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*Original Article*

# Signaling mechanisms coupled to CXCL12/CXCR4-mediated cellular proliferation are PTEN-dependent

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**Abstract:** A key difference between normal and malignant prostate cells in vitro and in vivo is that both alleles of PTEN are largely intact in normal benign prostate glands and cultured epithelial cells, whereas one or both alleles of PTEN are mutant or deleted in the majority of prostate tumors and malignant prostate cancer cell lines. Intact PTEN suppresses phosphorylation of Akt downstream of PI3K activation in non-transformed cells whereas Akt phosphorylation is unimpeded in malignant cells that are often PTEN-deficient. We have previously shown that activation of the CXCL12/CXCR4 axis transactivates the EGFR to promote pro-proliferative signaling preferentially through the Raf/MEK/Erk pathway in benign prostate epithelial cells. These cells demonstrate little basal pAkt and these levels do not increase with CXCL12 stimulation because PTEN is intact and fully functional. Thus, inactivation of PTEN may be the critical factor that modulates downstream signaling and the specific CXCL12-stimulated proliferative responses of non-transformed and transformed prostate epithelial cells. Based on these data, we hypothesize that the CXCL12/CXCR4-mediated activation of downstream pro-proliferative signaling through the Raf/MEK/Erk or PI3K/Akt pathways is modulated by PTEN status.

**Keywords:** Prostate, CXCL12, CXCR4, PTEN, BPH

## Introduction

Many prostate cancer cell lines and human tumors exhibit PTEN (phosphatase and tensin homologue deleted on chromosome 10) inactivation due to gene deletions and/or mutations [1, 2]. A recent study demonstrated deletion of at least one copy of PTEN in almost 70% of primary prostate tumors, but no deletions in benign samples examined, suggesting that inactivation of PTEN is perhaps the most common genetic defect observed in human prostate tumors [1]. PTEN is a critical regulator of Akt activity and thereby plays a central role in the mediation of PI3K signaling through Akt. Akt activation has been identified as an excellent predictor of poor clinical outcome in prostate cancer [3]. Other studies have demonstrated concurrent activation of Akt and inactivation of Erk in high-Gleason-grade prostate cancer [4]. Moreover, activated Akt has been directly correlated, and activated Erk inversely correlated, with a high proliferative index (measured by Ki67 staining) in human prostate tumors [5]. As reviewed by McAubrety et al., the PI3K/Akt and Raf/MEK/Erk pathways interact with each other to regulate growth, and cancer cells inactivated for PTEN may exhibit suppression of the Raf/MEK/Erk cascade due to the ability of activated Akt to inactivate different Rafs [6]. If so, activation or inactivation of PTEN may, indeed, dictate which signaling pathways can be activated by CXCL12 to mediate proliferation and gene transcription in non-transformed versus transformed prostate epithelial cells.

CXCL12 is secreted at sub-nanomolar quantities by aging human prostate stromal fibroblasts and stimulates the proliferation of both non-transformed and transformed prostate epithelial cells [7, 8]. Previous studies from our laboratory showed that, when stimulated with sub-nanomolar levels of CXCL12 comparable to those secreted by aging prostate stroma, non-

transformed N15C6 cells transiently and robustly activate Erk, whereas transformed LNCaP cells did not [7]. Moreover, experiments utilizing the mitogen-activated protein kinase kinase 1 (MEK1) inhibitor, U0126, showed that Erk activation was absolutely required for CXCL12-mediated cellular proliferation in non-transformed N15C6, but only minimally required for transformed LNCaP prostate epithelial cells [9]. Gene expression profiling using Affymetrix Human Genome U133 Plus 2.0 Arrays showed that both cell lines demonstrated differential expression of > 500 transcripts, but only 85 genes were similarly transcribed, in response to CXCL12 stimulation. In addition, pre-treatment of the cells with U0126 showed that the CXCL12-mediated transcriptional responses was largely Erk-dependent in non-transformed N15C6 cells and were less Erk-dependent, or perhaps Erk-independent, in transformed LNCaP cells [9]. Taken together, these results suggested that CXCL12-mediated proliferative and transcriptional responses were likely governed by multiple signaling pathways in non-transformed versus transformed prostate epithelial cells.

A key difference between normal and malignant prostate cells in vitro and in vivo is that both alleles of PTEN are largely intact in normal benign prostate glands and cultured epithelial cells, whereas one or both alleles of PTEN are mutant or deleted in the majority of prostate tumors and malignant prostate cancer cell lines [1, 2, 10-13]. PTEN suppresses phosphorylation of Akt downstream of PI3K activation in non-transformed cells whereas Akt phosphorylation is unimpeded in malignant cells that are often PTEN-deficient. Thus, inactivation of PTEN may be the critical factor that modulates downstream signaling and the specific CXCL12-stimulated proliferative responses of non-transformed and transformed prostate epithelial cells. Based on these data, we hypothesize that the CXCL12/CXCR4-mediated activation of downstream pro-proliferative signaling through the Raf/MEK/Erk or PI3K/Akt pathways is modulated by PTEN status.

## Materials and methods

### *Cell culture*

N15C6 cells were produced through the immortalization of primary human prostate epithelial cultures by transduction with a recombinant retrovirus encoding the E6 and E7 genes of human papillomvarius 16 and were maintained as previously described [9, 14]. RWPE-1 cells were produced through the immortalization of primary human prostate epithelial cultures by transduction with human papillomavirus 18 [15]. Both cell lines grow continuously in culture but do not form colonies in soft agar or tumors in immuno-compromised mice. PC3 cells, a widely-used transformed prostate epithelial cell line originating from a prostate cancer dural metastasis, were acquired from the American Type Culture Collection (ATCC# CRL-1435), were maintained in 10% RPMI media and 0.5 lg/ml fungizone.

### *Establishment of stable PTEN-knockdown cells*

Stable knockdown of the PTEN gene was accomplished by chemical transfection of an shPTEN expressing vector or a scrambled hairpin vector followed by selection with puromycin. Briefly, N15C6 or RWPE-1 cells were plated in complete medium at $6\times10^5$ cells/well in 6 well plates and grown for 48 hours. Using the Fugene HD transfection reagent (Roche Diagnostics), cells were transfected with shPTEN vector (T1379209, TI379210, TI379211, or TI379212) or scrambled vector control (TR-30012) (OriGene). Following passage of cells into new plates, selection with puromycin began and knockdown of PTEN protein expression was followed by Western blot. Maximal knockdown without excessive cell death occurred at 6 ug/ml puromycin; cell lines were maintained under this selection

### *Cellular proliferation assays*

Cellular proliferation was measured using the WST-1 cell proliferation reagent (Roche Diagnostics), a tetrazolium salt which is cleaved by mitochondrial enzymes to yield a formazan compound. The rate of reaction increases as cell number increases and can be used as an indicator of cell proliferation. Cells were plated at 10,000 cells per well in 100 ul serum-free media in 96 well plates. Cells were also plated in complete medium as a positive control. Inhibitors, DMSO, and CXCL12 (R&D Sytems) were added to the medium at the time of plating as indicated per experiment. Plates were incubated at 37 Celsius for 24 hours, followed by addition of 10 ul WST-1 reagent. Plates were

incubated for 60 minutes and the colorimetric reaction was read at 450 nM.

*Apoptosis assays*

Apoptosis was measured using the Cell Death Detection ELISA Plus kit (Roche Diagnostics), which uses a sandwich ELISA technique to detect histone-DNA complexes formed during apoptosis. Cells were plated in serum-free media at 10,000 cells/well in 96 well plates with inhibitors, DMSO, and CXCL12 added at the time of plating. After 24 hours of incubation, cells were lysed and the resulting cytoplasmic fraction recovered. Cytoplasmic fractions were assayed for histone-DNA complexes per kit protocol.

*Invasion assays*

For each assay, 1,500 cells were seeded into the upper chamber and cellular migration through the membrane was measured after 24 hrs of exposure to 20 nM CXCL12 or control PBS using Boyden chambers (BD Biosciences). All experiments were performed in triplicate and graphed as averaged values across multiple assays.

*Protein purification and immunoblot analysis*

Cells were grown to 60% confluence followed by pre-treatment with vehicle or inhibitor for 60 minutes and subsequent CXCL12 treatment for indicated times. Cells were collected and lysed with ice cold RIPA buffer; lysates were quantitated using the Bradford assay and prepared for denaturing electrophoresis. Following transfer to nitrocellulose, the protein blots were probed with rabbit antibodies from against phospho-Akt (#9271), phospho-Erk1/2 (#9101), PTEN (#9559), total Akt (#9272), total Erk1/2 (#9102), and GAPDH (#2118), all from Cell Signaling Technologies.

*Construction of tissue microarray (TMA) and immunohistochemistry*

A TMA was constructed from 31 radical prostatectomy specimens to represent benign prostate tissues as previously described [7]. Antigen retrieval was performed in citrate buffer (10 milimolar, pH 6.0) using microwave pressure cooker. Antibodies used for immunohistochemistry included those that detected CXCR4 (Abcam, ab2074; 1:50) at 1:50 dilution; Akt (Santa Cruz Biotechnology, H-136; 1:50), pAkt (Cell Signaling Ser473, 1:100), Erk (Santa Cruz Biotechnology C-16; 1:100), pErk (Cell Signaling Thr202/Thr2014; 1:100), and Ki67 (Dako, MIB-1; 1:100). Digital images were acquired with the BLISS Imaging System (Bacus Laboratory, Lombard, IL). Immunostaining intensity was recorded as absent (1), weak (2), moderate (3), or strong (4). Scoring was performed in a blinded fashion using the Profiler web based telepathology system (http://pvdb.path.med.umich.edu/htma/profiler/index.jsp).

*Statistical analysis*

Data was assessed by t-test or analysis of variance with $p < .05$ considered statistically significant.

## Results

*Basal PTEN protein expression in prostate epithelial cells*

Previous studies have reported abundant PTEN protein expression in 22Rv1 and BPH-1 normal prostate epithelial cells but little or no expression in LNCaP and PC3 prostate epithelial cancer cells [13, 16]. As seen in **Figure 1A**, N15C6 normal prostate epithelial cells also express abundant PTEN protein. Based on these observations, the RWPE and N15C6 normal prostate epithelial cells were chosen for further studies investigating the role(s) of PTEN in CXCL12/CXCR4-mediated cellular proliferation.

*PTEN knock-down increases basal and CXCL12/CXCR4-stimulated Akt phosphorylation*

RWPE and N15C6 were transfected and puromycin-selected separately for clones expressing shPTEN or scrambled sequence (SS) control sequences. Greater than 50% PTEN protein knockdown was observed in both cell lines though knock-down appeared more complete for RWPE-1 than N15C6 cells (**Figure 1B**). N15C6 (**Figure 1C**) and RWPE (**Figure 1C**) cells knocked-down for PTEN exhibited higher levels of basal and CXCL12-stimulated Akt phosphorylation, which was ablated upon treatment with the P13K inhibitor, Wortmanin (**Figure 1E** and **1F**). N15C6, but not RWPE, cells exhibited higher levels of basal and CXCL12-stimulated Erk phosphorylation, which was ablated upon treat-

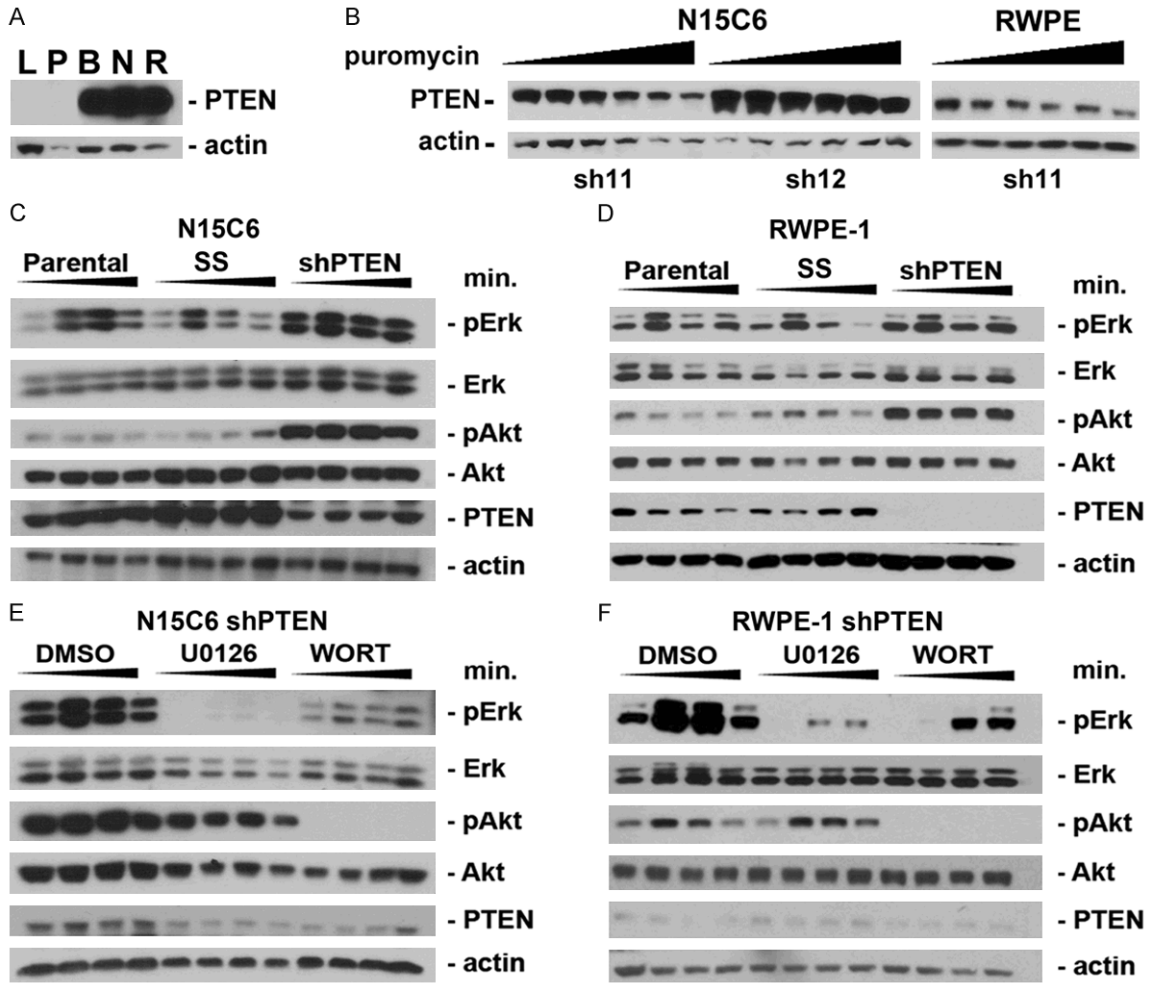


**Figure 1.** PTEN deficiency dysregulates CXCL12/CXCR4-mediated intracellular signaling. (A) Immunoblot of prostate cancer cells LNCaP (L) and PC3 (P), and benign prostate epithelial cells BPH-1 (B), N15C6 (N) and RWPE-1 (R) demonstrate loss of PTEN protein in LNCaP and PC3 but retention in all 3 benign cell lines. (B) N15C6 and RWPE-1 cells were transfected with shPTEN vector TI379211 (sh11) or TI379212 (sh12) and PTEN protein knock-down was followed by immunoblot after increasing concentrations of puromycin. Following passage of cells into new plates, selection with puromycin began and knockdown of PTEN protein expression was followed by Western blot. Maximal knockdown without excessive cell death occurred at 6 ug/ml puromycin; Parental and SS cells demonstrate increased levels of pErk in response to CXCL12 whereas shPTEN cells demonstrated higher levels of basal pErk and pAkt. (D) RWPE-1 parental, scrambled sequence (SS) control, and shPTEN cells (as indicated) and treated as in (C) All cells demonstrated increased levels of pErk in response to CXCL12 whereas shPTEN cells demonstrated higher levels of basal pAkt. (E) N15C6 shPTEN cells were treated with vehicle (DMSO), the MEK/Erk inhibitor U0126 or the PI3K inhibitor Wortmannin for 0, 10, 20 or 60 minutes and 10 pM CXCL12. U0126 ablated Erk phosphorylation in U0126-treated cells, and phosphorylation was partially restored with Wortmannin treatment. Wortmannin treatement ablated Akt phosphorylation. (F) RWPE-1 shPTEN cells were treated as in (E) and responded with similar signaling profiles as in (E).

ment with the MEK inhibitor, U0126 (**Figure 1E** and **1F**).

*CXCL12/CXCR4-stimulated proliferation is PTEN- and MEK/Erk dependent*

WST assays demonstrated that parental and scrambled sequence control N15C6 and RWPE cells proliferate to levels 20-30% higher than un-stimulated cells over a 24 hour period in the presence of low (1-100 pM) concentrations of CXCL12 (**Figure 2**). Dimethyl sulfoxide (DMSO), which can be toxic in vitro [17], dampened, but did not abrogate CXCL12-promoted proliferation. However, PTEN knock-down N15C6 and RWPE-1 cells failed to proliferate in response to CXCL12, suggesting that activation of the P13K/Akt pathway repressed CXCL12-medi-

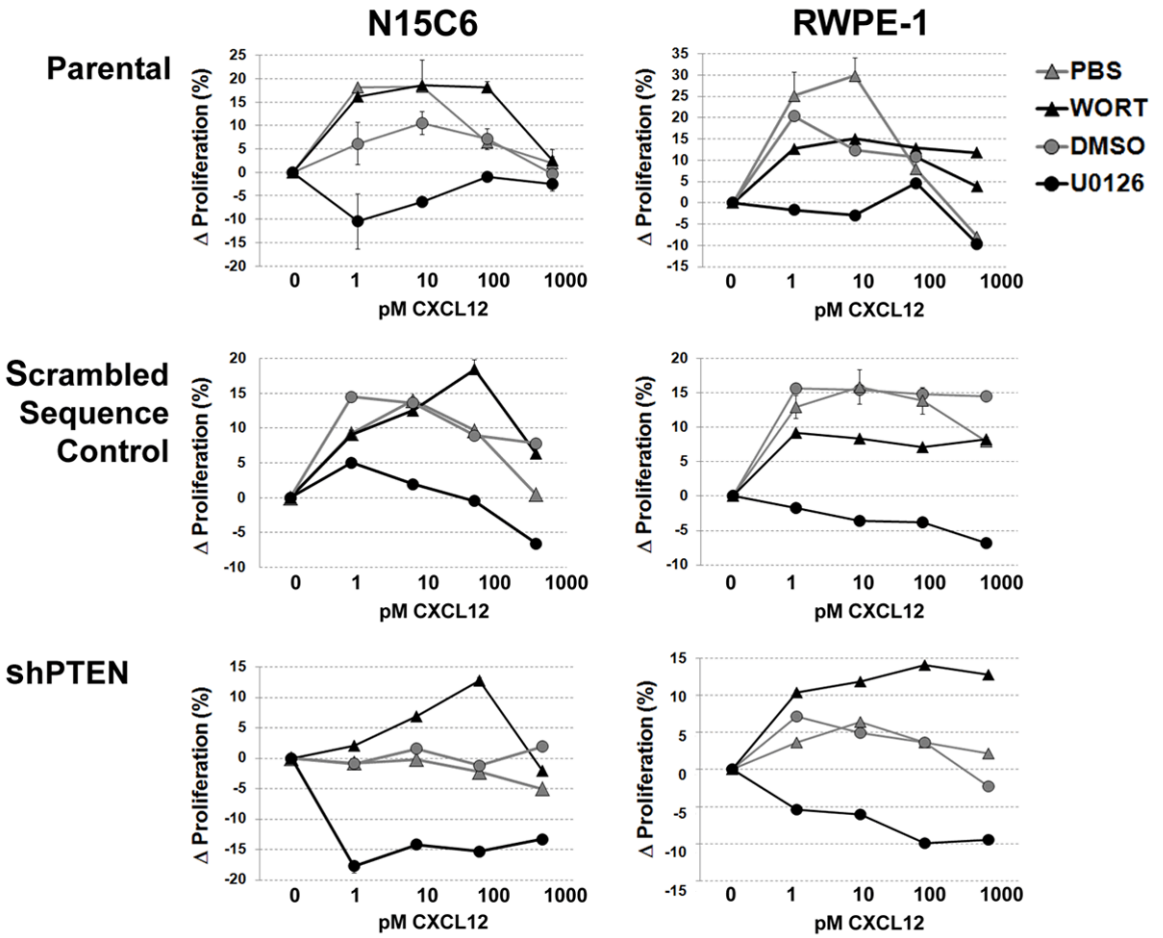


**Figure 2.** PTEN deficiency ablates CXCL12/CXCR4-mediated proliferative responses. N15C6 (left) or RWPE-1 (right) parental, scrambled sequence control (SS) or shPTEN cells (as indicated) were treated with ascending doses of CXCL12 (as indicated) and assessed for proliferative responses. Parental and SS N15C6 and RWPE-1 cells proliferated to levels 20-25% higher than basal levels at 1-10 pM CXCL12. This effect was dampened but not ablated by DMSO. Parental and SS N15C6 and RWPE-1 cells also proliferated when pre-treated with Wortmannin. However, pre-treatment with U0126 ablated CXCL12/CXCR4-mediated proliferative responses and reduced these responses below basal levels in both cell lines. PTEN deficiency repressed CXCL12/CXCR4-mediated proliferative responses for both N15C6 and RWPE-1 shPTEN cells (as indicated). However, this repression was partially lifted by pre-treatment with Wortmannin. Both N15C6 and RWPE-1 shPTEN cells were unable to respond proliferatively to CXCL12/CXCR4 axis activation and basal proliferation was repressed in both PTEN deficient cell lines.

ated proliferation. CXCL12-mediated proliferative responses were modestly restored in both PTEN knock-down cell lines upon pre-treatment with Wortmannin, which represses P13K/Akt signaling (**Figure 2**). This proliferative response was likely due to MEK/Erk signaling, as pre-treatment with U0126 completely abrogated CXCL12-mediated proliferative responses and repressed cellular proliferation to levels 10-20% below parental and SS cell basal levels (**Figure 2**). Moreover, pre-treatment with Wortmannin partially restores MEK/Erk signaling in CXCL12-treated shPTEN cells (**Figure 1E** and **1F**). Taken together, these data suggest that activation of the MEK/Erk signaling pathway, but not the P13K/Akt pathway, is intrinsically coupled to robust CXCL12-mediated proliferation.

P13K/Akt pathway stimulation is associated with cellular survival. Therefore, it would be reasonable to expect that PTEN knock-down cells might exhibit lower levels of apoptosis than PTEN competent cells. The overall percentage of cells undergoing apoptosis over a 24 period was decreased by ~10% for scrambled sequence control and PTEN knock-down RWPE-1 cells compared to parental cells (**Figure 3**). Apoptotic

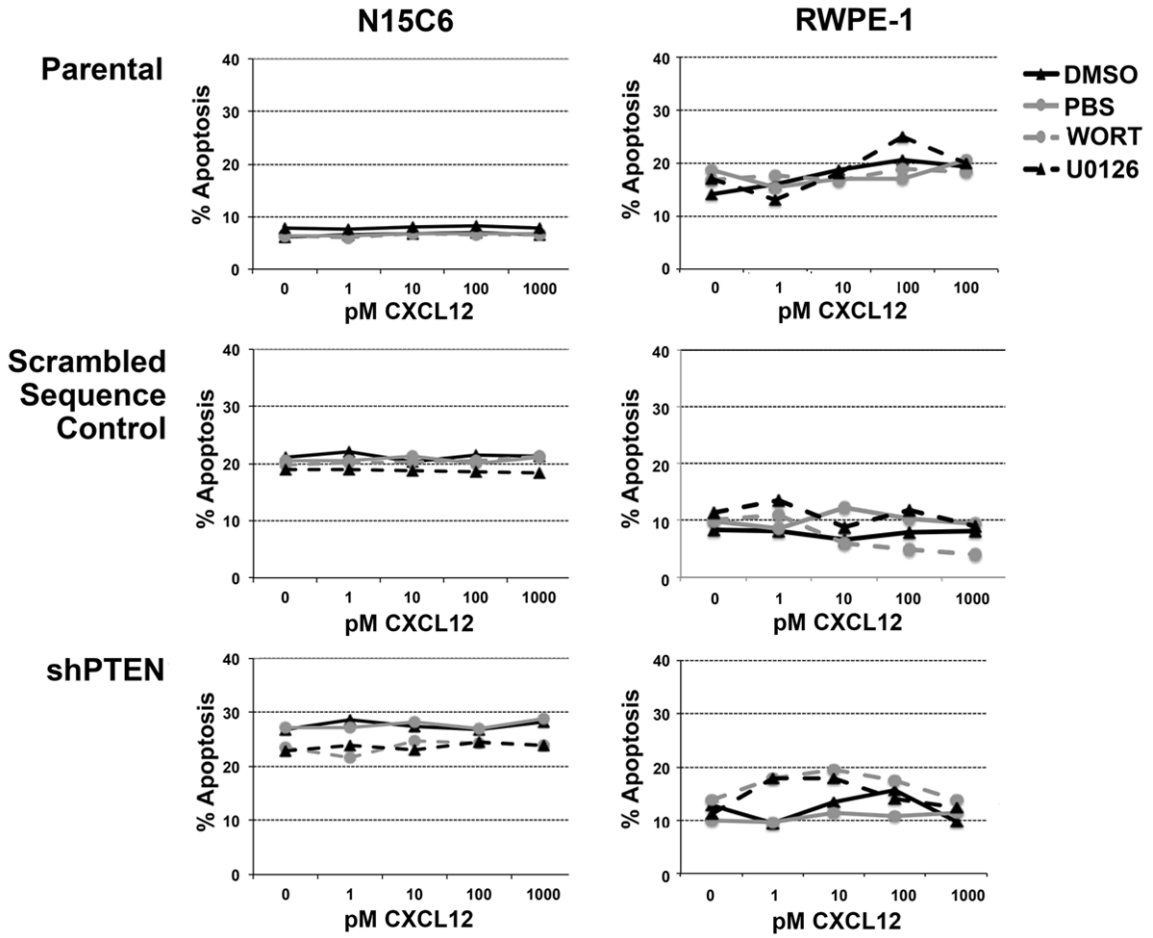


**Figure 3.** PTEN deficiency alone in insufficent for reduced apoptosis. N15C6 (left) or RWPE-1 (right) parental, scrambled sequence control (SS) or shPTEN cells (as indicated) were treated with ascending doses of CXCL12 (as indicated) and assessed for apoptosis. Untransfected parental N15C6 demonstrated a low, < 10%, apoptotic rate whereas SS and shPTEN cells demonstrated an overall higher 20-25% apoptotic rate. RWPE-1 cells demonstrated a higher basal apoptotic rate of 10-25% which was somewhat reduced in SS and shPTEN cells. Treatment with CXCL12 did not reduce or increase the apoptotic rate in any cells tested, and pre-treatment with U0126 or Wortmannin resulted in minimal affects of the apoptotic rate.

rates were not significantly affected by CXCL12 treatment or pre-treatment with the inhibitors Wortmannin and U0126. Apoptotic rates increased for scrambled sequence control and PTEN knock-down compared to parental N15C6 cells, and these rates were unaffected by CXCL12 treatment or pre-treatment with the inhibitors Wortmannin and U0126 (**Figure 3**). The increased apoptotic rate in transfected N15C6 cells is unexpected but may be due to inherent differences in viability in response to transfection.

*PTEN downregulation Is insufficient for malignant transformation*

PTEN knock-down clearly dysregulated CXCL12/CXCR4 axis-mediated N15C6 and RWPE-1 proliferative responses. In order to determine whether these effects were associated with malignant transformation, RWPE-1 cells, which demonstrated the most completed PTEN ablation, were assessed for ability to migrate through a porous membrane, which is a measure of cellular motility and invasiveness. RWPE-1 parental, scrambled sequence control, and PTEN knock-down cells were seeded onto a membrane in the upper chamber of a modified Boyden chamber assay and assessed for their ability to migrate towards serum-free media supplemented with PBS or 20 nM CXCL12 over a 24 hr period. As shown in **Figure 4**, none of these cells were able to migrate through the membrane. In contrast, PC3 prostate cancer cells were able to migrate and

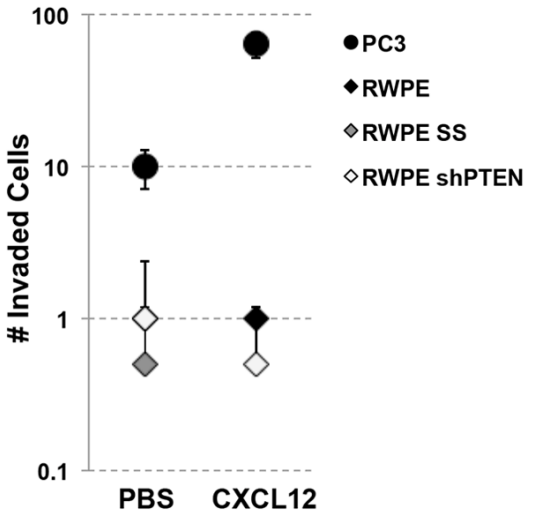


**Figure 4.** PTEN deficiency alone in insufficent for malignant transformation. Motility and invasiveness are traits associated with the malignant phenotype. PC3 cells or RWPE-1 parental, SS or shPTEN cells were seeded on the upper surface of a porous membrane and assessed using a modified Boyden chamber assay for their ability to migrate towards complete media supplemented with PBS or 20 nM CXCL12 over a 24 hour period. PC3 cells migrated towards complete media and CXCL12-supplemented media at 10X and 100X, respectively, higher rates than RWPE-1. This showed that PTEN deficiency alone was insufficient for expression of the malignant phenotype by this measure in RWPE-1 cells.

accumulate on the other side of the membrane (**Figure 4**). These data showed that, although ablation of PTEN expression in benign RWPE-1 cells was sufficient for dysregulation of CXCL12/CXCR4-mediated responses, it was insufficient for malignant transformation.

*Human BPH tissues are marked by a benign proliferative protein signature*

We had previously reported that human BPH tissues, particularly epithelial BPH nodules, express higher levels of CXCR4 than normal prostate tissues though the difference was not statistically significant [7]. Using the same tissue microarray (TMA) from those experiments we have now analyzed pErk, pAkt, and Ki67 levels in normal prostate and BPH tissues. As shown in **Figure 5**, both pErk and Ki67 expression levels were significantly higher in BPH compared to normal tissues. In contrast, pAkt levels were not significantly different between BPH and normal prostate glands (**Figure 5**). Taken together, these data are consistent with

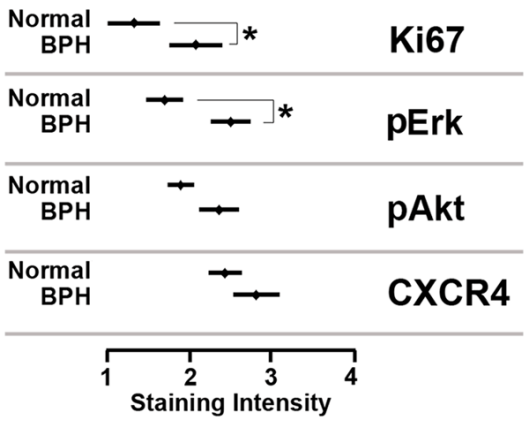


**Figure 5.** Human BPH tissues exhibit a benign proliferative protein signature. Immunohistochemical analysis of a tissue microarray was performed to assess expression levels of the proliferative antigen Ki67, pErk, pAkt, and CXCR4. The staining intensities of pErk and Ki67 expression levels were significantly higher in BPH compared to normal tissues. In contrast, pAkt levels were not significantly different between BPH and normal prostate glands.

a benign proliferative signaling mechanism that is coupled to MEK/Erk- rather than PI3K/Akt-signaling.

## Discussion

The intent of the studies reported here was to test the hypothesis that CXCL12/CXCR4-mediated activation of downstream pro-proliferative signaling is modulated by PTEN status. The results of these studies clearly demonstrate that PTEN status largely dictates downstream signaling consequent to CXCL12/CXCR4 activation in benign prostate epithelial cells. Parental and shControl N15C6 or RWPE-1 benign prostate epithelial cells responded proliferatively to CXCL12/CXCR4 axis activation in a MEK/ERK-dependent and PI3K-independent manner. In contrast, shPTEN cells lost the CXCL12/CXCR4-mediated proliferative response, which was exacerbated when MEK/Erk signaling was inhibited but was partially restored when PI3K signaling was inhibited. These results are consistent with our overall hypothesis and suggest that: 1) PTEN acts as a 'switch' to direct signal transduction downstream of CXCL12/CXCR4 axis activation; and, 2) CXCL12-mediated proliferative responses are coupled to MEK/Erk signaling in benign prostate epithelial cells. Moreover, there is some evidence from our studies that PTEN expression actively represses MEK/Erk signaling.

Our group previously published studies showing that benign N15C6 prostate epithelial cells, but not malignant PC3 prostate cancer cells, exhibited transactivation of EGFR and downstream MEK/Erk signaling in response to CXCL12/CXCR4 axis activation. Moreover, EGFR transactivation was required for CXCL12/CXCR4-mediated cellular proliferation in N15C6 cells [18]. This previously reported data is consistent with new information reported here, and shows that CXCL12/CXCR4-stimulated intracellular signaling is EGFR-dependent. In turn, EGFR preferentially signals intracellularly, and produces proliferative responses, through the MEK/Erk pathway in PTEN competent cells.

Immunohistochemical analysis reported here correlated high pErk and Ki67 levels, suggesting that BPH tissues express a MEK/Erk signaling signature concurrent with a high proliferative index. Data reported by others showed that expression of activated Akt and activated ERK are inversely correlated in human prostate tumors [3, 4]. As reviewed by McCubrey et al., the PI3K/Akt and Raf/MEK/Erk pathways interact with each other to regulate growth, and suggests that cancer cells inactivated for PTEN may exhibit suppression of the Raf/MEK/Erk cascade due to the ability of activated Akt to inactivate different Rafs [6]. Along with the data reported here, these studies demonstrate that PTEN incompetence may actively suppress EGFR-mediated Raf/MEK/Erk signaling through multiple mechanisms and alter cellular response to anti-proliferative therapeutics.

The results of these studies have high therapeutic relevance. A recent study showed that cultured prostate epithelial cells lacking PTEN (e.g., PC3, LNCaP) were partially resistant to the anti-proliferative effects of the EGFR small molecule inhibitor, Gefitinib, but that sensitivity could be improved by co-inhibiting PI3K activity. Moreover, cells with at least one intact copy of PTEN (e.g., DU145) were more sensitive to the anti-proliferative effects of Gefitinib than PTEN-deficient cells, and that this sensitivity was modulated by the MEK/ERK pathway [19] These findings have been mirrored in the results of a randomized phase II clinical trial examining the efficacy of Gefitinib for hormone refractory prostate cancer (HRPC). The results of this trial were disappointing, and the study coordinators concluded that a better understanding of which cell growth and signaling pathways were modulated by EGFR alone or in combination with other major signaling proteins was necessary to develop more efficacious therapeutics [20]. These data suggest that the utilization of tyrosine kinase inhibitors (TKIs) may be more effective in PTEN-competent benign proliferative conditions, such as BPH, than potentially PTEN deficient malignant proliferative diseases such as PCa.

It should be noted that PTEN deletion failed to transform normal benign RWPE-1 prostate epithelial cells or render either N15C6 or RWPE-1 cells more resistant to apoptosis. These results may indicate that PTEN knock-down was incomplete in these cells, hence, the cells were 'leaky' for PTEN activity, or that PTEN deficiency alone is insufficient for manifestation of the malignant phenotype and apoptosis resistance. Regardless, these data indicate that PTEN competency may dysregulate intracellular signaling but other genetic or environmental factors may be required for cellular transformation.

In summary, the data reported here shows that PTEN acts as a 'switch' to direct signal transduction downstream of CXCL12/CXCR4 axis activation, and that CXCL12-mediated proliferative responses are coupled to MEK/Erk signaling in benign prostate epithelial cells in vitro. Examination of human tissues shows that pErk and Ki67 levels are correlated and denote a proliferative profile in PTEN competent BPH tissues. The clinical relevance of these studies is the utilization of tyrosine kinase inhibitors (TKIs) may be more effect in PTEN-competent benign proliferative conditions, such as BPH.

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