lab has previously demonstrated that PCa DTCs mimic HSC biology in the bone marrow (4). ATF1 is a canonical regulator of cAMP signaling, which is the primary means of signal activation following adrenergic stimulation. Together, literature supports these three factors as central to quiescence, and, in the case of this study, reactivation of PCa cells.

The experiments of this study found that downregulation of GAS6 expression by norepinephrine results in significant changes in the bone microenvironment that are conducive towards reactivation of dormant PCa cells. The dot blot data specifically identified 12 cytokines that were altered by NE stimulation, of which GAS6 was ultimately selected through analyses of transcription factors demonstrating significant changes in E2F, ATF1, and RAR activity after stimulation with soluble GAS6. GAS6 has been previously shown to induce dormancy through signaling through its receptors Axl and Mer

(5). The fact that GAS6 was selected through an unbiased bioinformatic analysis strengthens these results. The results of these studies agree with previous reports demonstrating that GAS6 can activate tumor cell dormancy both *in vitro* and *ex vivo* (4,5,32,33). While GAS6 has been established to regulate immune homeostasis (34), its role within the complex signaling environment of the healthy HSC niche is currently unknown. Cackowski and coworkers have suggested that GAS6 can act on PCa cells through MERTK to induce dormancy (35). The current study provides evidence of down-regulation of GAS6 expression by NE in osteoblasts, and that the effect on GAS6 is necessary for NE to reactivate dormant tumor cells that are in co-culture with OBs.

Our analysis also suggested Flt3 ligand as a potential mediator of NE signaling. FLT3L has been previously shown to promote egress of stem cells from the bone marrow (36)**,** and therefore may be related to quiescence in DTCs. Investigation of the effects of FLT3L on dormant tumor cells did not suggest that signaling through FLT3 would result in dormancy in tumor cells alone. It is possible that FLT3 signaling in combination with other factors not measured in the dot blot, for example TGFβ, may yield differing results. We note that GAS6 has been shown to act in a similar manner, however signaling through soluble GAS6 is sufficient for cell cycle arrest in PCa cells (5)**.** Other altered factors, such as upregulated CCL5 or downregulated GM-CSF, may also play a role, however the results of this study suggest that these may not be directly tied to adrenergic signaling.

In summary, the experiments in this chapter identified GAS6 as an indirect mediator of adrenergic signaling leading to reactivation of dormant PCa cells. GAS6 was consistently downregulated by adrenergic signaling, which was shown to be necessary

for reactivation of dormant PCa cells. The next steps are to identify a mechanism through which GAS6 is downregulated by NE signaling, which is the focus of Chapter 5.

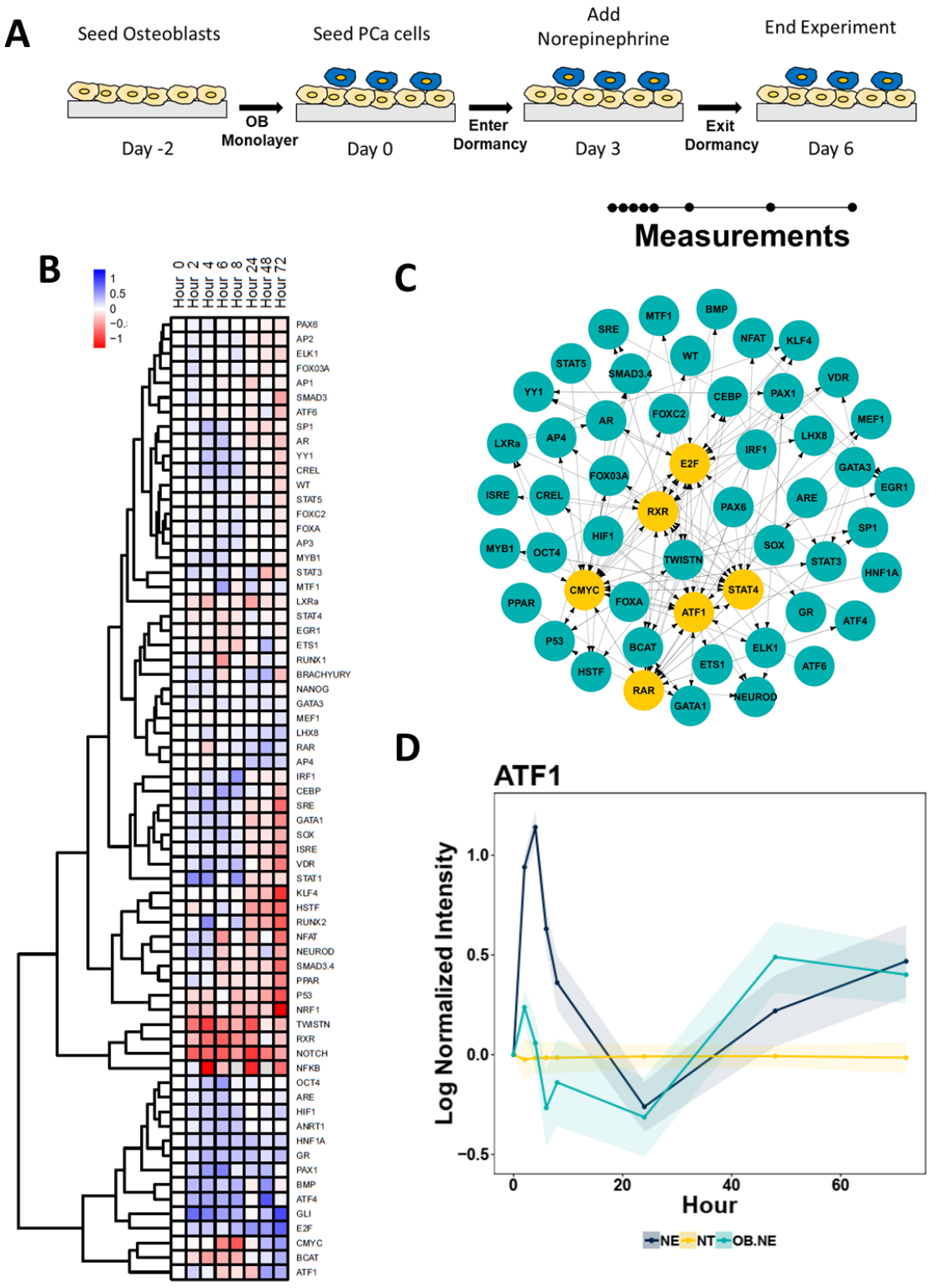

Figure 4.1: Co-Culture dampens NE effects on ATF signaling A) Schematic of TRACER experiment. B) Hierarchical clustering of time course TF activity data. C) Results from network analysis of TF activity data. Yellow nodes are in the top 10% by eigenvector centrality. D) ATF1 activity in PC3 cells cultured alone (blue) or in co-culture with MC3T3 cells (OB.NE, green). Shading indicates standard error.

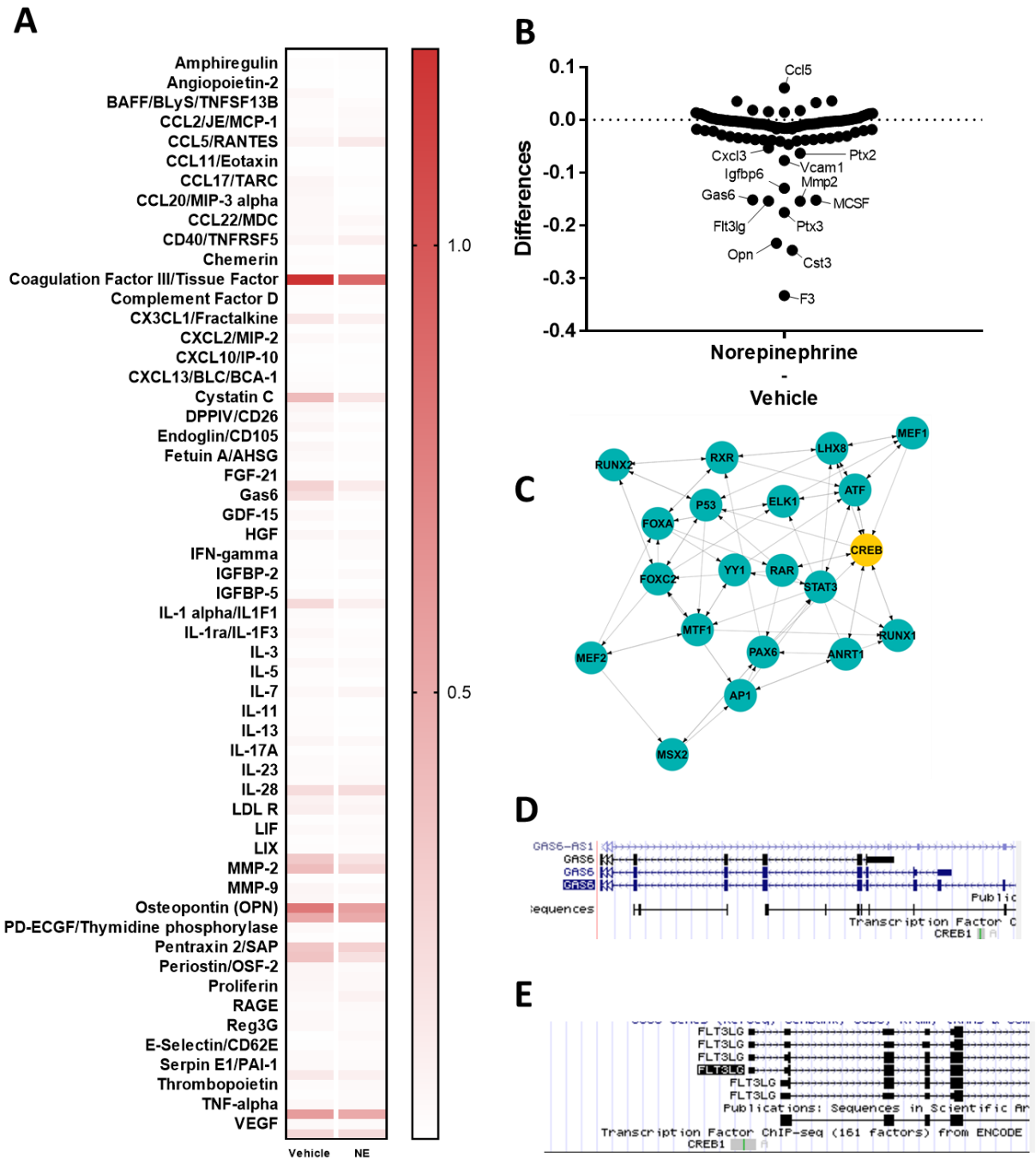

Figure 4.2: GAS6 and FLT3L expression in OBs is directly targeted by NE. A) Heatmap of protein expression values for 111 different cytokines from the dot blot. B)Difference in protein expression between NE and vehicle treated MC3T3 cells. Labeled points are statistically significant. C) CREB was identified as a central to network (yellow) mediating NE signaling in OBs. D,E) GAS6 and FLT3L were found in the ENCODE database to have experimentally verified binding of CREB1 to their promoter regions..

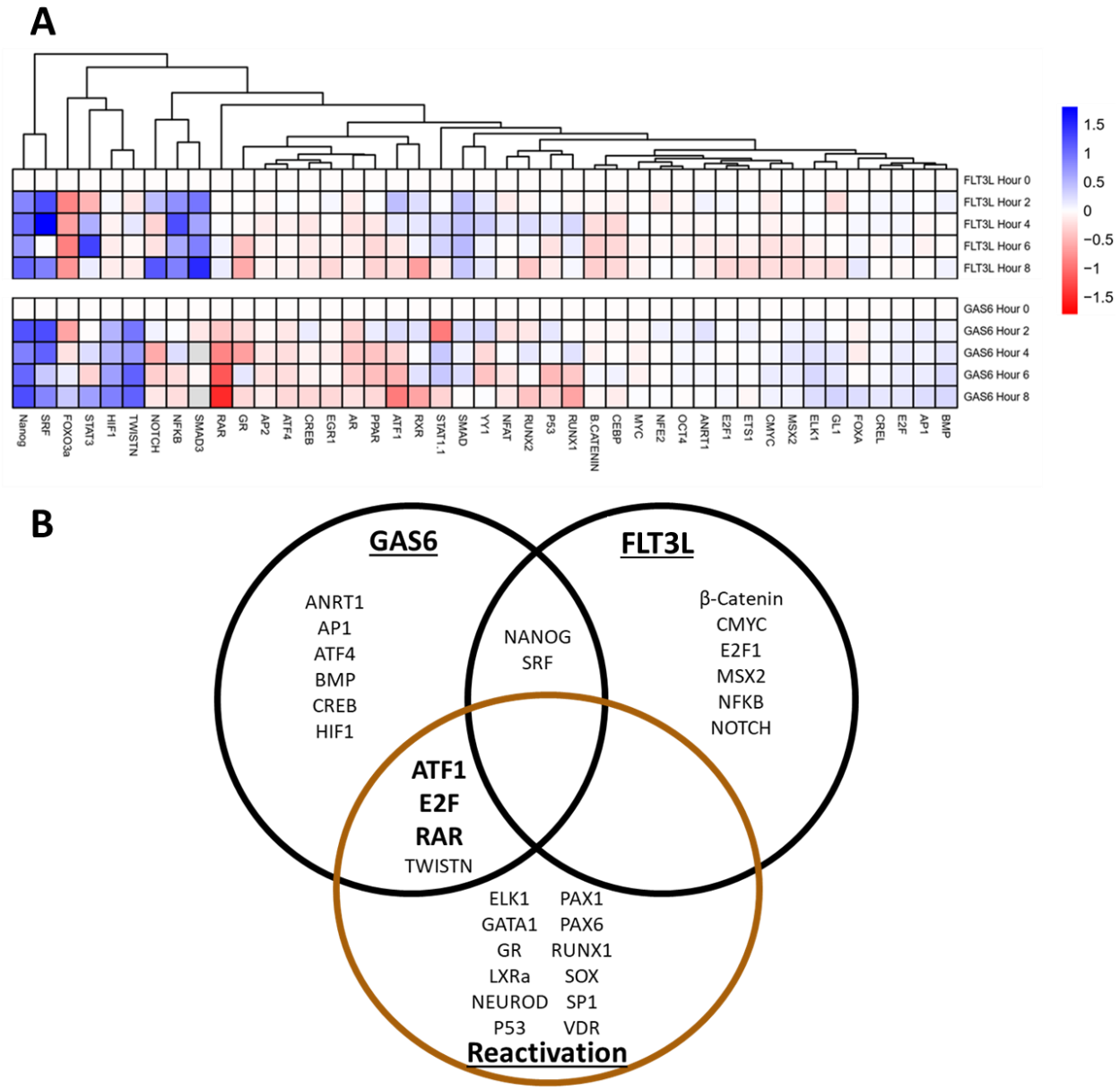

Figure 4.3: GAS6 drives indirect responses of NE in PCa cells A) Heatmaps for dynamic TF activity after stimulation with FLT3L (top) or GAS6 (bottom). B) Venn diagram of significantly altered TF activity for reactivation (bottom), GAS6 stimulation (left) or FLT3L stimulation (right). Bolded factors were central to reactivation of PCa cells in co-culture.

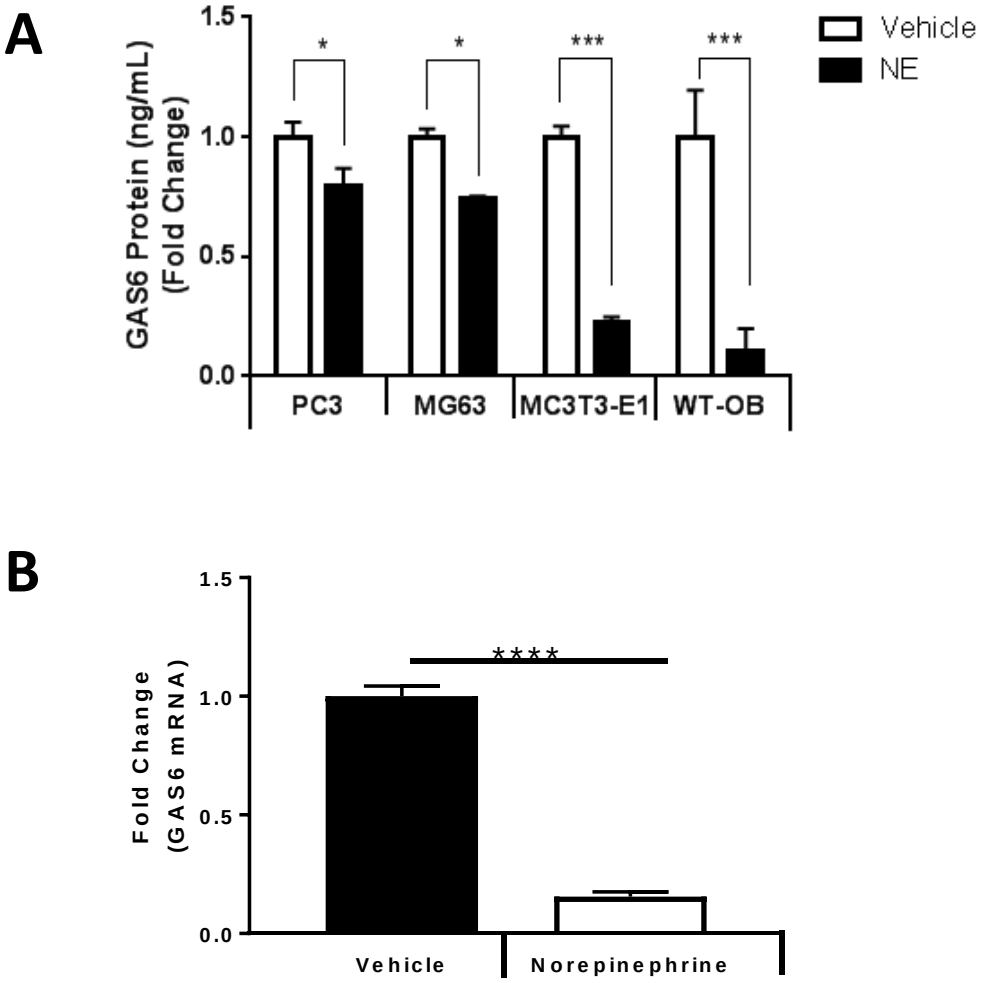

Figure 4.4: NE reliably downregulated GAS6 in a variety of PCa and OB cell lines. A) ELISA of stimulated cell lines in culture showed significant downregulation of secreted GAS6 protein after NE stimulation. B) mRNA isolated from the bone marrow NE-treated mice showed significantly downregulated GAS6 expression compared with vehicle treated animals

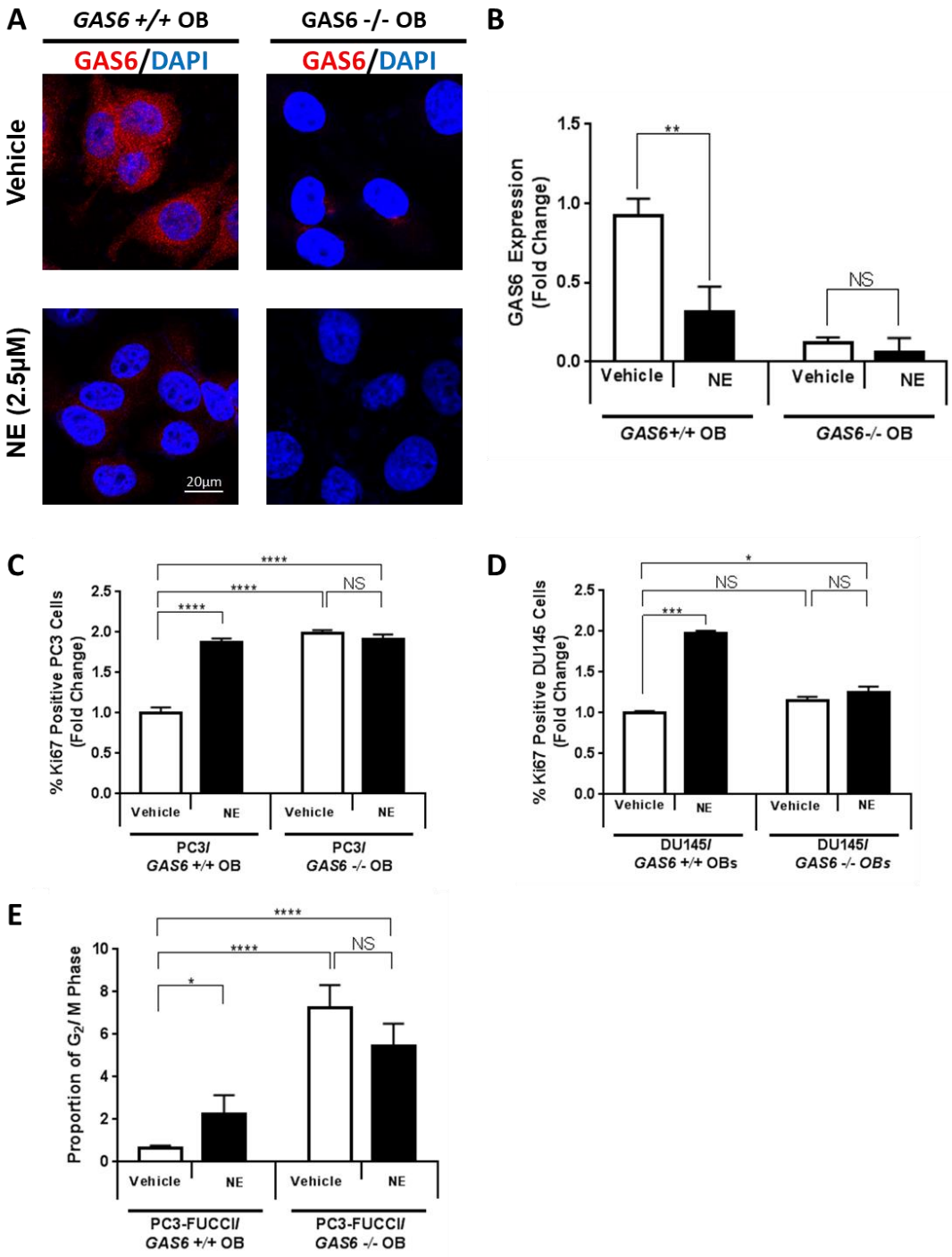

Figure 4.5: NE effects cellular proliferation in GAS6 competent co-cultures (A-B) *GAS6* expression in *GAS6*$^{+/+}$ OBs cultured under conditions with NE or vehicle control was determined by immunocytochemistry and quantified (N=3; student's t-test). *GAS6*$^{-/-}$ OBs were cultured under the same conditions, serving as a negative control. (C) PC3 cells and (D) DU145 cells were co-cultured with *GAS6*$^{+/+}$ and *GAS6*$^{-/-}$ OBs in the presence of NE or vehicle control. Ki67 expression was determined by FACS (N=3; ANOVA). (E) PC3-FUCCI cells were co-cultured with *GAS6*$^{+/+}$ and *GAS6*$^{-/-}$ OBs in the presence of NE or vehicle control. Cell cycle phase was determined by FUCCI vector expression using flow cytometry (N=3; ANOVA).

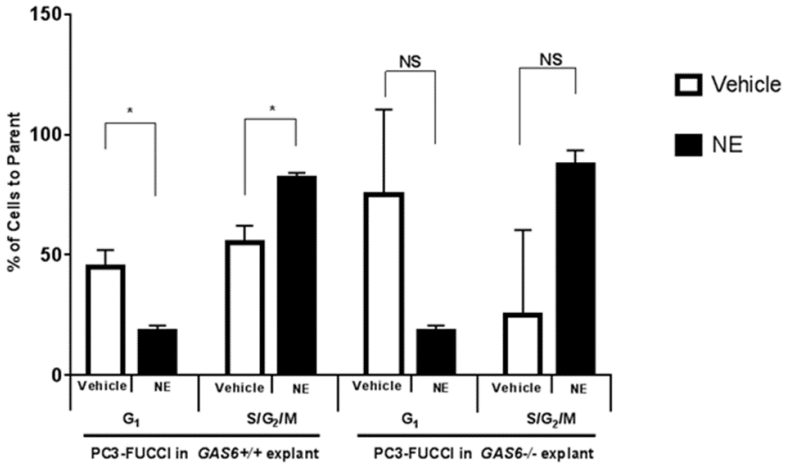

Figure 4.6: NE effects cellular proliferation in GAS6 competent *ex vivo* cultures. PC3-FUCCI cells were isolated following injection into *ex vivo GAS6*$^{+/+}$ or *GAS6* $^{-/-}$ femur explants and cell cycle was determined using flow cytometry (N=3; Student's t-test).

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Chapter 5

Norepinephrine downregulates GAS6 through β-adrenergic signaling and ATF4

Introduction

Bone plays an important endocrine role within the body's general homeostasis. Part of this endocrine function is elucidated in the regulation of resorption (hemopoietic-lineage derived, osteoclasts) and replacement (mesenchymal-lineage derived, osteoblasts) of a rich mineralized bony matrix. Much research has previously established this regulation can occur via endocrine, paracrine, and mechanical factors; however, in the early 2000s a new line of investigation into obesity, leptin signaling, and bone density provided insight into a new bone-modulatory method: hypothalamic neurons and sympathetic signaling (1,2).

Innervation of the skeleton is a complex map with both evolutionary function and homeostatic feedback. The nervous system is present to collect information from the environment and interior compartment organs and coordinates response through both efferent pathways and somatic motor synapses. One specialized unit of the nervous system is the autonomic nervous system, which functions to ensure homeostasis and provide adaptive response to various stressors by interfacing between the external and internal sections. The autonomic peripheral nervous system can be further divided into sympathetic and parasympathetic branches. The sympathetic branch is mediated by the neurotransmitter, Norepinephrine (NE), which is the molecular agonist of α- and β-

adrenergic receptors on target cells. The parasympathetic branch is mediated by the neurotransmitter, Acetylcholine (ACh), which is the molecular agonist of muscarinic and nicotinic receptors on target cells.

The sympathetic mediator, NE, is synthesized from amino acid, tyrosine, through a number of coordinated steps (3). First, tyrosine is converted to L-DOPA by enzyme tyrosine hydroxylase, which is also the rate limiting step in catecholamine synthesis. L-DOPA is then converted to dopamine by L-aromic amino acid decarboxylase. Dopamine is transferred to an intracellular vesicle and converted to the final product of NE by dopamine beta-hydroxylase. NE can then be released onto target cells from the intracellular synaptic vesicles by fusion of the vesicles to the neuronal membrane. Not only is the release of NE tightly regulated through enzymatic steps and fusion of the carrier vesicles, but the process following release of NE is also well-regulated. NE is often co-released with neuropeptide Y, a potent inhibitor of NE, and released into a synaptic space with metabolizing enzymes, monoamine oxidase and catechol-O-methyltransferase. In addition, pre-synaptic nerve terminals have NE transport proteins that reuptake NE from the area of stimulus.

The primary bone metastatic sites of interest in this thesis are the femur and tibiae. The femoral and sciatic nerves are the primary nerve conduits to the lower extremity skeletal space, and carry motor fibers, sensory fibers, and autonomic fibers. Most sympathetic fibers travel along the main arteries feeding these skeletal bones through nutrient foramina (4-6). Though much of the sympathetic nerve distribution map within the lower extremity skeleton is unestablished, recent immunofluorescence approaches have been used in combination with tyrosine-hydroxylase antibodies to identify sympathetic

networks that wrap around blood vessels in the femoral bone marrow with a spiral-like morphology (7,8). Interestingly, the highest density of sympathetic and sensory fibers were associated with areas that felt the largest quantities of mechanical stress and have the highest metabolic rate/bone turnover.

The targets of sympathetic synapses remains an area of ongoing study. One group has investigated mechanical stress related-osteocytes, describing tyrosine hydroxylase-immunoreactive fibers associated with osteocytes and their surrounding Volkmann's canals (9). Because the field has had difficulty identifying single synaptic targets within the bone, one growing consensus suggests that diffusion mechanisms may provide activation of multiple target cells away from the immediate nerve terminal. This theory suggests that sympathetic nerve activity and released NE can act on multiple bone marrow niche cell types and have a more coordinated response either due to broad expression of adrenergic receptors or intercellular junction-based communication (10).

The connection between the nervous system and the multifaceted functions of healthy bone are clear, however less is known regarding the specific regulatory events that connect sympathetic nervous system signaling to bone endocrine function. In Chapter 5, GAS6 was identified as a secreted factor within osteoblasts (OBs) that mediated reactivation of quiescent PCa cells within the bone marrow. GAS6 has numerous functions in the body, and the specific function of interest for this work is its connection to PCa dormancy within the bone. The goal of this chapter is to identify a mechanism through which NE signaling leads to downregulation of GAS6 in osteoblasts, which was previously shown to mediate escape from dormancy. We focused on the use of pharmacological blockade to identify families of receptors and transcription factors that

may be targeted clinically, with the hopes of finding a path towards targeting these sympathetic interactions within prostate cancer patients.

Methods

Cell Culture

Human PCa cell lines (PC3) were obtained from American Type Culture Collection (Rockville, MD). Primary murine osteoblasts were established from C57BL/6J (WT) and GAS6 knockout mice (GKO-C57BL/6J ($GAS6^{-/-}$)) mice as previously reported. The murine osteoblastic cell line MC3T3-E1 Subclone 4, was obtained from ATCC (CRL-2593). All prostate cancer cell lines were cultured with RPMI 1640 (Life Technologies, Carlsbad, CA), and murine or human osteoblasts were grown in αMEM or DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, GEMINI Bio-Products, Sacramento, CA, 1% penicillin-streptomycin (P/S, Life Technologies) and maintained at 37ºC, 5% CO2, and 100% humidity.

CREB and receptor inhibition

MC3T3 OBs were cultured under standard conditions with a general CREB inhibitor (Millipore Sigma, CAT#538341), or with the α-adrenergic receptor antagonist phentolamine, or with the β-adrenergic receptor antagonist propranolol.

Immunoprecipitation

MC3T3 OBs were cultured under standard conditions with 2.5uM NE for 2 Days. All manufacture instructions were subsequently followed for ChIP sequencing assay (Qiagen, Cat# 334471). Antibodies were used for ATF4 (Cell Signaling, Cat#D4B8) and CREB (Cell Signaling, Cat#9197T). Primers used for ATF4 (ThermoFisher, Cat#Mm00515325) and CREB1 (ThermoFisher Cat#Mm00501607).

In vivo regulation of GAS6

C57BL6/J mice were treated with either 1 mg/kg NE, 3 mg/kg propranolol, or combination for three days. Femurs and tibias bilaterally isolated at the end point of the study. Marrow from these bones were flushed with 1X PBS three times, sequentially until the bone was lucent and white, and no marrow color remnants remained. Then stromal layer was flushed with RLT+Bmerc into a cell shredder column. RNA was processed as described previously.

Results

NE signaling to GAS6 occurs through ATF4 binding

Since TRACER identified the CREB family of factors as mediating the response to NE in osteoblasts, we next searched for the specific TFs that mediated this response. Use of a general CREB inhibitor (666-15) at increasing doses abrogated the effects of NE (Figure 5.1A). Interestingly, use of CREB inhibitor increased expression of GAS6 compared to vehicle control conditions, suggesting that these mechanisms were interconnected (Figure 5.1B). PCR of members of the CREB family found three factors (ATF4, ATF5 and CREB1) as significantly expressed in MC3T3 cells (Figure 5.1C). We selected two of these factors, ATF4 and CREB1, to evaluate using chromatin immunoprecipitation (CHiP) (11). These factors were selected based on a literature search, which is summarized in Table 5.1. This assay demonstrated that in fact NE signaling in osteoblastic cells decreased the binding activity for ATF4 and CREB1, however ATF4 more significantly altered compared with CREB1 (Figure 5.1D).

Adrenergic signaling is connected to GAS6 through β-adrenegic signaling

We next sought to identify which receptors significantly relayed NE signaling to the GAS6 promoter. PCR for adrenergic receptors in MC3T3 cells and bone marrow cells isolated from a C57Bl6/J mouse show expression of the α1, α2 and β2 adrenergic receptors, with the β2 receptor most strongly expressed and the only receptor whose expression was significantly altered by adrenergic stimulation (Figure 5.2A). Pharmacological targeting of the β family of adrenergic receptors significantly altered the effects of NE on GAS6 mRNA expression in a dose dependent manner (Figure 5.2B). Conversely, inhibition of the α family of receptors had varied results (Figure 5.2C). Interestingly, propranolol alone was sufficient to increase GAS6 mRNA expression in MC3T3 cells in a dose dependent manner (Figure 5.2D). These results suggest that the β receptors, specifically β2, were responsible for transmitting adrenergic signaling in response to NE altering GAS6 expression. These results extended *in vivo*, in which OBs isolated from the bone marrow had NE-mediated downregulation of GAS6 that could be abrogated by administering propranolol (5.2E).

Finally, we sought to connect CREB activity to β-adrenergic signaling. This was accomplished through measuring changes in CREB activity through luciferase assay following adrenergic stimulation in the presence of propranolol (Figure 5.3). The concentration of cAMP following adrenergic signaling was significantly decreased through addition of propranolol in a dose dependent manner (Figure 5.3A), to a low of 34% of the vehicle control. CREB activity was also decreased significantly, from a high of a 7-fold increase to a low of a 1.7 fold increase relative to untreated cells (Figure 5.3B). Together, these results indicated that treatment with propranolol can successfully block the major activator of adrenergic signaling, cAMP, and its downstream effects on transcription.

Discussion

These studies suggested that beta adrenergic signaling was primarily responsible for connecting NE to GAS6 downregulation. Previously, the Taichman lab has shown metastasis, lodging, and dormancy of prostate cancer within the confines of the hematopoietic stem cell niche. Normal binding partners to the bone-metastatic prostate cancer cells occurred through binding of Annexin-2 receptors on adjacent osteoblasts within the hematopoietic stem cell niche. Interestingly, circulating HSCs and their progenitors enter and exit from bone marrow into circulation with expression of the chemokine CXCL12 regulated by circadian NE secretion by the sympathetic nervous system. Through a series of chemical inhibitory experiments, Mendez-Ferrer (2008) identified that sympathetic nerve fibers delivered NE locally to adrenergic β3 receptors on stromal cells, leading to downregulation of CXCL12 and subsequent release of hematopoietic stem cells into circulation. These experiments suggested that the β2 receptor was more important than other adrenergic receptors in osteoblasts. The Mendez-Ferrer study focused exclusively on stromal cells, which included cell types other than osteoblasts, while the studies in this chapter focused exclusively on osteoblasts, which are the major source of dormancy-inducing GAS6 in the bone marrow. No β3-adrenergic receptor mRNA was detected through qPCR, indicating that this receptor is not strongly expressed in osteoblasts (not shown). It is possible other sources of GAS6 in the bone marrow may exist, however the strategy of targeting signaling through propranolol blockade would be valid in these cases as well.

In the context of activated sympathetic stimulus, secreted NE signaled through the osteoblastic adrenergic β2 receptors and increased ATF4 transactivation function by

phosphorylation via protein kinase A. ATF4 transactivation function induces RANKL expression in osteoblasts, resulting in osteoclastic differentiation and increased bone remodeling. Towards this point, adrenergic beta-2 receptor knockout mice have a bone phenotype present of increased bone formation and decreased bone remodeling compared to the wild type counterparts. This study found decreased ATF4 binding at the GAS6 promoter through CHiP following adrenergic stimulation. This is somewhat counterintuitive, as NE is expected to increase ATF4 phosphorylation, and an increase in ATF4 activity was observed in Chapter 4. Of note, the results of these studies suggested that, while ATF4 activity was altered, abundance was similar after stimulation with NE. ATF4 is likely bound to the GAS6 promoter at baseline and helps drive expression in OBs prior to NE stimulation. Adrenergic signaling could potentially lead to ATF4 (along with other CREB proteins) being recruited to other sites within the DNA that are direct targets of adrenergic signaling (for example, IL6 or VEGF), which would decrease the available transcription factor molecules to activate GAS6. The studies of this chapter did not examine other DNA binding sites, however future experiments in which protein-DNA interactions were directly imaged during NE stimulation may yield an answer to this question.

Pharmacological methods for targeting adrenergic signaling in patients with hypertension have garnered attention as potential correlative factors for long term success of PCa therapies (12). Several potential mechanisms for this correlation exist, such as limiting invasion and metastasis from the primary tumor (13) or sensitizing the cells to other therapies (14). Less is known about the effects of propranolol on the fate of DTCs. The studies presented here suggested adrenergic signaling affects GAS6

expression through β-adrenergic receptors, most likely the β2 receptor. Of note, β-adrenergic receptor blockade *in vitro* not only abrogated the effects of NE on GAS6 expression but were sufficient to increase GAS6 mRNA in cultured osteoblasts. These results suggest that therapeutic manipulation of β-adrenergic may be an effective method to increase GAS6 signaling within the bone marrow, and therefore may have benefit to control recurrence in high-risk patient. Clearly more investigation is required to link prolonged exposure to beta-blockers *in vivo* to GAS6 signaling and the potential as a therapy in prostate cancer.

In conclusion, adrenergic signaling downregulates GAS6 expression through signaling form the β-adrenergic receptors and activation of ATF4, leading to decreased residency at the GAS6 promoter region. This effect could be abrogated through blockade of either CREB phosphorylation of β-adrenergic signaling using the beta-blocker propranolol. Given the correlation between beta-blocker use and improved survival in prostate cancer, these studies suggest a potential mechanism for limiting recurrence in these patients through increased GAS6 expression and provide preliminary data for propranolol as a potential adjuvant to traditional therapies upon chemical recurrence of PCa.

Table 5.1: CRE binding proteins associated with NE signaling mechanisms

CBBP	Cell Types	Function	Citations
ATF2	• Ubiquitous	• Binds with AP1 • Regulates JNK pathway	Hayakawa, J., Mittal, S., Wang, Y., Korkmaz, K.S., Adamson, E., English, C., Omichi, M., McClelland, M. and Mercola, D., 2004. Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress. *Molecular cell*, *16*(4), pp.521-535. Gupta S, Campbell D, Derijard B, Davis RJ. Transcription factor ATF2 regulation by the JNK signal transduction pathway. SCIENCE-NEW YORK THEN WASHINGTON-. 1995 Jan 20:389-.
ATF4	• Osteoblast-specific	• SNS mediated bone resorption • Glucose metabolism in osteoblasts	Yang, X. and Karsenty, G., 2004. ATF4, the osteoblast accumulation of which is determined post-translationally, can induce osteoblast-specific gene expression in non-osteoblastic cells. Journal of Biological Chemistry, 279(45), pp.47109-47114. Elefteriou, F., Ahn, J.D., Takeda, S., Starbuck, M., Yang, X., Liu, X., Kondo, H., Richards, W.G., Bannon, T.W., Noda, M. and Clement, K., 2005. Leptin regulation of bone resorption by the sympathetic nervous system and CART. Nature, 434(7032), pp.514-520.
ATF5	• MSC • Adipocyte derived SCs	• Osteogenic differentiation • Apoptosis (via CHOP protein)	Leong, D.T., Abraham, M.C., Gupta, A., Lim, T.C., Chew, F.T. and Hutmacher, D.W., 2012. ATF5, a possible regulator of osteogenic differentiation in human adipose-derived stem cells. Journal of cellular biochemistry, 113(8), pp.2744-2753. Leong, D.T., Abraham, M.C., Chew, F.T., Lim, T.C. and Hutmacher, D.W., 2007. ATF5, a possible regulator of osteogenic differentiation in adult mesenchymal stem cells. Journal of stem cells & regenerative medicine, 2(1), p.110.
CREB1	• Ubiquitous	• Involved in skin cancer, histocytomas • Depression-link • Tcell function • Col24a1 in osteoblast	Altarejos, J.Y., Goebel, N., Conkright, M.D., Inoue, H., Xie, J., Arias, C.M., Sawchenko, P.E. and Montminy, M., 2008. The Creb1 coactivator Crtc1 is required for energy balance and fertility. Nature medicine, 14(10), pp.1112-1117. Zubenko, G.S., Hughes, H.B., Stiffler, J.S., Brechbiel, A., Zubenko, W.N., Maher, B.S. and Marazita, M.L., 2003. Sequence variations in CREB1 cosegregate with depressive disorders in women. Molecular psychiatry, 8(6), pp.611-618.
CRTC2	• hypothalamic neurons • B-cell function • Hepatocytes	• CREB coactivator linking hepaticstress and fasting gluconeogenesis • Corticotrophin-releasing hormone regulation via hypothalamic neurons	Lerner, R.G., Depatie, C., Rutter, G.A., Screaton, R.A. and Balthasar, N., 2009. A role for the CREB co-activator CRTC2 in the hypothalamic mechanisms linking glucose sensing with gene regulation. EMBO reports, 10(10), pp.1175-1181.

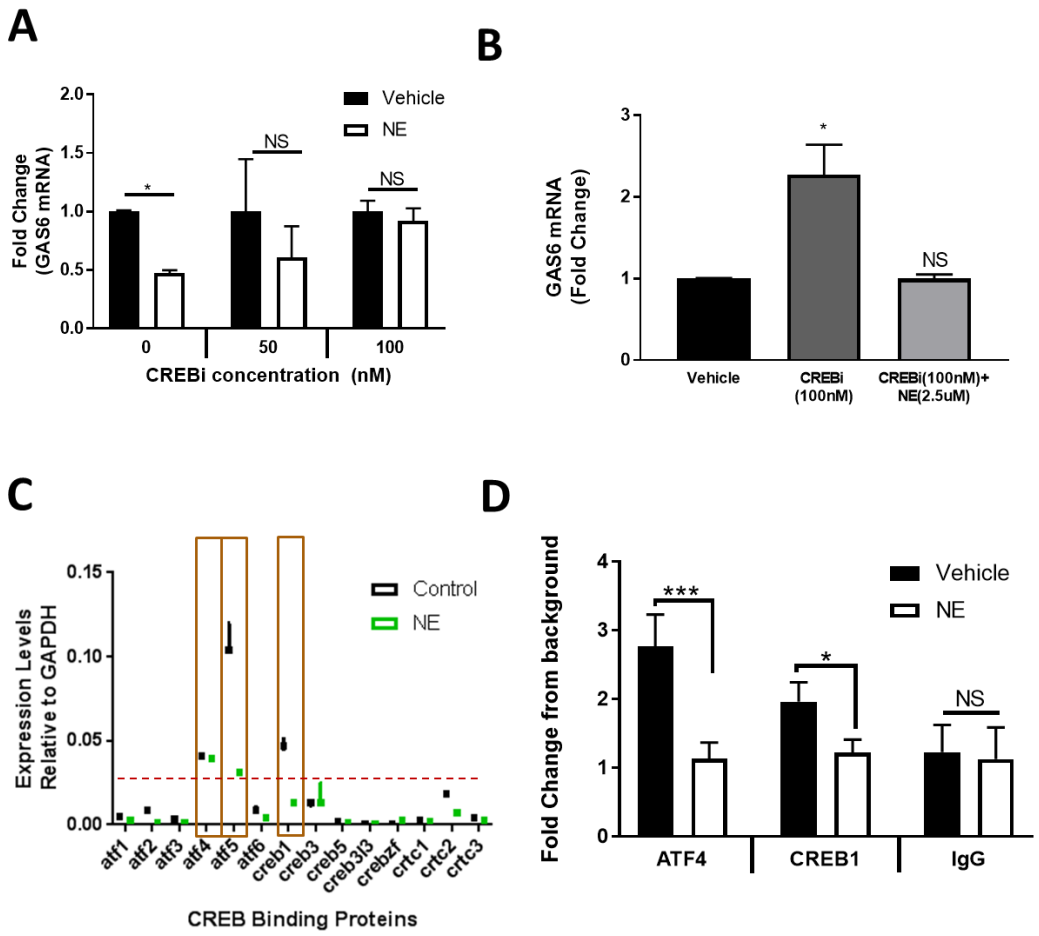

Figure 5.1: NE decreases CREB binding protein ATF4 in osteoblasts. A)Dose dependent CREB inhibition of NE-stimulated GAS6 downregulation. B) High dose of CREB inhibition increases GAS6 mRNA expression. C) PCR panel of CRE binding proteins. Boxes indicate factors expressed above 1% of GAPDH level. D) CHiP showed decreased binding of ATF4 and CREB1 to the GAS6 promoter after GAS6 stimulation

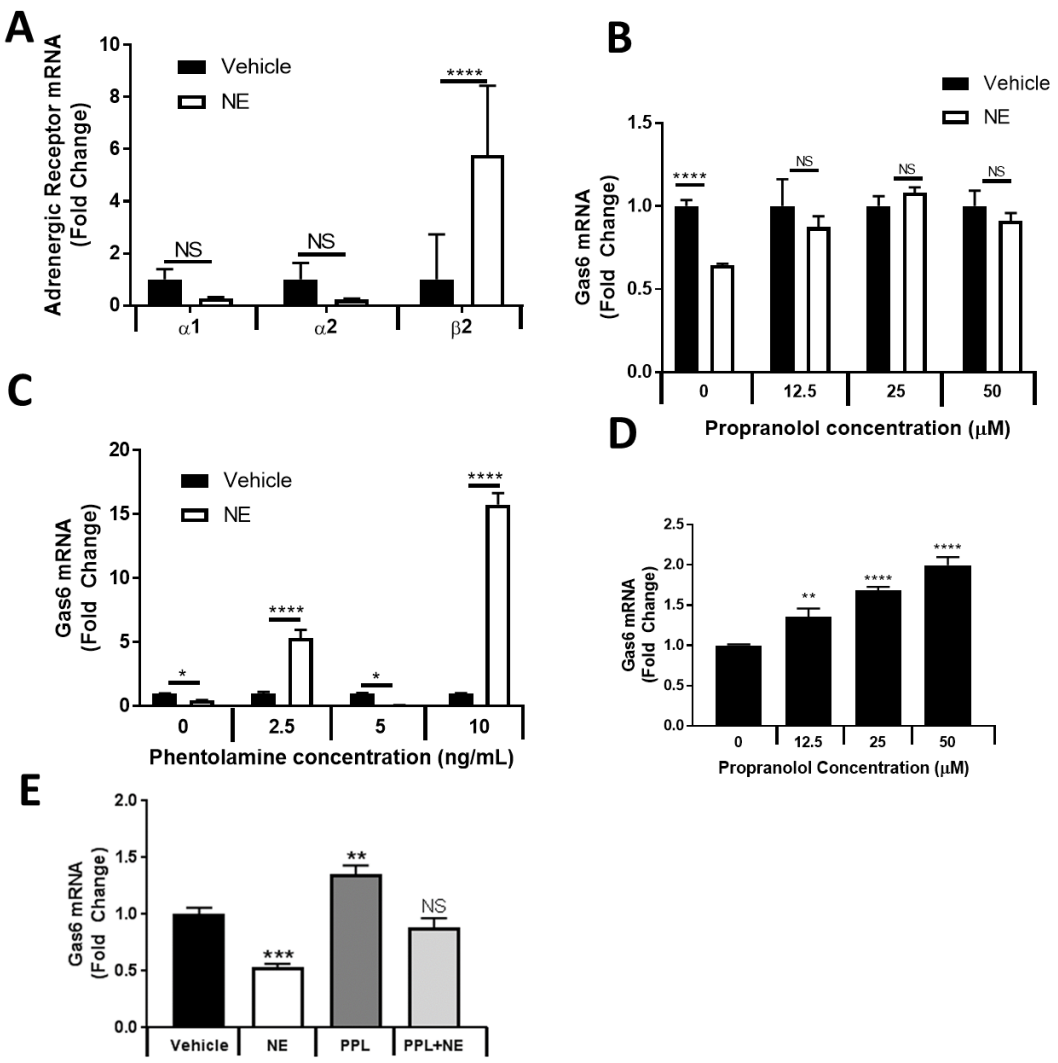

Figure 5.2: Signaling of NE occurs through beta adrenergic receptors in osteoblasts A)The β2 adrenergic receptor was most strongly associated with NE stimulation B) Dose dependent inhibition of GAS6 downregulation by propranolol. C) Increasing doses of phentolamine caused mixed changes in NE-mediated downregulation of GAS6. D) Increasing doses of propranolol increased GAS6 mRNA expression in MC3T3 cells. E) The effect of propranolol on GAS6 expression extended to OBs isolated from the marrow space of a Balb/c mouse.

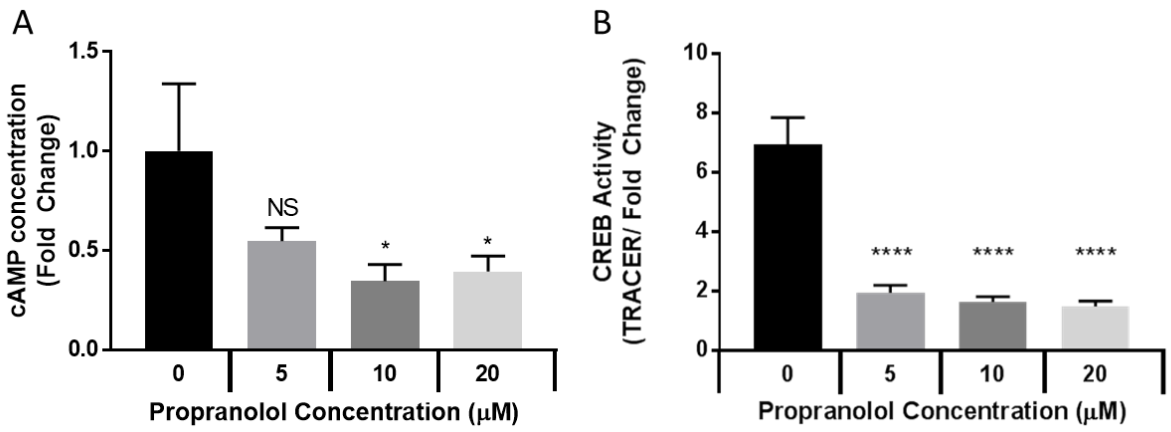

Figure 5.3: Propranolol decreases intracellular cAMP concentration. A) Propranolol decreased relative changes in cAMP concentration after NE stimulation in a dose dependent manner. B) Propranolol blocked the effects of NE on CREB activation by luciferase assay. * $p<0.05$, **** $p<0.001$.

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Chapter 6

Future Work and Conclusions

Future Work

This dissertation established a link between adrenergic signaling, PCa reactivation and GAS6 was established for DTCs in the bone. One of the most intriguing questions raised by these experiments is the role of GAS6 in normal bone homeostasis, and how this signaling pathway may be responsible for aberrant signaling other diseases that affect the bone marrow. In order to begin a preliminary investigation into this topic, some brief characterization of the long bones of GAS6$^{-/-}$ mice.

Hematoxylin and eosin staining of femur sections from GAS6$^{-/-}$ mice showed increased adiposity compared to age-matched wild-type (WT) mice (Figure 2A). Immunohistochemistry confirmed increased localization of adipose markers (Perilipin-1) in the femur sections of GAS6$^{-/-}$ mice (Figure 1B). Osmium staining of these bones further demonstrated increased adiposity to be observed and quantified through μCT scan (Figure 6.1A). The percent of bone volume attributed to adipose significantly increased in the samples isolated from the GAS6$^{-/-}$ animals compared to WT, from 4% to 22% in the proximal region and from 1% to 9% in the growth plate region of the tibia (Figure 6.1B).

Further studies using μCT bone scans demonstrated decreased trabeculation of the long bones in GAS6$^{-\backslash-}$ mice (Figure 6.2A), and decrease in the bone volume/total volume ration (Figure 2B), potentially indicating an increase in osteoclastic activity in

these bones. TRAP staining of sections of WT and GAS6$^{-/-}$ mice femurs (Figure 6.2C, 6.2D) indicated an increase in osteoclast number at these sites in the GAS6 deficient bone. The number of osteoclasts per field nearly doubled (from 6 in the WT to 11 in the GAS6$^{-/-}$ mice, (Figure 6.2C).

Together, these experiments suggest a second mechanism where by GAS6 levels may have an indirect impact on DTC fate. This conclusion is derived from the observations of altered bone homeostasis, encompassing the increased adiposity in the GAS6$^{-/-}$ bones along with increased osteoclast number. Increased osteoclast activity has long been associated with bone metastasis (1). The relationship between bone marrow adiposity and PCa dormancy is less well understood, however some recent works suggests these cells promote a pro-metastasis microenvironment (2). Increased bone adiposity is a hallmark of several metabolic diseases, including diabetes (3,4) and osteoporosis (5), both of which are also associated with decreased bone strength and volume. Interestingly, diabetic patients have been found to have decreased serum GAS6 (6), suggesting a potential link between these phenotypes. Treatments for diabetes such as metformin have been found to improve cancer outcomes in patients with prostate cancer (7,8), and further evidence exists relating untreated diabetes to increased mortality (9). Clearly further study into the mechanism is needed, but one potential link could be related to changes in GAS6 within the bone leading to decrease proliferative potential of DTCs and relatively fewer metastases in these patients. These results represent a promising future direction for this project that would yield important insights into the connections between bone homeostasis and quiescent stability in prostate cancer disseminated tumor cells.

Conclusions

The work in this dissertation focused on the connection between sympathetic signaling and reactivation of PCa DTCs in the bone marrow. Specifically, this work focused on the action of norepinephrine, which had been previously shown to mobilize hematopoietic stem cells in a similar microenvironment (10). The first aim of these studies was to establish if adrenergic signaling could in fact stimulate cell cycle re-entry of quiescent PCa cells. Two *in vitro* models, serum starvation and OB coculture, both supported the hypothesis that adrenergic signaling promoted proliferation in quiescent PCa cells. These results were measured through traditional proliferation assays (e.g. Ki67 stain) along with cell cycle reporters (FUCCI vectors) and western blots for cell cycle mediators, all of which supported the conclusion that NE stimulation can cause cell cycle re-entry. Additionally, we examined two different “native” bone marrow environments, one consisting of an *ex vivo* culture of a murine femur and a second using an intratibial injection in a living mouse. Both of these models also supported the hypothesis, leading to the conclusion that adrenergic signaling can lead to cell cycle re-entry.

The focus of the next aim was to identify indirect factors that influenced cell cycle re-entry. The previous experiments had demonstrated direct effects of adrenergic signaling on cell cycle mediators p21, p27 and ERK. Additionally, qPCR had suggested adrenergic signaling affected dormancy-involved cytokines in OBs, however a thorough characterization was lacking. Transcription factor analysis identified three factors (ATF1, E2F, and RAR) involved in reactivation; all of these factors had been previously implicated in NE signaling and cell cycle arrest. A subsequent dot blot and bioinformatic analysis identified GAS6 as a likely mediator of the indirect action of NE on osteoblasts. GAS6

was found to alter all three of the essential transcription factors and also be downregulated consistently across several models of OB and bone marrow environments. Testing the effects of NE on the cell cycle state of PCa cells cultured with either OBs lacking GAS6 expression or in a $GAS6^{-/-}$ *ex vivo* model found no effects of adrenergic signaling on the cell cycle. Thus, the conclusion was made that GAS6 mediated the indirect of effects of NE on the cell cycle of PCa cells in marrow.

Finally, a mechanism through which NE regulated GAS6 was sought, as this would provide a potential clinical route to targeting these interactions (Figure 6.3). ATF4 and CREB1 were both found to bind to the GAS6 promoter. NE stimulation significantly decreased their residency at this site, with ATF4 more strongly downregulated compared with CREB1. This led to the conclusion that ATF4 was the primary transcriptional mediator of the NE-GAS6 pathway. Testing of pharmacological blockades of the adrenergic receptors found that propranolol, a β-adrenergic receptor antagonist, could successful block the downregulation of GAS6 by NE in a dose dependent manner. These results agree with some clinical observations suggesting propranolol may be an effective drug to treat some cancer patients. These studies potentially suggest a patient cohort that may benefit from targeted therapy that aims to limit the downregulation of GAS6 by adrenergic signaling, specifically PCa patients with chemical recurrence but no measurable metastatic disease.

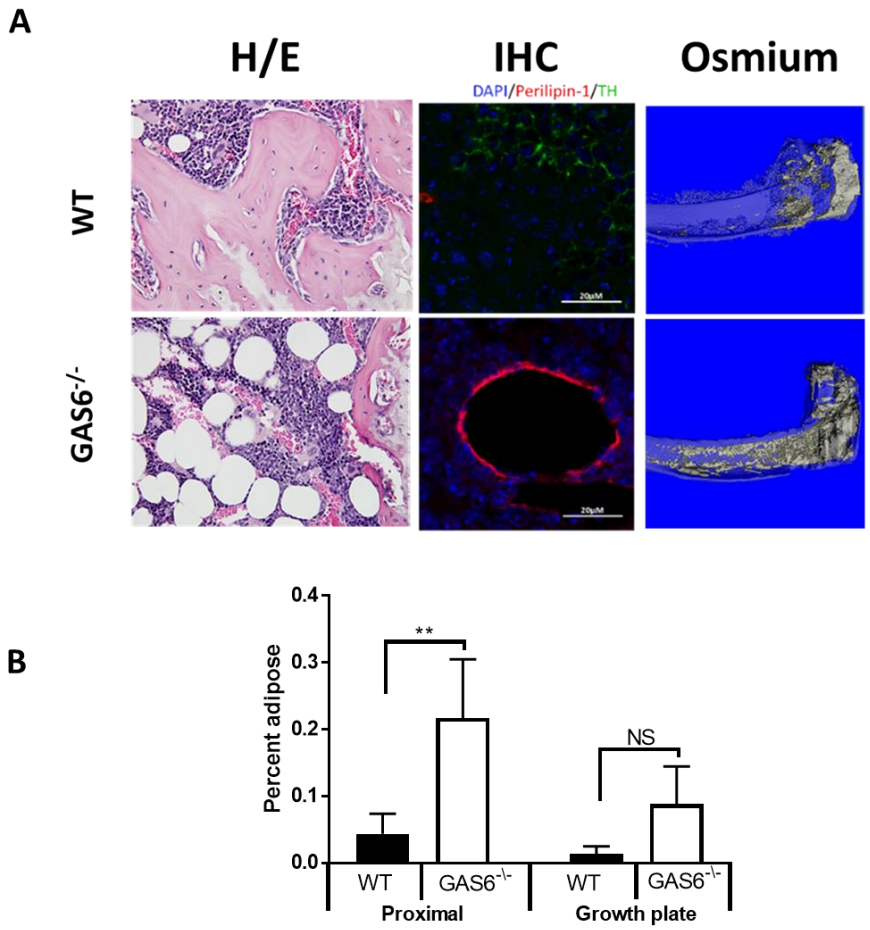

Figure 6.1: GAS6 knockout mice exhibit abnormal bone phenotype A: Representative histology, immunostaining and osmium-staining µCT for WT and KO bones shows increased adiposity B: Quantification of osmium staining shows statistically significant increases in adiposity after GAS6 knockout. GKO = GAS6$^{-/-}$ mouse. WT = wild type mouse.

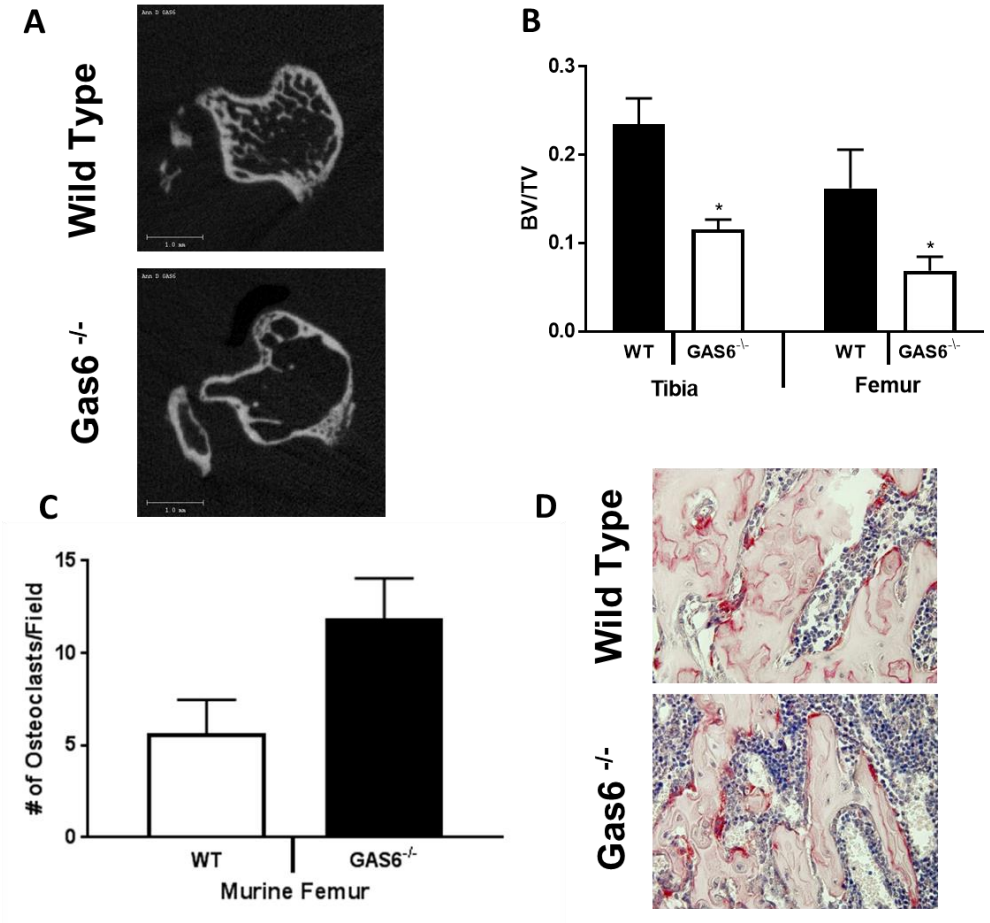

Figure 6.2: GAS6 knockout mice exhibit low bone volume and increased bone turnover A: µCT cross section of WT and GAS6KO femurs B: Bone volume/total volume (BV/TV) of femurs and tibias of WT and KO animals C: Osteoclasts per image from TRAP staining of WT and KO animals D: Representative TRAP staining of bone sections for WT and KO animals

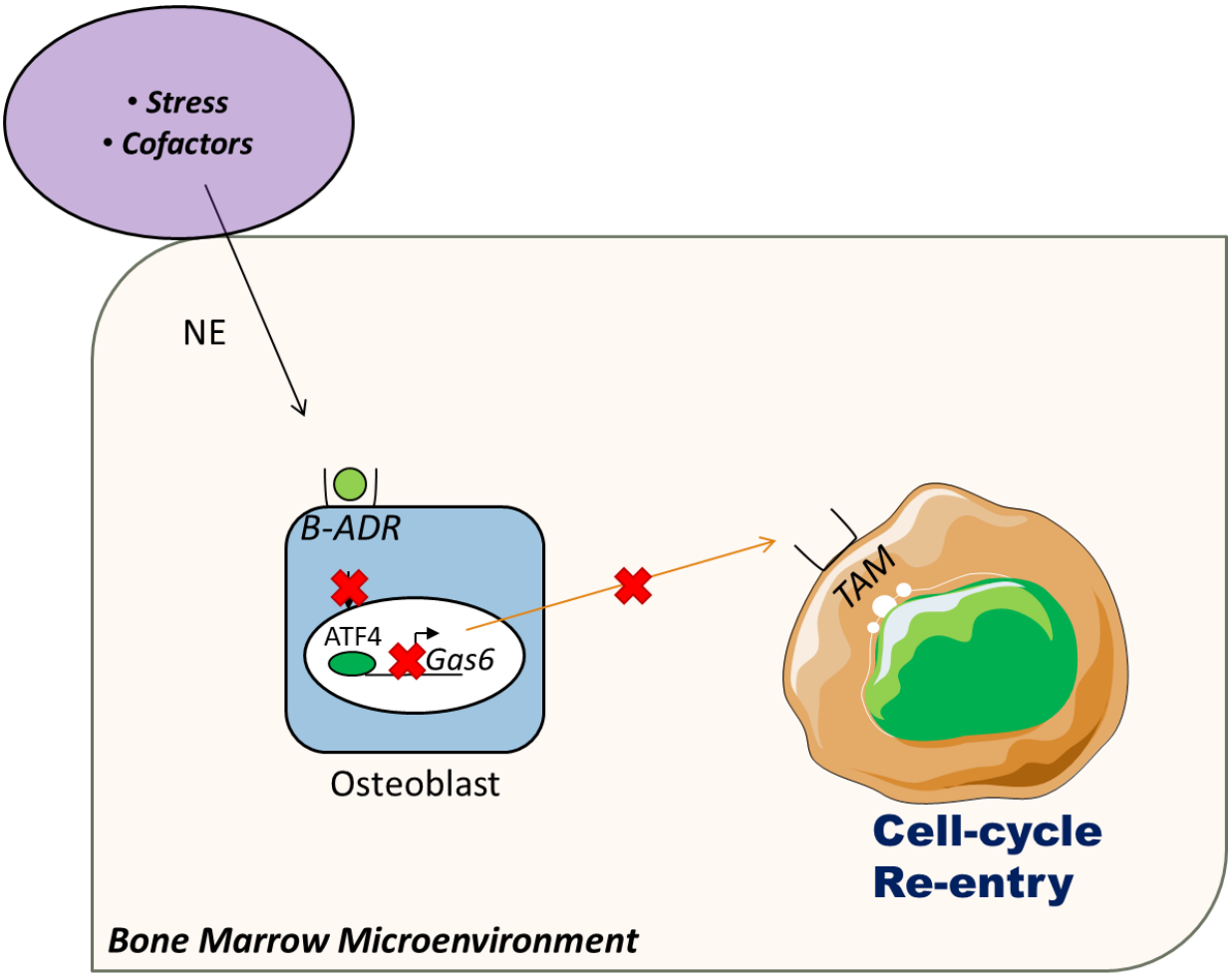

Figure 6.3 Model of the relationship between adrenergic signaling and PCa cell-cycle re-entry. NE can cause reactivation of dormant PCa cells through downregulation of GAS6, which proceeds through β-adrenergic and ATF4 signaling.

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