Adrenergic Blockade Promotes Maintenance of Dormancy in Prostate Cancer Through Upregulation of GAS6

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ABSTRACT

Men diagnosed with localized prostate cancer can develop metastases many years after initial treatment, resulting in a poor prognosis. The purpose of this study was to investigate the mechanisms by which signaling through norepinephrine (NE) may incite relapse of quiescent prostate cancer. We used an unbiased bioinformatics pipeline to examine mechanisms for recurrence related to sympathetic signaling in the bone marrow. A transcription factor cell array identified ATF1, RAR, and E2F as key nodes in prostate cancer cells exiting quiescence through adrenergic signaling. Subsequent secretome analysis identified GAS6 as affecting activity of these three factors, leading to cell cycle reentry. GAS6 expression was downregulated in osteoblasts through activation of the cAMP pathway and was targeted *in vitro* and *in vivo* using pharmacological agents (propranolol and phentolamine). Propranolol increased expression of GAS6 by osteoblasts, and phentolamine significantly inhibited expression. Propranolol treatment was sufficient to both increase GAS6 expression in marrow osteoblasts as well as eliminate the effects of NE signaling on GAS6 expression. These results demonstrate a strong correlation between adrenergic signaling, GAS6 expression, and recurrence in prostate cancer, suggesting a novel therapeutic direction for patients at high risk of metastasis.

Introduction

Prostate cancer (PCa) remains the most common noncutaneous cancer in men and is the result of about 26,000 deaths per year in the United States, almost all of which are due to metastatic disease [1]. Upon dissemination to secondary sites, such as the bone, PCa cells can undergo one of three fates: 1) apoptosis due to incompatibility with the microenvironment; 2) colonization and proliferation, resulting in metastatic tumors; or 3) cell cycle arrest and dormancy [2]. The mechanisms regulating dormancy of these disseminated tumor cells (DTCs) when they enter the bone marrow or lymph node microenvironments have been a considerable source of scientific debate [3]. Late recurrence (more than 5 years after curative therapy) accounts for 20% of all recurrences, and the presence of DTCs in marrow is a poor predictor of clinical outcomes [4,5]. However, the signaling mechanisms within the bone marrow microenvironment which control proliferation of DTCs are poorly understood.

We have previously demonstrated that PCa DTCs replace resident stem cells in marrow [6] and are subject to similar signaling within the bone marrow microenvironment. Extracellular signaling from soluble factors such as GAS6 [7], TGFβ2 [8], BMP7 [9], or WNT5A [10] all can induce DTC dormancy through a variety of intracellular signaling mechanisms. Intracellular factors, such as signaling from p38 MAPK, ERK1/2, or NR2F1 [11], 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 [12]. 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 showed that adrenergic signaling through norepinephrine (NE) may drive dormant DTCs to reenter the cell cycle [13]. Adrenergic signals can act directly on primary tumor cells to promote their proliferation and metastasis [14], and circadian fluctuations in NE within the bone marrow have been shown to mediate hematopoietic stem cell

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activation and entry into circulation [15]. For dormant PCa cells, intrinsic and extrinsic models of dormancy suggest that adrenergic signaling has both direct activity on DTCs as well as indirect activity on their microenvironment, which may also alter the proliferative phenotype of these cells. For a direct effect, NE can alter expression of several key cell cycle regulators including p21, p27, p38, and ERK, which are known to regulate cell cycle reentry. However, the mechanisms regulating the indirect action of NE on the microenvironment remain largely unknown. This study sought to identify the mechanisms through which adrenergic signaling leads to proliferation of quiescent tumor cells in marrow. By identifying how NE alters the production of niche-derived factors which regulate DTC dormancy, we hope to elucidate opportunities to regulate DTC dormancy for therapeutic gain.

Methods

Cell Culture

Human PCa cell lines (PC3) were obtained from American Type Culture Collection (Rockville, MD). The murine preosteoblastic cell line MC3T3-E1 subclone 4 was obtained from American Type Culture Collection (CRL-2593). These cells 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 (GEMINI Bio-Products, Sacramento, CA) and 1% penicillin-streptomycin (Life Technologies) and maintained at 37°C, 5% CO_2, 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 [16]. Viral supernatant was collected after 48 hours in culture and concentrated using PEG-it (Systems Biosciences). Virus was resuspended in phosphate-buffered saline and stored at −80°C until use.

Reporter Arrays

A transcriptional activity cell array (TRACER) was used to identify transcription factors (TFs) leading to adrenergic signaling-mediated reentry into the cell cycle as previously described [17–19]. For co-culture experiments, PC3 cells were infected with a library of reporter viruses, cultured for at least 2 days, and subsequently plated at a low density onto a confluent monolayer of MC3T3-E1 cells in a black 384-well plate. Three days later, 2.5 μM NE was added to the culture, and TF activity was measured after 2, 4, 6, 8, 24, 48, and 72 hours using an IVIS Spectrum (Perkin Elmer). For cytokine induced activity, cells were infected 2 days prior to assay and treated with either 500 ng/ml GAS6 or 2 ng/ml FLT3LG and measured every 2 hours for 8 hours. TRACER data were processed as previously described [19]. Briefly, activity measurements were background subtracted, normalized to the empty control reporter, and log2 transformed prior to analysis [20]. 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 or mean ± standard error, where appropriate. Statistical analysis was performed using the *limma* R package [21]. *P* values were adjusted using the Benjamini-Hotchberg method for false discovery rate correction [22].

Network Analysis

NTRACER (networks for TRACER) was used to analyze connections between dynamic TF activity measurements, as previously described [19,23]. This method uses a combination of inference methods (PLSR [24], similarity index [25], linear ordinary differential equations based on TIGRESS [26], random forest [27], ARACNE [28], CLR [29], MRNET [30]) 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* [31].

Dot Blot

MC3T3-E1 cells were cultured under standard conditions until confluent, at which point they were treated with either 2.5 μM NE or vehicle control. Cells were cultured under these conditions unperturbed for 48 hours, and protein was collected for dot blot analysis. Lysates were prepared in cOmplete lysis M (Roche #04719956001) supplemented with proteinase inhibitor Mini cOmplete Tablets (Roche #04705378) and phosphatase inhibitor PhosSTOP EASYpack Tablets (Roche #04906837001). Protein concentration was calculated using the BCA protocol (Sigma). Twenty micrograms of total protein was added to dot blot nitrocellulose sheets, and subsequent incubation and wash steps were followed according to manufacturer's 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). Differences between treatment groups were evaluated using two-way analysis of variance (ANOVA) with false discovery rate correction.

Quantitative Polymerase Chain Reaction (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 the 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), ATF4 (Hs00909569_g1; Mm00515325_g1), Flt3l (Hs00957747_m1; Mm00442801_m1), and CRE binding (CREB)1 (Hs00231713_m1; Mm00501607_m1). GAPDH (Hs02786624_g1; Mm99999915_g1) was used as internal controls for the normalization of target gene expression.

CREB Inhibition

MC3T3-E1 cells were cultured under standard conditions with a general CREB inhibitor (Millipore Sigma, cat. #538341) at the indicated concentrations for 48 hours. RNA was processed using an RNEasy kit (Qiagen), converted to cDNA, and assayed through quantitative PCR using above-described methods.

Immunoprecipitation

MC3T3-E1 cells were expanded until confluent and treated with either 2.5 μM NE or vehicle control for 48 hours. DNA was subsequently extracted. All manufacturer instructions were subsequently followed for chromatin immunoprecipitation (CHiP) assay (Qiagen, cat. #334471). Antibodies were used for ATF4 (Cell Signaling, cat. #D4B8) and CREB1 (Cell Signaling, cat. #9197T). Primers were used for ATF4 (ThermoFisher, cat. #Mm00515325) and CREB1 (ThermoFisher cat. #Mm00501607). Data are presented as differences in promoter region amplification relative to IgG control and were statistically compared using two-way ANOVA with Sidak's multiple-comparisons test.

In Vivo Regulation of GAS6

Marrow osteoblasts were isolated using the method described by Balduino et al. [32]. C57BL6/J mice were treated with 1 mg/kg NE, 3 mg/kg propranolol, or combination for 3 days. Femurs and tibias were

bilaterally isolated at the end point of the study. Marrow from these bones was flushed with $1\times$ phosphate-buffered saline three times, sequentially until the bone was lucent and white and no marrow color remnants remained. Then, stromal layer was flushed with RLT + β-mercaptoethanol into a cell shredder column. RNA was processed using an RNeasy Kit (Qiagen) and above-described methodology for quantitative PCR.

Survival Data

Data from a previously defined cohort of consecutive patients with clinically localized prostate cancer [33] who underwent radical prostatectomy at the University of Michigan were assessed and augmented with internal registry data to capture medication use (IRB #HUM00162207). Time to recurrence was measured from date of prostatectomy to the time of measured elevated PSA (>2 ng/ml measured more than 6 weeks after surgery). Patients were placed in the adrenergic antagonist cohort if they had been prescribed adrenergic antagonists at any point prior to recurrence or censoring. Specific drugs were classified as α1, β1, nonselective β, or nonselective β/α1 antagonists based on manufacturer-described specificities for different compounds. Association of adrenergic use in prostate cancer patients who underwent prostatectomy with biochemical recurrence-free survival was modeled with a time-dependent Cox proportional-hazards model.

Results

Adrenergic Signaling Can Reactivate Dormant Cells Through RAR and ATF1 Signaling

TRACER was used 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 preosteoblasts (MC3T3-E1 cells) serves as surrogates for the osteoblasts in the bone marrow, while sparsely plated PC3 cells are used as a model of PCa dormancy (Figure 1A). TRACER can be used to easily 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. Transcriptional activity was monitored for 3 days after administration of NE to the MC3T3-E1 cells, during which significant alterations in TF activity were observed. Specifically, 16/66 TFs had their activity altered over 3 days of NE stimulation, leading to cell cycle reentry (Figure 1B).

We used NTRACER to discern central TFs controlling reactivation of PCa cells [19,34]. This network analysis identified five factors as central to reactivation: E2F, RAR, ATF1, CMYC, and STAT4 (Figure 1C). Of these factors, only ATF1, RAR, and E2F were significantly different than the no NE control during the experiment, leading to the conclusion that these three factors were primarily responsible for the observed effects. All three reporters showed upregulated activity after 72 hours of treatment with NE, with additional significant downregulation of ATF1 at 24 hours and upregulation of E2F at 24 hours.

NE Causes Downregulation of Dormancy-Inducing Cytokine GAS6

Adrenergic signaling may reactivate dormant PCa cells through both direct effects on the cells themselves and 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 1D). However, despite ATF1 being central to reactivation (Figure 1C), co-culture was found to significantly limit the immediate effects of NE stimulation (Figure 1D). We therefore focused on identifying indirect mechanisms of PCa reactivation by studying how adrenergic signaling alters cytokine secretion by osteoblasts (MC3T3-E1 cells) using a dot blot comprised of 111 different mouse cytokines (Figure 2, A and B). A total of 12 cytokines were differentially expressed in the NE-treated group compared with vehicle control (P < .05), with 1

upregulated cytokine (CCL5) and 11 downregulated cytokines. Downregulated cytokines included GAS6, M-CSF, and osteopontin (Figure 2B).

We next employed TRACER to identify transcriptional regulators of NE signaling in MC3T3-E1 cells (Figure 2C). 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 upregulated relative to untreated control. NTRACER was subsequently employed to discover connections between dynamic TF activity during adrenergic stimulation to identify the predominant factors regulating the impact of NE on osteoblasts. CREB protein family was identified as mediating the response to NE in MC3T3-E1 cells through eigenvector centrality of the inferred network (Figure 2C).

Next, we examined the ENCODE database to identify canonical CREB1 binding sites promotor regions of the 12 cytokines identified by the dot blot which were altered in osteoblasts following NE treatment. From this database, we identified CREB1 binding sites in the promotor regions of 5 of the 12 factors (GAS6, FLT3LG, IGFBP6, PTX3, and VCAM1) identified in the dot blot. Of these, only GAS6 and FLT3LG had canonical CREB binding sites in their promotor regions (Figure 2C).

We screened the TF reporter library against signaling from soluble GAS6 or FLT3LG in PC3 cells and compared these results to significantly altered factors from the reactivation experiment (Figure 1) to validate a role for either cytokine in reentry into the cell cycle in co-culture (Figure 2D). GAS6 signaling significantly altered activity of reporters in the CREB protein family (downregulated CREB and ATF4, upregulated ATF1), along with others, for a total of 12 factors of the total 43 screened. FLT3LG signaling significantly altered 8 of 43 factors. Of these factors, GAS6 and FLT3LG had alterations in NANOG and SRF in common, however, in opposite directions (upregulation of SRF and downregulation of NANOG for GAS6 and the inverse for FLT3LG). FLT3LG 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, suggesting that GAS6 was a prime candidate for mediating indirect effects of NE on dormant PCa cells in co-culture (Figure 2D).

We next sought to confirm transcription of GAS6 by TFs from the CREB protein family. Use of a general CREB inhibitor at increasing doses abrogated the effects of NE on GAS6 mRNA expression in MC3T3-E1 cells during 48 hours of treatment (Figure 3A). We hypothesized from this result that treatment of MC3T3-E1 cells with NE decreased CREB protein binding to the GAS6 promoter. Quantitative reverse-transcription PCR of the CREB factor family following NE treatment found that three family members (ATF4, ATF5, and CREB1) were significantly expressed in MC3T3-E1 cells (>1% GAPDH level, dashed line in Figure 3B), while four factors (ATF2, ATF5, CREB1, and CRTC1) had mRNA expression significantly altered by NE stimulation. We selected the two factors that were both significantly expressed and included in the TRACER analysis, ATF4 and CREB1, to evaluate using CHiP [35]. Treatment of MC3T3-E1 cells with NE for 48 hours significantly decreased the binding activity for ATF4 by 59% (P < .001) and CREB1 by 38% (P < .05) at the GAS6 promoter region relative to an untreated control, indicating that NE treatment decreased binding of relevant CRE TFs at the GAS6 promoter (Figure 3C).

B-Adrenergic Signaling Leads to Downregulation of GAS6

Adrenergic signaling is pharmacologically targeted through adrenergic receptor inhibitors. We therefore sought to establish a relationship between these drugs and GAS6 expression in osteoblasts. PCR for adrenergic receptors in MC3T3-E1 cells showed expression of the α1, α2, and β2 adrenergic receptors and no expression of the β1 receptor. The β2 receptor was most strongly expressed and the only receptor whose expression was significantly altered by adrenergic stimulation (Figure S1). We examined the effects of escalating doses of both the general β-adrenergic receptor
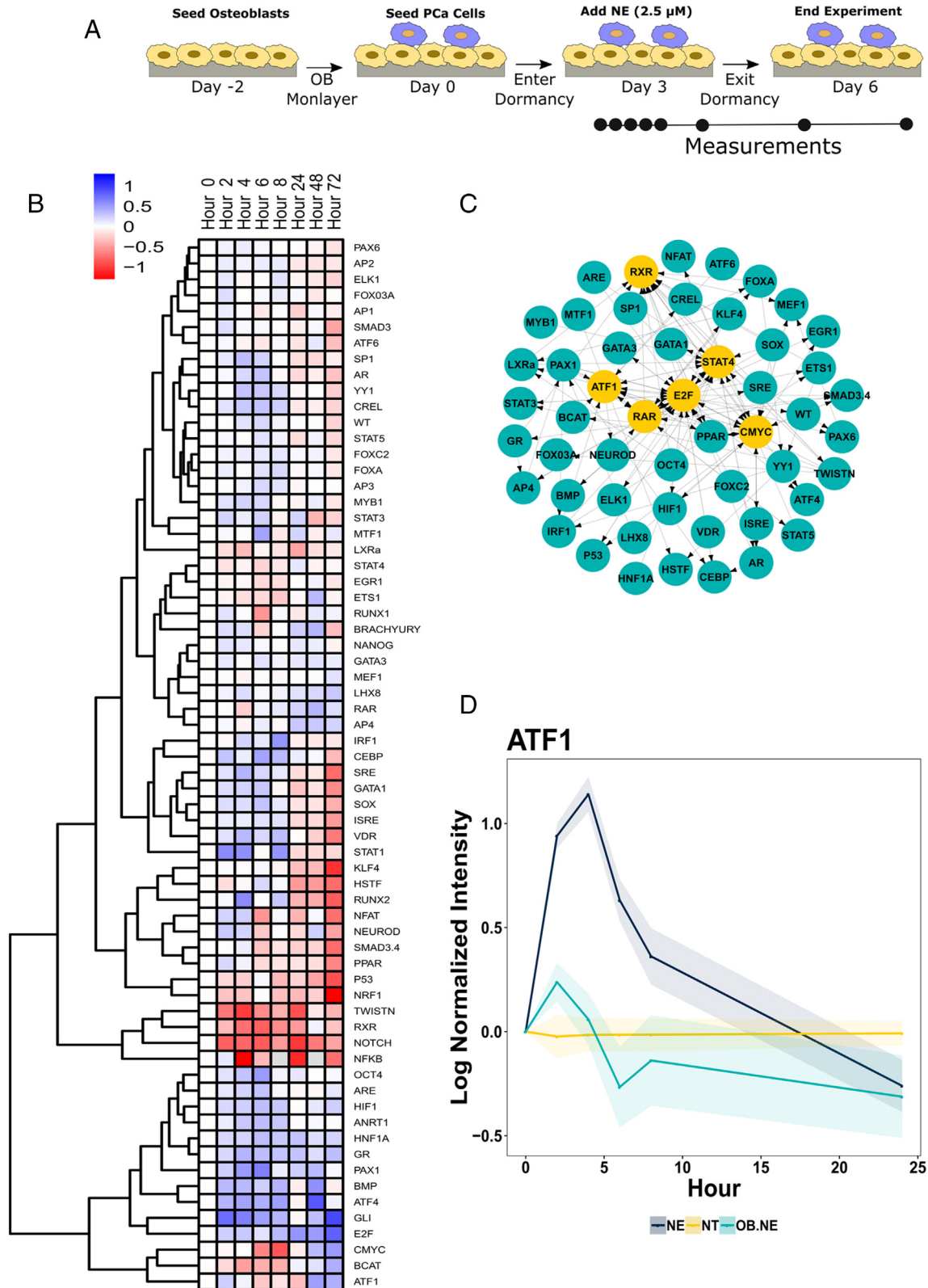
Figure 1. TRACER measures reactivation dynamics in quiescent PCa. (A) Schematic of TRACER experiment. (B) Hierarchical clustering of time course TF activity data. (C) Results from network analysis of TF activity data. Yellow nodes are in the top 10% by eigenvector centrality. (D) ATF1 activity in PC3 cells cultured alone (blue) or in co-culture with MC3T3 cells (green).

antagonist propranolol and the general α-adrenergic receptor antagonist phentolamine on the expression of GAS6 in MC3T3-E1 osteoblasts (Figure 4, *A* and *B*). Both antagonists altered GAS6 expression in a dose-dependent manner, however, in opposing directions. Propranolol increased

GAS6 mRNA expression up to a maximum of two times the vehicle control, while phentolamine decreased GAS6 mRNA to a minimum of 2% of the vehicle control. Propranolol additionally inhibited the effects of NE on GAS6 mRNA expression in a dose-dependent manner (Figure 4C), while
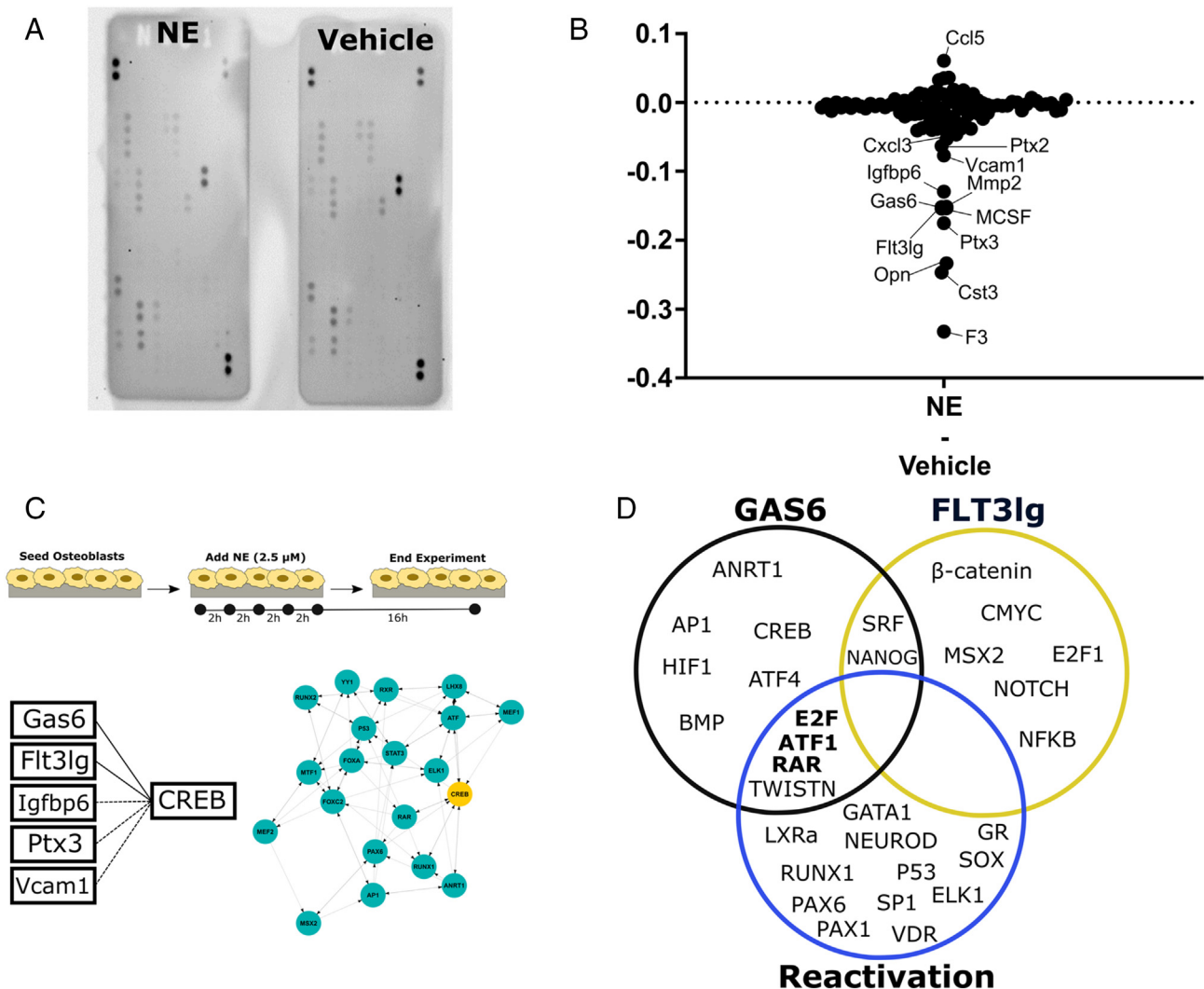
Figure 2. GAS6 mediates indirect effects of NE on dormant PCa cells. (A) Dot blot data comparing NE-treated MC3T3-E1 cells to vehicle control. (B) Difference in protein expression between NE- and vehicle-treated MC3T3 cells. Labeled points are statistically significant. (C) CREB (central to network, yellow) was found in the ENCODE database to bind to 5 of the 12 significant cytokines. Solid line denotes canonical binding site; dashed line is noncanonical binding. CD Venn diagram of significantly altered TF activity for reactivation (bottom), GAS6 stimulation (left), or FLT3LG stimulation (right).

phentolamine did not show a consistent effect on GAS6 downregulation by NE signaling (Figure S2).

Since the effects of adrenergic signaling on GAS6 expression were mediated by CREB (Figures 2 and 3), we sought to confirm inhibition of CREB activation by propranolol following NE treatment. Here, CREB activity was monitored by luciferase assay following adrenergic stimulation in the presence of propranolol, a nonselective β-adrenergic signaling antagonist (Figure 4, D and E). The concentration of cAMP following adrenergic signaling was significantly decreased through addition of propranolol in a dose-dependent manner (Figure 4D), 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 4E).

We further examined the ability of β-adrenergic blockade to inhibit downregulation of GAS6 by NE in marrow osteoblasts _in vivo_ (Figure 4F). Similar to the previous _in vitro_ observations (Figure 4C), systemic administration of NE to C57BL/6 mice downregulated the expression of GAS6 in marrow osteoblasts. Interestingly, administration of propranolol alone increased GAS6 mRNA expression by 36% over the vehicle control, consistent with _in vitro_ observations (Figure 4A). Administration of both NE and propranolol to these mice led to no statistically significant change in GAS6 mRNA expression relative the vehicle control. Together, these results

indicate that propranolol may block the major activator of adrenergic signaling, cAMP and its downstream effects on transcription.

Discussion

Patients with clinically localized prostate cancer can experience relapse years after initial treatment, the cause of which is unknown. TF analysis of quiescent PCa cells reactivated through adrenergic signaling suggested that three factors (ATF1, RAR, and E2F) were primarily responsible for the change in phenotype. The E2F family of TFs is responsible for cell cycle regulation and DNA synthesis [36]. 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 [11]. Further, RAR has been demonstrated to be involved in quiescence of hematopoietic stem cells [37], which is interesting in that we have previously demonstrated that PCa DTCs closely mimic HSC biology in the bone marrow [6]. ATF1 is a canonical regulator of cAMP signaling, which is the primary means of signal activation following adrenergic stimulation. Together, these three factors appear to be critical central regulators of quiescence and reactivation of PCa cells.
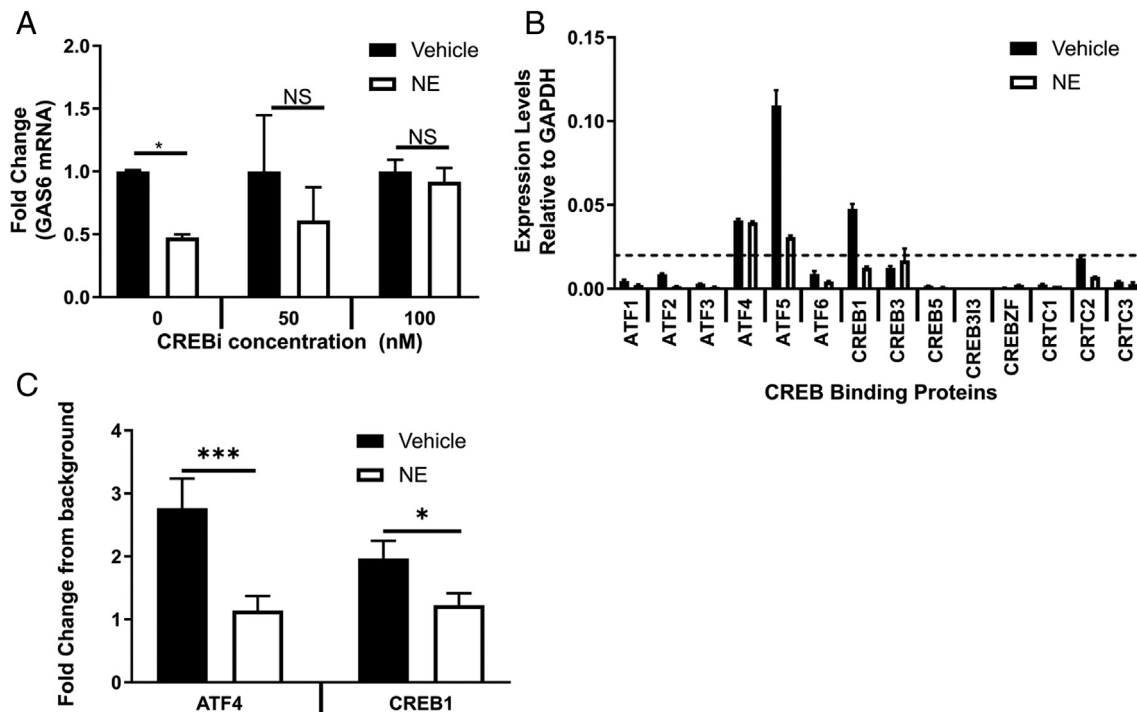
Figure 3. ATF4/CREB1 bind to the GAS6 promoter and control NE response. (A) Dose-dependent CREB inhibition of NE-stimulated GAS6 downregulation. (B) PCR panel of CREB proteins. Boxes indicate factors expressed above 1% of GAPDH level. (C) CHiP showed decreased binding of ATF4 and CREB1 to the GAS6 promoter after GAS6 stimulation. * = $P < .05$, *** = $P < .001$, from two-way ANOVA (A) or t test (C).

We also found that downregulation of GAS6 expression by NE resulted in significant changes in the bone microenvironment that are permissive for reactivation of dormant PCa cells. The dot blot data identified 12 cytokines that were altered by NE stimulation, of which GAS6 was ultimately proven through analyses of TFs as stimulating significant changes in E2F, ATF1, and RAR activity. GAS6 has been previously shown to induce dormancy through signaling primarily through its receptor AXL [7]. The fact that GAS6 was selected through an unbiased bioinformatic analysis strengthens these results as previous work demonstrates that GAS6 can activate tumor cell dormancy both *in vitro* and *ex vivo* [6,7,38,39].

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 β2 receptor knockout mice have a bone phenotype of increased bone formation and decreased bone remodeling compared to the wild-type counterparts [40]. We found decreased ATF4 binding at the GAS6 promoter through CHiP following adrenergic stimulation. ATF4 is likely bound to the GAS6 promoter under basal conditions, which facilitates GAS6 expression in OBs prior to NE stimulation. Adrenergic signaling may 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 TF molecules to activate GAS6. While we did not examine other DNA binding sites, future experiments in which protein-DNA interactions were directly imaged during NE stimulation may yield greater insight into the molecular mechanism which regulates GAS6 expression following NE stimulation.

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 [41]. Investigation of the effects of FLT3L on dormant tumor cells did not suggest that signaling through FLT3 would alter the proliferative response of PCa tumor cells alone. It is possible that FLT3 signaling in combination with other factors may produce different responses. 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 [7]. 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.

These studies suggested that β-adrenergic signaling was primarily responsible for connecting NE to GAS6 downregulation. Previously, we have shown that metastasis, lodging, and dormancy of prostate cancer within the confines of the hematopoietic stem cell niche occur through binding of annexin-2 receptors on adjacent osteoblasts within the hematopoietic stem cell niche [42]. 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 et al. [15] 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. Our 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 our investigations focused primarily on osteoblasts, which are a major source of dormancy-inducing GAS6 in the bone marrow. Expression of GAS6 in stromal cells may indeed be controlled through β3-adrenergic receptors, leading to the need for a nonselective β-adrenergic receptor antagonist to target all possible sources within bone marrow.

Pharmacologic methods for targeting adrenergic signaling in patients with hypertension have garnered attention as potential correlative factors for long-term success of PCa therapies [43–47]. The studies presented here suggested that 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 (Figure 4A). These results suggest that therapeutic manipulation of β-adrenergic may be an effective method for increase GAS6 signaling within the bone marrow and therefore may have benefit to control recurrence in
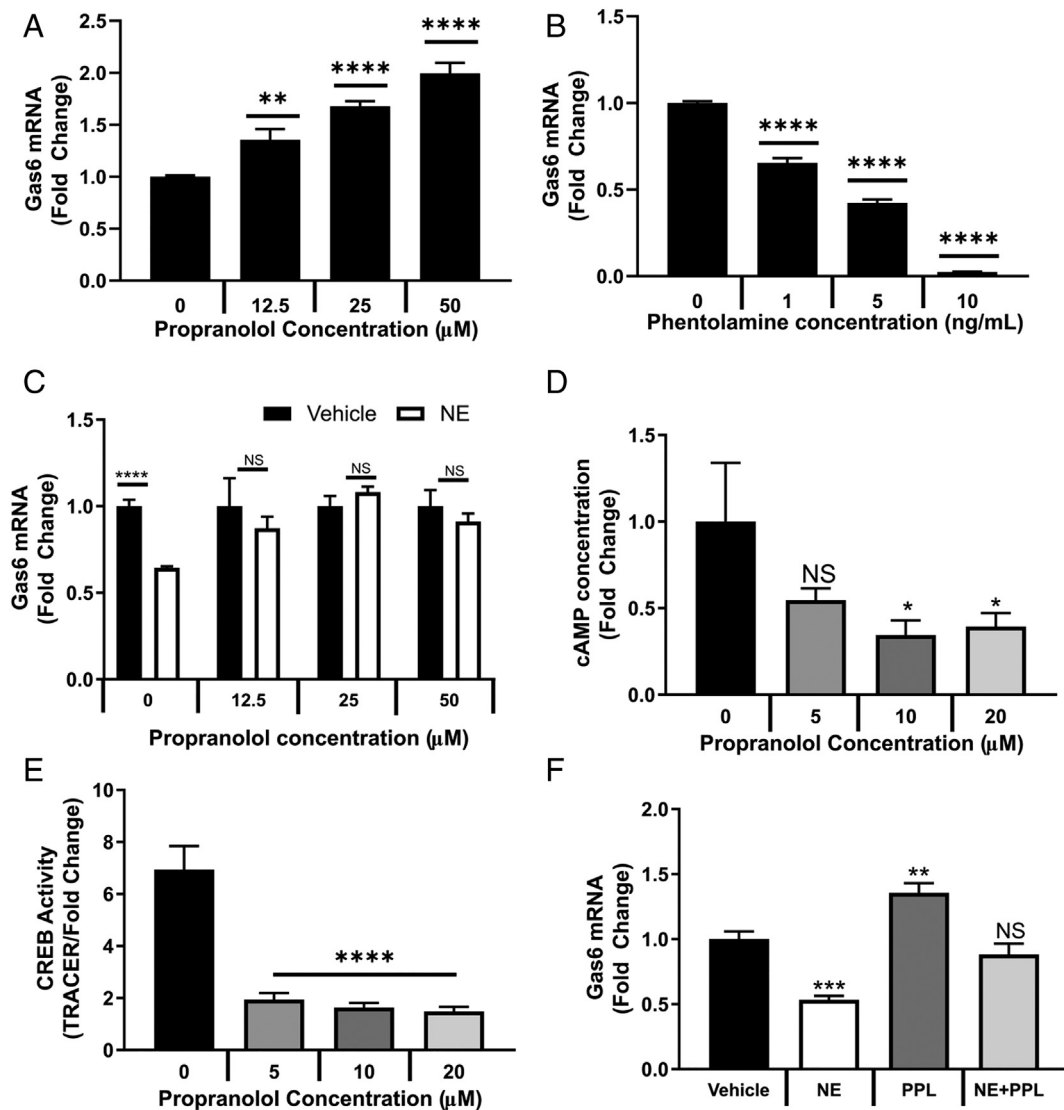
Figure 4. NE causes downregulation of GAS6 through β2 adrenergic receptor signaling. (A) Effects of propranolol concentration on GAS6 expression without NE treatment. (B) Effect of phentolamine concentration on GAS6 expression without NE treatment. (C) Dose-dependent inhibition of GAS6 downregulation by propranolol. (D) Increasing doses of propranolol decreased cAMP concentration. (E) Propranolol inhibited CREB activation by luciferase assay. (F) Mice administered NE exhibited downregulation of bone marrow osteoblast GAS6 mRNA, dependent on β-adrenergic signaling. * = $P < .05$, ** = $P < .01$, **** = $P < .0001$ from two-way ANOVA.

high-risk patients. We also observed a decrease in GAS6 mRNA expression following treatment with the pan α-adrenergic receptor antagonist phentolamine (Figure 4B). The α- and β-adrenergic receptor families have opposing effects on CREB protein activation [48], and our observations are consistent with a role of these proteins in connecting adrenergic signaling to GAS6 expression.

Our data suggested that PCa patients treated for localized disease who were also taking β2 antagonists would potentially be protected from recurrence. We attempted to test this hypothesis using a previously defined cohort of 2289 patients who underwent prostatectomy between November 1999 and June 2013 and not previously treated with hormone therapy [33]. This cohort represented the majority of PCa patients treated at the University of Michigan during this timeframe that fit the study parameters. Patient outcomes were analyzed for the effects of adrenergic antagonists on time to biochemical recurrence. We found that the sample size for patients taking adrenergic antagonists in this cohort was limited (Table S1). The overall hazard ratios from these data were consistent with our results, with a potential benefit for relapse-free survival for β2-antagonists (0.607, Table S2) and potential detriment from α-antagonists (1.271, Table S2); however, these

results were not statistically significant. Therefore, these results suggest that a prospective, multicenter study may be needed to fully elucidate the effects of adrenergic blockade on PCa recurrence.

In conclusion, we found that adrenergic signaling downregulates the cytokine GAS6 through β-adrenergic and CREB signaling, leading to reactivation of dormant prostate cancer cells. This mechanism can be targeted in patients at risk for metastatic recurrence, as this population showed increased relapse-free survival while taking adrenergic blockade medications. While we focused on the mechanisms of adrenergic-mediated reactivation in bone-metastatic prostate cancer, our results may be relevant to other tumor types in which cells lay dormant for years in the marrow, and may inform other cancer treatments involving GAS6 and its signaling pathways, such as those that target tumors that have developed AXL-mediated resistance [49–52].

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Declaration of Competing Interests

The authors do not have any conflicts of interest to disclose related to this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100781.

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