REGULATION OF PHOSPHATASE HOMOLOGUE OF TENSIN PROTEIN EXPRESSION BY BONE MORPHOGENETIC PROTEINS IN PROSTATE EPITHELIAL CELLS

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ABSTRACT

Phosphatase homologue of tensin (PTEN) is the key endogenous inhibitor of phosphoinositide signaling and is the most commonly mutated gene in human prostate cancer. The bone morphogenetic proteins (BMPs) are secreted developmental signaling molecules known to promote differentiation in the prostate. BMP ligands have been shown to inhibit prostate cancer cell line proliferation and tumor growth and expression of BMPs, BMP ligands, receptors and signaling effectors are diminished in prostate cancer. A previous report in the colon led us to investigate the potential mechanistic relationship between PTEN and BMP signaling in prostate epithelial cells. We show here that BPM signaling positively regulates PTEN in normal and malignant prostate cells by increasing mRNA expression and stabilizing PTEN protein. Further, we show that BMP attenuates prostate cell growth at least in part through its effects on PTEN. BMP treatment did not further inhibit the growth of conditional PTEN over-expressing cells, and stable shRNA-PTEN transfectants were refractory to BMP-mediated growth inhibition. Loss-of-function of PTEN in prostate cancer cells may render them insensitive to the normal differentiating and growth-inhibitory effects of BMPs. These data are the first to identify a mechanistic linkage between BMP signaling and PTEN in normal prostate epithelial cells and to suggest coordinate dysregulation in prostate cancer.

Keywords: Prostate, epithelium, bone-morphogenetic protein, phosphatase homologue of tensin analogue, protein stability

INTRODUCTION

Phosphatase homologue of tensin (PTEN) is one of the most commonly associated mutated genes in prostate cancer, and its mutation is tightly correlated to poor clinical prognosis (Sircar et al., 2009). This protein functions as an endogenous intracellular modulator of the phosphoinositide-3-kinase (PI_3K)-Akt pathway and therefore plays a central role in regulating proliferation in many epithelial cells (Cantley and Neel, 1999). A key regulatory step in this cascade is phosphorylation of phosphoinositide-bisphosphate (PIP_2) to generate PIP_3 by PI_3K, resulting in activation of phosphoinositide-dependent kinases (PDK) and activation of Akt by phosphorylation (Cantley and Neel, 1999). PTEN acts as a phosphoinositide phosphatase to reverse this reaction and inhibit PI_3K-Akt signaling (Cantley and Neel, 1999). Therefore reduced expression or function of PTEN in cancer permits unregulated Akt activation and increased tumor cell proliferation (Groszer et al., 2001; Halvorsen et al., 2003). Numerous mitogens and cytokines impinge upon this pathway by activating secondary adaptor molecules such as src, focal adhesion kinase (FAK), and insulin-like receptor substrate (IRS) that initiate the PI_3K-Akt signaling cascade (Frost and Lang, 2007; Arcaro et al., 2007; Xia et al., 2004; Ozes et al., 2001).

Studies of growth regulatory pathways in the developing prostate have identified a number of important morphogens including fibroblast growth factors, hedgehog ligands, cytokines including interleukins and transforming growth factor beta, Notch, wnt/beta-catenin and the bone morphogenetic proteins (BMPs) (Prins and Putz, 2008). The BMPs are members of the TGF-beta superfamily and over 20 BMP ligands have been identified (Gazzerro and Canalis, 2006). The BMPs act by binding heterodimers of BMPR type II and type I receptors activating members of the SMAD family of signaling intermediates, most particularly SMADs 1, 5, and 8 (Gazzerro and

Canalis, 2006). The readout of BMP signaling is phosphorylation of SMADs and changes in transcription of specific target genes (Gazzerro and Canalis, 2006). Previous studies have shown that the ligands BMP-4 (and its homolog BMP-2) and BMP-7 are expressed in the developing prostate and exert important effects on prostate growth and proliferation (Lamm et al., 2001; Grishina et al., 2005). Interestingly, expression of BMP-2, 4, and 7 ligands, BMP receptors and intracellular signaling components is diminished in prostate cancer as compared to normal tissue, and BMPs have been shown to inhibit prostate cancer cell line proliferation and tumor growth (Miyazaki et al., 2004; Brubaker et al., 2004; Kim et al., 2004).

Although it is widely accepted that genetic mutation of PTEN can contribute to tumor tumorgenesis, recent studies suggest regulation of PTEN expression and activity may also contribute to tumor growth (Tamguney and Stokoe, 2007). For example, studies have shown that PTEN protein expression is reduced in a significant number of breast cancers (Engin et al., 2006). Although, the precise mechanism behind reduced PTEN protein levels in these cancers is poorly understood, repression of PTEN expression as well as changes in the activity and stability of PTEN protein have been proposed. Multiple signals could lead to positive or negative modulation of PTEN expression, including TGFβ signaling, UV radiation, and EGR-1 and PPAR signaling (Li and Sun, 1997; Stiles, 2009). Interestingly, BMP signaling has been demonstrated to sustain PTEN expression in the colon (Waite and Eng, 2003). Given the concurrent loss of expression of PTEN and BMP commonly observed in prostate cancer, we sought to investigate a possible mechanistic relationship between these pathways.

RESULTS

We treated E6 and BPH-1 prostate epithelial cells with a concentration-response curve to BMP-4 and measured its effect on growth rate and PTEN protein expression. BMP-4 inhibited proliferation of E6 and BPH-1 cells in a concentration-dependent manner **[Figure 1]** and this inhibitory effect correlated with BMP-induced PTEN protein expression. Treatment with BMP-4 significantly induced expression of PTEN protein in E6 (6-fold) and BPH-1 (2.5-fold). **[Figure 2]** This induction was concentration-dependent; the maximal effect in both cell lines was observed from treatment with 10 ng/ml BMP-4. Induction of canonical BMP signaling was confirmed by determination of activated (phosphorylated) SMAD (isoforms 1, 5, 8). BMP-4 treatment and the associated induction of PTEN reduced PI_3K-Akt signaling as evidenced by decreased phosphorylation of Akt (serine 473): BMP-4 reduced activated Akt by 39 (± 12) % in E6 cells, and by 33 (± 14) % in BPH-1 cells. **[Figure 2]**. Both the induction of PTEN and inhibition of proliferation by BMP-4 were completely blocked by the BMP antagonist NOGGIN.

The LNCaP prostate cancer cell line is generally considered to be PTEN negative and no induction of PTEN expression or change in Akt activation was observed in LNCaP cells treated with BMP-4 (data not shown). Overexpression of PTEN in LNCaP-derived C4-2 cells consistently reduces proliferation, suggesting that PTEN activation is sufficient to inhibit growth (Wu et al., 2007). C4-2 cells engineered to express active PTEN from a doxacyclin-driven promoter exhibit a concentration-dependent inhibition of cell proliferation by doxacyclin (Wu et al., 2007). We treated these cells with increasing concentrations of doxacyclin in semi-log increments in the presence or absence of 10 ng/ml BMP-4 to examine the interaction of BPM-4 and PTEN **[Figure 3A]**. BMP-4 did not inhibit proliferation of cells lacking significant promoter-driven PTEN expression (0 to 0.1 mg/ml doxacyclin). Intermediate concentrations of doxacyclin (0.3 μg/ml and 1.0 μg/ml doxacyclin) produced partial growth inhibition that was significantly augmented by

BMP treatment as evidenced by a log-fold leftward shift in the concentration-response curve. However, cells lacking significant promoter-driven PTEN expression (0 to 0.1 mg/ml doxacyclin) did not respond to BMP, and BMP failed to produce further growth inhibition in cells with full PTEN promoter-driven expression (3 mg/ml doxacyclin). The effect of BMP on growth inhibition was mirrored by PTEN protein induction. **[Figure 3B]** These data indicate that partial promoter-driven PTEN expression is necessary for BMP-4 to significantly affect growth rate in C4-2 cells.

We next sought to determine if the growth inhibitory effects of BMP-4 are dependent upon PTEN. Transfection of E6, and BPH-1 cells with an siRNA construct, producing a minimum of 70% expression knockdown, abrogated BMP-mediated growth inhibition **[Figure 4]**. BMP signaling was unaffected, as evidenced by SMAD 1, 5, 8 phosphorylation, but PI_3K-Akt signaling activity was increased. PTEN knockdown increased basal Akt phosphorylation and abrogated the decrease in Akt phosphorylation by BMP-4. Non-silencing scrambled siRNA constructs had no effect on BMP-mediated growth inhibition. PTEN knockdown had an expected measurable inductive effect on PI_3K-Akt signaling as measured by phosphorylated Akt. These data indicate that the growth-slowing effect of BMP-4 on prostate cell lines is at least in part due to its ability to induce PTEN expression.

We next sought to determine if the effect of BMP-4 on PTEN expression is transcriptional, post-transcriptional, or translational, or post-translation. E6 cells treated with BMP-4 exhibit time-dependent induction of PTEN mRNA levels, with significant induction evident at 8 hours of treatment. **[Figure 5A]** This is not associated with an increase in mRNA stability for PTEN message, indicating that BMP-4 is likely inducing PTEN at the transcriptional level. In addition, BMP-4 treatment is associated with a rapid decrease in phosphorylated PTEN protein levels

(residues 380, 382, and 383), despite an evident increase in total PTEN levels. **[Figure 5C]**. To investigate the effect of BMP treatment on PTEN protein stability, we treated E6 cells pre-exposed to either BMP-4 or vehicle with cyclohexamide (5 μM) for 0, 0.5, 1, 1.5, 2, 3, 4, 8, or 24 hours and measured PTEN protein levels. We found that 24 hours of BMP pretreatment caused a doubling in PTEN half-life (15.1 hours to 28.4 hours, $p=0.03$, $n=4$). **[Figure 5D]** These data indicate that BMP-4 has a dual effect on PTEN expression that involves increased expression and enhanced protein stability.

DISCUSSION

To our knowledge, this is the first paper to report on the mechanism by which bone morphogenetic proteins mediate prostate cell proliferation rates. The data indicate that treatment of prostate cell lines with BMP-4 results in increased cellular PTEN concentrations and a corresponding decrease in PI_3K-Akt signaling. The data further show that PTEN increase is at least in part responsible for the decreased proliferation rate observed in prostate cell lines since siRNA knockdown of PTEN expression attenuates BMP-mediated decreases in cellular proliferation. PTEN increase involves both transcriptional and post-translational mechanisms, as a rapid increase in PTEN mRNA and an increase in PTEN protein stability were observed after BMP-4 treatment. Experiments with conditional PTEN over-expressing cell lines further support a role for PTEN stability in cellular proliferation, as these cells required previous promoter-driven PTEN expression in order to respond to BMP-4 by PTEN expression increases and decreases in proliferation. Further, a decrease in phosphorylation of PTEN at residues 380, 382, and 383 are suggestive of enhanced PTEN activity, as these residues have been implicated in enzymatic

activity in previous reports (Rahdar, et al., 2009). Future studies should be directed toward detailing the enzymatic effects of BMP signaling on PTEN function.

The BMP signaling pathway has critical roles in embryogenesis, organogenesis, cell growth, differentiation, cell migration and cell death (Miyazaki et al., 2003). Due to the diversity of these biological functions, it is not surprising that BMPs have been associated with control of proliferation and differentiation in normal and malignant prostate epithelial cells (Miyazaki et al., 2004; Brubaker et al., 2004; Kim et al., 2004). BMP-4 and BMP-7 are androgen-regulated genes that play critical roles in the proper regulation of prostate development by inhibiting epithelial proliferation, ductal budding and branching (Grishina et al., 2005). Specifically, BMP signaling is believed to promote differentiation of prostate epithelial cells, thereby repressing proliferative capacity (Grishina et al., 2005; Cook et al., 2007). In addition, the BMP antagonist Noggin is expressed in the developing mouse prostate and neutralizes inhibition of epithelial proliferation by BMP4 allowing ductal budding and regulation of bud outgrowth (Cook et al., 2007). Our data suggest that in depth study of a possible interplay of BMP and PTEN / PI_3K-Akt signaling is warranted to further the mechanistic understanding of BMP signaling in prostate development.

With regard to prostate cancer however, divergence in expression and function among the BMPs is evident in published reports and there are considerable inconsistencies in published results regarding the biological function of BMPs in prostate cancer cells. For example, BMP-6 expression is upregulated in prostate cancers relative to noncancerous epithelium (Darby et al., 2008), while BMP-2, BMP-4, and BMP-7 expression is decreased (Masuda et al., 2004). Yet, BMP-4, BMP-6 and BMP-7 have all been consistently detected in prostate cancer bone metastases and expression of proteins involved in the BMP signaling pathway are often observed in

metastatic prostate cancer (Feeley et al., 2005). While several reports show a growth-reducing and differentiating effect of BMPs similar to what occurs in prostate development, other findings support a tumor-promoting role (Yang et al., 2005; Ye et al., 2007). These inconsistencies illustrate a pronounced uncertainty in this field, and the data presented in this manuscript may contribute to resolving some of these uncertainties. Specifically, the PTEN status of a particular tumor may be critical in determining how that tumor may respond to BMP expression: a PTEN-positive cell may respond in growth inhibitory/pro-differentiation direction while a PTEN-negative cell may respond in the opposite direction.

Invasion and migration are processes of particular interest regarding BMP signaling in prostate cancer. Yang et al have reported that BMP-7 increased invasiveness and migration on PC-3 cells, yet Feeley et al. reported no effects of BMP-7 on migration or invasion of these same cells (Feeley et al., 2006). Dai et al showed that BMP-7 (and BMP-2/4) did not alter invasion and migration of LuCaP and C4-2B cells (Dai et al., 2005). Yet, Ye et al have reported that inhibition of BMP-7 expression in PC-3 cells increases the invasive potential and migration of cells while reducing their proliferation (Ye et al., 2007). This process was dependent upon the loss of BMP signaling antagonists noggin and follistatin. It would be interesting to know how PTEN expression affects the results in this model, and future studies should be directed at determining if BMP-mediated invasion and metastasis of high-grade prostate cancer cells is modulated by the PTEN-Akt pathway.

While it is well-established that PTEN loss of function or suppression of expression is associated with prostate cancer growth and progression, recent papers have begun to define a role for PTEN in prostate cell migration and metastases (Wu et al., 2007). Cell migration in PTEN-

negative C4-2 cells is inhibited by ectopic PTEN expression and is dependent upon activity of the lipid phosphatase function of PTEN and corresponding decrease in PI_3K-Akt signaling (Wu et al., 2006).[23] Similarly, PTEN-deficient cells exhibit increased cell motility, and PI_3K-Akt signaling plays an important role in the regulation and directionality of cell migration (Shukla et al., 2007). In addition, bladder cancer cells exhibit PI_3K-Akt-dependent epidermal growth factor-mediated chemotaxis (Gu et al., 1999; Theodorescu et al, 1998). Our data demonstrate that BMP signaling, known to play a critical role in cell migration and metastases, regulates PTEN expression. This further implicates PTEN expression status of the tumor as a possible intermediary factor in how a tumor responds to BMP, and may explain inconsistencies in the literature involving prostate cancer responses to BMP signaling.

Our work shows that a strong signaling interrelationship exists between two signaling molecules known to be lost or down-regulated during prostate cancer growth and progression: the BMP and PTEN pathways. BMP-4 causes the induction of PTEN expression in prostate epithelial cells and is involved in stabilizing PTEN protein, resulting in significantly increased PTEN protein levels and the slowing of cell proliferation. In addition, previously published reports have implicated BMP signaling and the PI3K/Akt pathway in cell migration and metastases of prostate tumors, therefore future studies regarding the effect of BMP mediated PTEN expression on these critical processes should yield interesting results. Further future studies modulating both the BMP and PTEN pathways may have clinical relevance in the understanding and treatment of prostate cancer.

MATERIALS / SUBJECTS AND METHODS

Cells and culturing conditions: E6 prostate epithelial cells were kind gifts from Dr. David Jarrard, Department of Urology, University of Wisconsin Madison, and were cultured in conditions as previously published (Schwarze et al., 2002).[21] The immortalized cells were screened for HPV16 E6 protein expression by Western blot analysis and were cultured for over 20 passages to confirm immortality. BPH-1 cells were kindly provided by Dr. Simon Hayward, Vanderbilt University, Nashville, TN and were grown and maintained in conditions previously published (Hayward et al., 1995). The doxycyclin (DOX)-inducible C4-2 primary cell line (C4-2 pTetOn) cells were generated by Dr. Zhong Wu under the supervision of Dr. Dan Theodorescu and have been previously described (Wu et al., 2006). Cells were generated by transfection of pTRE2hyg PTEN-HA into C4-2 pTetOn cells; cells were maintained in 10% FBS/RPMI 1640 medium.

Growth rate determinations: Equivalent passages within each prostate cell line were plated at 5000 cells/well in 24-well plates. After a 24 hour attachment period, cells were grown in their prescribed media with 0, 1, 10, or 100 ng/ml BMP-4 (R&D systems, Minneapolis, MN) in the presence or absence of 500 ng/ml noggin (BMP antagonist-BMP and noggin were co-incubated 1 hour prior to addition to the cells to allow for binding). At time 0 and at 24 hour intervals, cell photos of each well were taken at consistent marked locations in quadruplicate. All cell growth experiments were performed 4 times (n=4) for each cell line. Cell numbers were determined for each treatment and are reported as number of cells per 20X field. Each point is the mean ± S.E.M. of the total cell counts.

Protein extraction, SDS-PAGE, and immunoblotting: Isolated prostate cells were homogenized in protease inhibitor-containing lysis buffer (150 mM NaCl, 10 mM Tris, 1 mM

EDTA, 1 mM benzenesulfonyl fluoride, and 10 µg/ml each of aprotinin, bestatin, L-leucine, and pepstatin A). Triton X-100 was added to a concentration of 1%, and the homogenate was incubated on ice for 60 minutes, followed by centrifugation for 20 min at 14,100*xG at 4C*. The supernatant was collected and total protein concentration was determined by BCA assay (Pierce, Rockford, IL). Proteins (20 µg/well) were resolved by electrophoresis in 4-20% gradient SDS-polyacrylamide electrophoresis gels. Proteins were transferred to PVDF membranes, blocked overnight [10 g/L nonfat dry milk, 10 g/L bovine serum albumin, and 0.5 g/L NaN_3 in 1x phosphate-buffered saline (PBS; 2.7 mM KCl, 1.5 mM KH_2PO_4, 136 mM NaCl, 8 mM Na_2HPO_4) + 0.05% (v/v) Tween 20] and incubated for 16 hours with one of the following primary antibodies: rabbit anti-PTEN (1:1000; Cell Signaling Technologies, Danvers, MA), rabbit anti-PO_4-S380,T382, T383-PTEN (1:250; Cell Signaling), rabbit anti-PO_4-T308/473-Akt (1:100, Cell Signaling), rabbit anti-PO_4 SMAD 1 [S463/465], 5[S463/465], 8 [S426/428] (1:1000, Cell Signaling). After washing six times in PBS + 0.05% Tween 20, the blots were incubated with goat anti-rabbit IgGs conjugated to horseradish peroxidase for one hour (1:200,000 dilution, Pierce, Rockford, IL) in 2.5 g/L nonfat dry milk, PBS, and 0.05% Tween 20. Peroxidase activity was detected via West Femto® chemiluminescence reagent as directed by the manufacturer (Pierce). Photo images were analyzed by densitometry and ratios of protein of interest to GAPDH were determined and compared between treatments.

Doxacyclin-inducible PTEN expression: pTetOn-PTEN or C4-2 control cells (described above) were plated at a density of 10,000 cells per well in 6 well plates (for protein expression analysis) or 5000 cells/well in 24-well plates (for growth analysis) and grown in 10% FBS/ RPMI 1640 medium. Analysis of PTEN protein expression: after reaching 90% confluency, cells were treated with concentrations of doxacyclin (0.03-3.0 µM in semi-log increments) and either 10 ng/ml

BMP-4 or vehicle (0.2% BSA in PBS) overnight (16 hours). Cells were collected and protein was extracted and analyzed as described previously. Analysis of growth rate: 5000 cells were plated per well in 24-well plates and allowed to attach for 24 hours. Cells were then treated with concentrations of doxacyclin (0.03-3.0 μM in semi-log increments) and either 10 ng/ml BMP-4 or vehicle (0.2% BSA in PBS) in 10% FBS/ RPMI 1640 medium. At time 0 and at 24 hour intervals, cell photos of each well were taken at consistent marked locations in quadruplicate. All cell growth experiments were performed 4 times (n=4) for each cell line. Cell numbers were determined for each treatment and are reported as number of cells per 20X field. Each point is the mean ± S.E.M. of the total cell counts.

siRNA knockdown of PTEN expression: Stable PTEN siRNA-expression clones of E6 and BPH-1 cell lines were generated using the Trans-Lentiviral^{TM} GIPZ packaging system and Expression Arrest^{TM} shRNAmir lentiviral delivery method using conditions and methods instructed by the manufacturer (Open Biosystems, Huntsville, AL). PTEN siRNA gene constructs employed human PTEN sequence clone RHS4430-99328837. Briefly, shRNA coding constructs supplied by Open Biosystems were cloned into pGIPZ expression plasmids (expressing selection factors puromycin resistance and GFP expression) using the protocol supplied and transformed into TLA-HEK293T packaging cells to generate live Lentivirus. After ampicillin selection of expressing clones, Lentiviral-producing packaging cells were grown and the lysed product was collected and used to infect prostate epithelial cell lines plated to 50% confluency. After infection, epithelial cells were selected for expression by puromycin-resistance and sorted by flow cytometry for GFP expression to ensure gene transduction. Effective PTEN expression knockdown on the siRNA clones was confirmed by a minimum of 70% expression knockdown by immunoblotting. Growth rate and PTEN inducibility experiments were performed in these cells and those

expressing a scrambled sequence siRNA previously shown not to effect gene expression by Open Biosystems.

RT-PCR of mRNA levels: Prostate cell lines were grown to 90% confluency in 12 well plates using cell line-specific conditions as described above and treated with either 10 ng/ml BMP-4 or vehicle (0.02% BSA in PBS) for 2, 4, 8, or 24 hours. Cells were harvested in 350 µl Qiagen RNeasy RLT lysis buffer plus 2-mercaptoethanol, RNA was extracted using the RNeasy kit as directed by the manufacturer (Qiagen, Valencia, CA), and complementary DNA was made using reverse transcription as previously described (Jerde and Bushman, 2009). RT-PCR for PTEN expression was performed as published (Jerde and Bushman, 2009) using the following primers: S27 forward TCTTTAGCCATGCACAAACG; S27 reverse TTTCAGTGCTGCTTCCTCCT; PTEN forward GAAGACCATAACCCACCACA; PTEN reverse TACACCAGTCCGTCCCTTTC. Cycle to threshold was calculated as previously described, and expression of PTEN was calculated as a ratio to ribosomal S27 expression. Comparisons between groups were made with analysis of variance (ANOVA), with $p<0.05$ indicative of significant difference.

PTEN mRNA decay assay: E6 prostate cell lines were grown to 80% confluency in 12 well plates using cell line-specific conditions as described above and treated with either 10 ng/ml BMP-4 or vehicle (0.02% BSA in PBS) for 16 hours. Cells were then treated with 5 µM actinomycin D, a transcriptional inhibitor. This concentration was shown to be optimal for inhibition of transcription in these cells in previous experiments. Separate wells of cells were harvested at time 0 and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 24 hours after addition of actinomycin in

350 µl Qiagen RNeasy RLT lysis buffer plus 2-mercaptoethanol, RNA was extracted using the RNeasy kit as directed by the manufacturer (Qiagen, Valencia, CA), and complementary DNA was made using reverse transcription as previously described (Jerde and Bushman, 2009). RT-PCR for PTEN expression was performed using the method and primers described above. Cycle to threshold was calculated, expression ratio of PTEN to ribosomal S27 expression was calculated for each sample. The amount of PTEN for each time point was calculated as a ratio to that at time 0, and a degradation curve was made and linear regression analysis performed (excel) to determine the half-life of each mRNA. Comparisons between BMP and vehicle-treated cells were made with analysis of variance (ANOVA) with $p<0.05$ indicative of significant difference.

PTEN protein stability assay: E6 prostate cell lines were grown to 80% confluency in 6 well plates using cell line-specific conditions as described above and treated with either 10 ng/ml BMP-4 or vehicle (0.02% BSA in PBS) for 16 hours. Cells were then treated with 1 µM cyclohexamide, a translational inhibitor. This concentration was shown to be optimal for inhibition of translation in these cells in preliminary experiments. Separate wells were harvested at time 0 and at 1, 2, 4, 8, 24, and 48 hours after addition of cyclohexamide in protease inhibitor-containing lysis buffer. Protein was extracted and immunoblotting for PTEN and GAPDH was performed for each sample as described above. The protein expression ratio of PTEN to GAPDH was calculated for each sample. The amount of PTEN for each time point was calculated as a ratio to that at time 0, and a degradation curve was made and linear regression analysis performed (excel) to determine the half-life of PTEN protein. Comparisons between BMP and vehicle-treated cells were made with analysis of variance (ANOVA) with $p<0.05$ indicative of significant difference.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. BMP-4 slows the growth of prostate cell lines. Growth curves of E6 **(A.)** and BPH-1 **(B.)** treated with vehicle (0.1% BSA), 1 ng/ml BMP-4, 10 ng/ml BMP-4, and 100 ng/ml BMP-4. Quantification reflects the number of cells per 20x field; data reflect the mean of 4 experiments with 4 determinations averaged in each experiment. Statistical considerations: * $p<0.05$ BMP-4 versus vehicle, ANOVA.

Figure 2. BMP treatment of prostate epithelial cell lines increases PTEN protein expression. A. Quantified data from immunoblots from 4 experiments: 10 ng/ml BMP-4 induced PTEN expression in E6 and BPH-1 cells, resulting in suppressed PI_3K-Akt signaling, as measured by phosphorylated T308/S472 Akt. Data presented are mean +/- sem. Statistical considerations: * $p<0.05$ BMP-4 versus vehicle. **B.** Quantified data from immunoblots from 4 experiments; data presented are mean +/- sem. Statistical considerations: * $p<0.05$ BMP-4 versus vehicle; ** $p<0.05$ BMP-4+Noggin versus BMP alone, analysis of variance (ANOVA). **C.** Immunoblots of prostate cells (**1-4**, E6; **5-8**, BPH-1) treated with either vehicle (0.1% BSA, lanes **1, 5, 9**), 10 ng/ml BMP-4 (lanes **2, 6, 10**), 100 ng/ml Noggin (lanes **3, 7, 11**), and BMP-4 pre-incubated (30 minutes) with Noggin (lanes **4, 8, 12**).

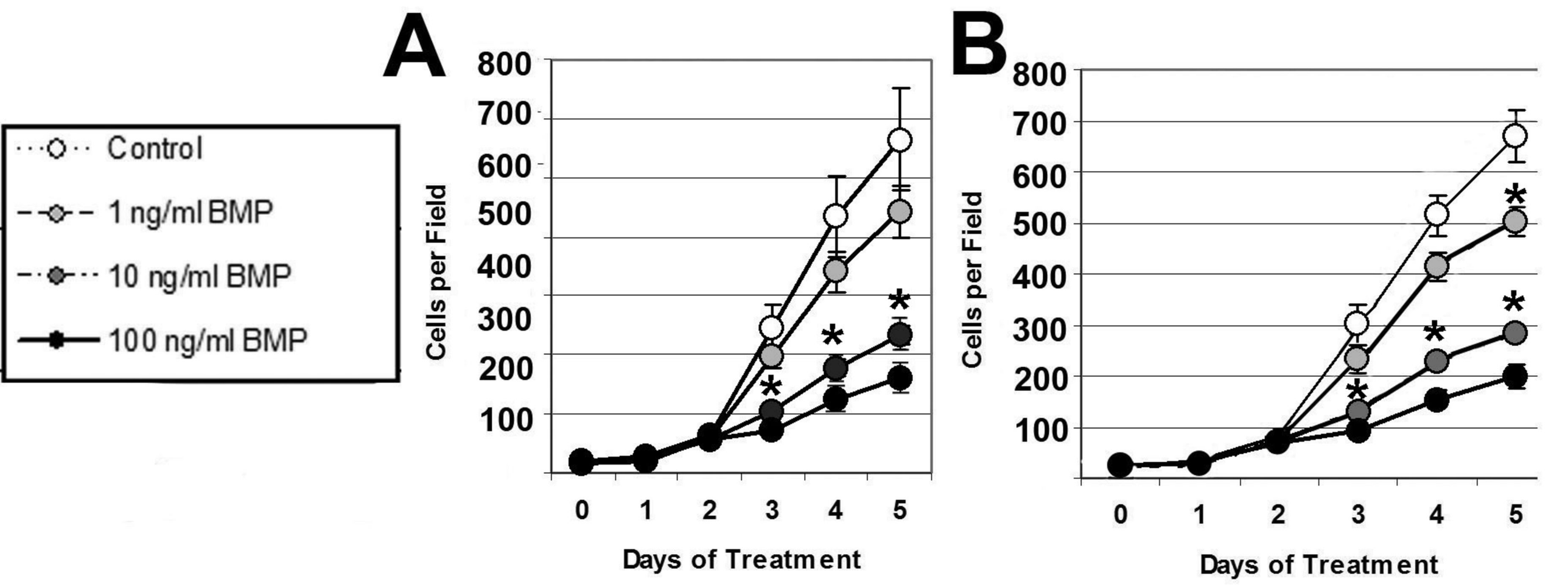
Figure 3. BMP-4 exerts PTEN-dependent effects on C4-2 growth. PTEN negative C4-2 cells were engineered to express a doxacyclin (dox)-inducible PTEN gene in which PTEN expression is dose-dependently induced by dox. Cells with little or no PTEN induction by dox were unable to respond to BMP-4 by either cell growth inhibition **[A]** or PTEN protein expression. **[B]** However,

upon moderate dox stimulation (0.1-1.0 μg/ml dox), BMP-4 repressed growth and enhanced PTEN expression.

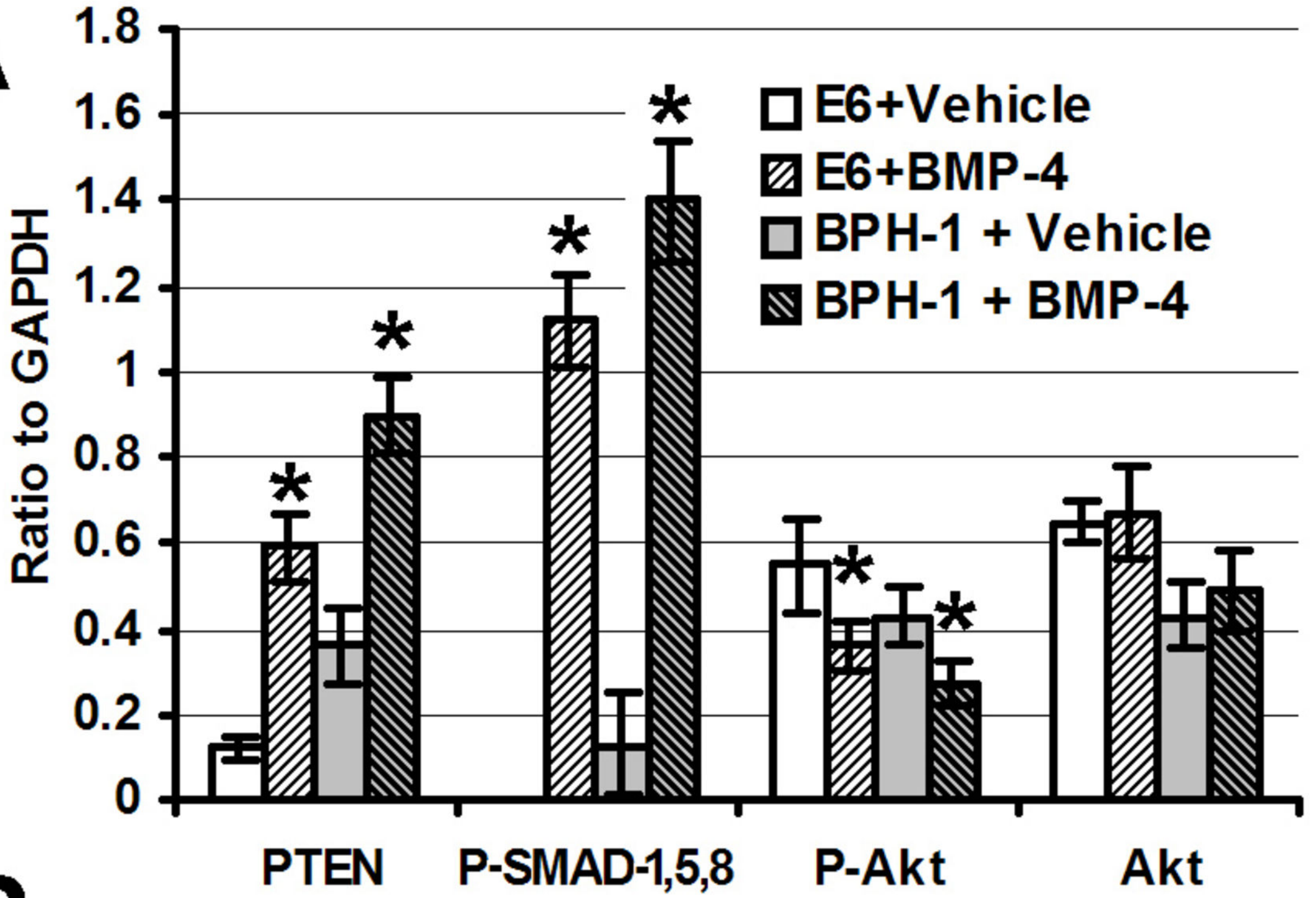
Figure 4. BMP-4 repressed cell growth in prostate epithelial cells is dependent on PTEN expression. **[A]** BMP-4 was unable to fully repress growth of E6, and BPH-1 stably transfected with siRNA constructs against PTEN relative to cells transfected with a scrambled non-silencing siRNA. Expression of the siRNA construct itself caused a substantial induction in growth rate further demonstrating the importance of PTEN in controlling growth rate in prostate epithelial cells. Data presented reflect the number of cells per 20x field; data presented are the mean of 4 experiments with 4 determinations averaged in each experiment. Statistical considerations: * $p<0.05$ BMP-4 versus vehicle, ANOVA. **[B]** siRNA knockdown of PTEN prevented BMP-mediated induction of PTEN expression and inactivation of Akt signaling, as determined by immunoblotting. Activation of SMAD signaling is unaffected, demonstrating that the BMP signaling axis remains intact in PTEN siRNA-transfected cells.

Figure 5. BMP-4 sustains PTEN protein expression in prostatic epithelial cells post-translationally by reducing PTEN protein degradation. **[A]** BMP-4 treatment of E6 cells induces PTEN mRNA expression gradually over 24 hours of treatment as determined by RT-PCR. BMP-4 treatment had no effect on PTEN mRNA stability, as determined by actinomycin D treatment **[B]**. However, BMP treatment of E6 cells causes a marked reduction in phosphorylated PTEN (P-S380, T382/383 PTEN) despite increasing amounts of total PTEN (t-PTEN) **[C]**, suggesting that PTEN protein conformation and activity may be affected by BMP. PTEN protein stability is significantly enhanced by BMP-4 treatment as determined by cyclohexamide treatment **[D]**.

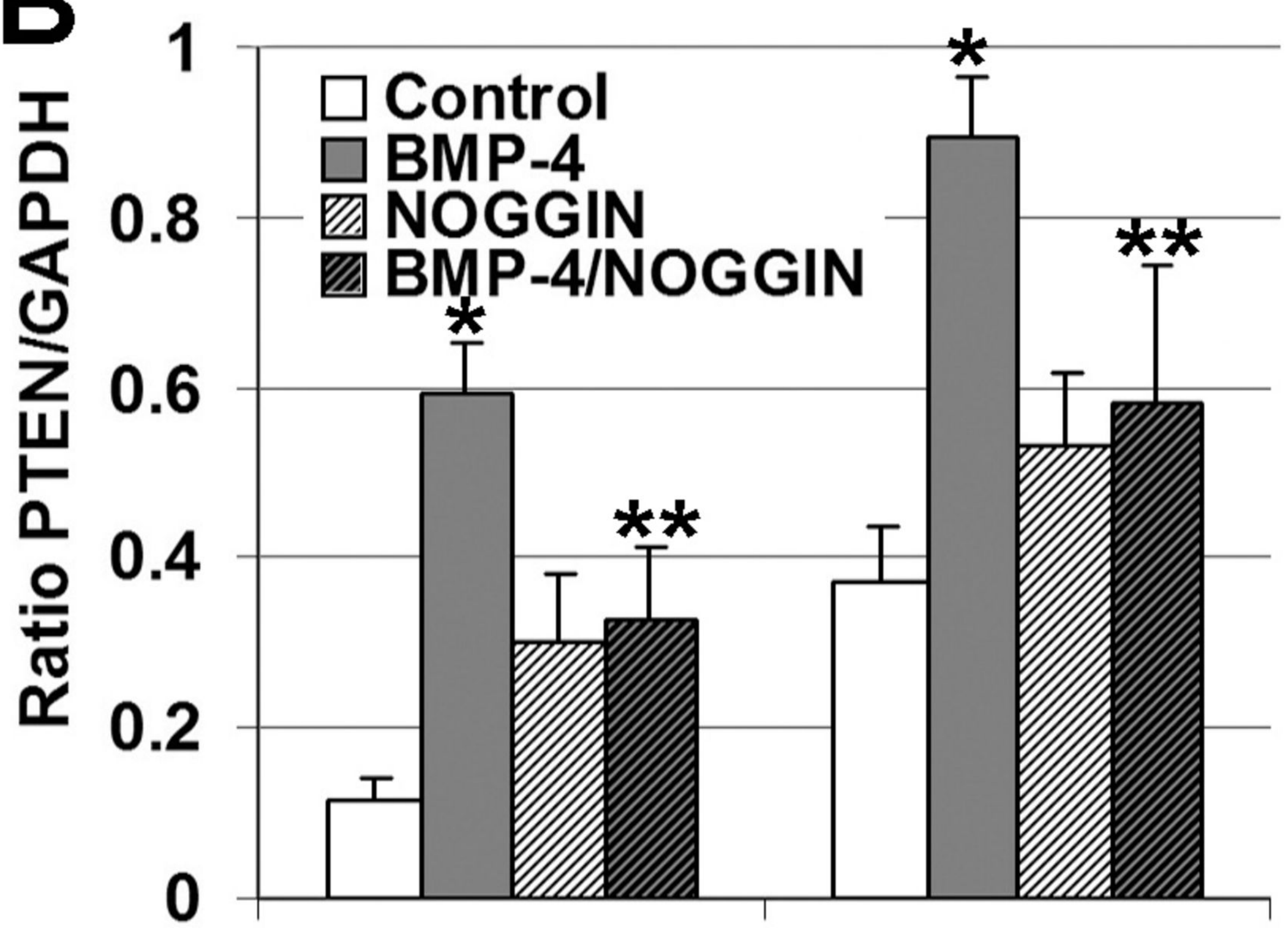
Inhibition of the 26S proteosome with 10 μM MG-132 results in similar protein stabilization effects on PTEN (not shown).


A
B
Control
1 ng/ml BMP
10 ng/ml BMP
100 ng/ml BMP
Cells per Field
800
700
600
500
400
300
200
100
0
1
2
3
4
5
Days of Treatment
*

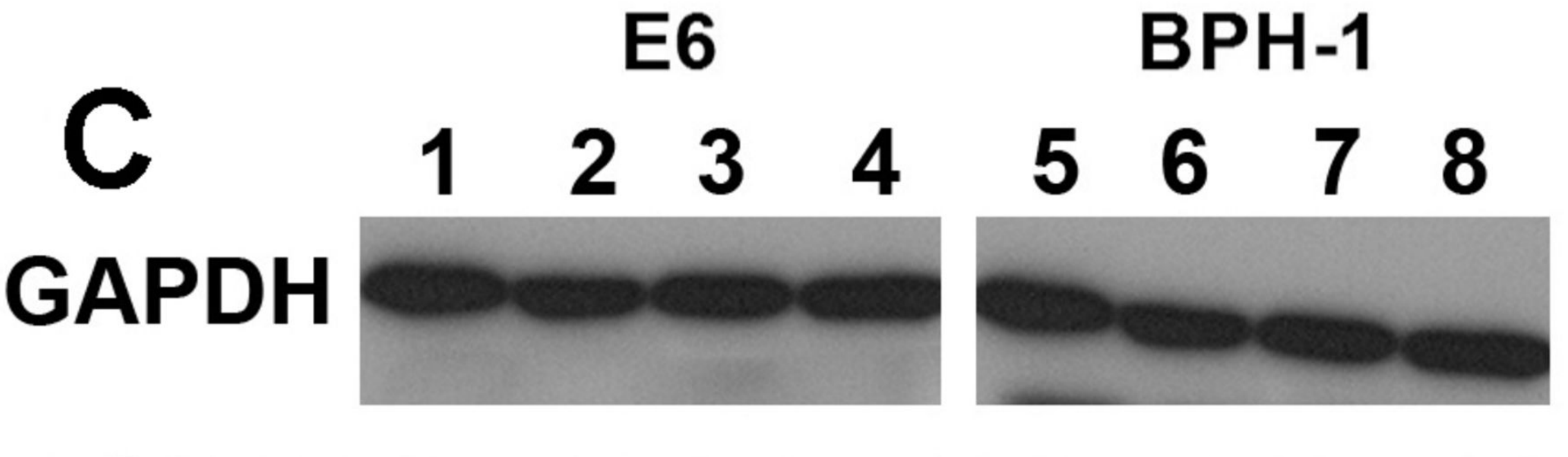
A


Ratio to GAPDH
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
E6+Vehicle
E6+BMP-4
BPH-1 + Vehicle
BPH-1 + BMP-4
PTEN
P-SMAD-1,5,8
P-Akt
Akt

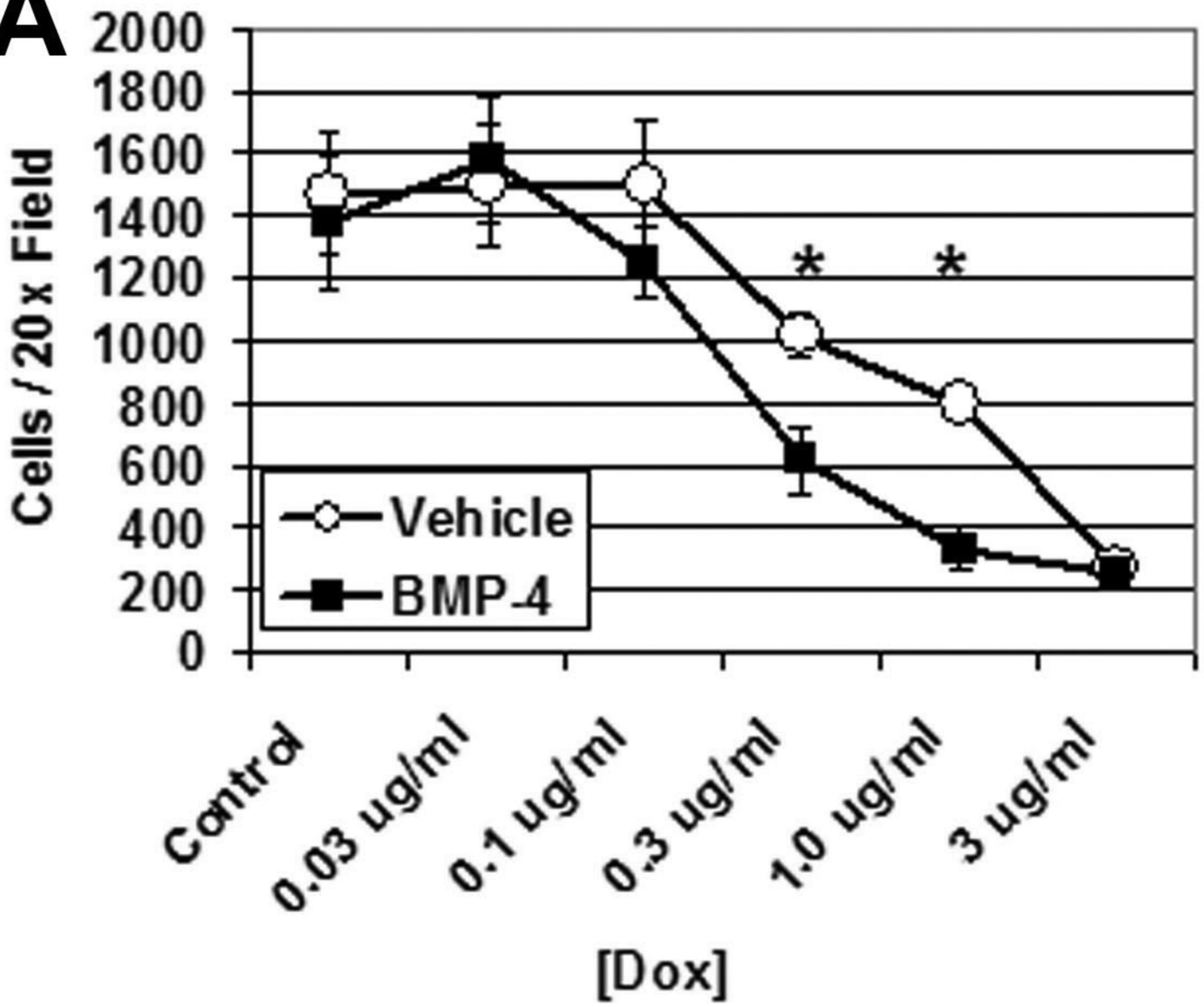
B


Ratio PTEN/GAPDH
1
0.8
0.6
0.4
0.2
0
Control
BMP-4
NOGGIN
BMP-4/NOGGIN

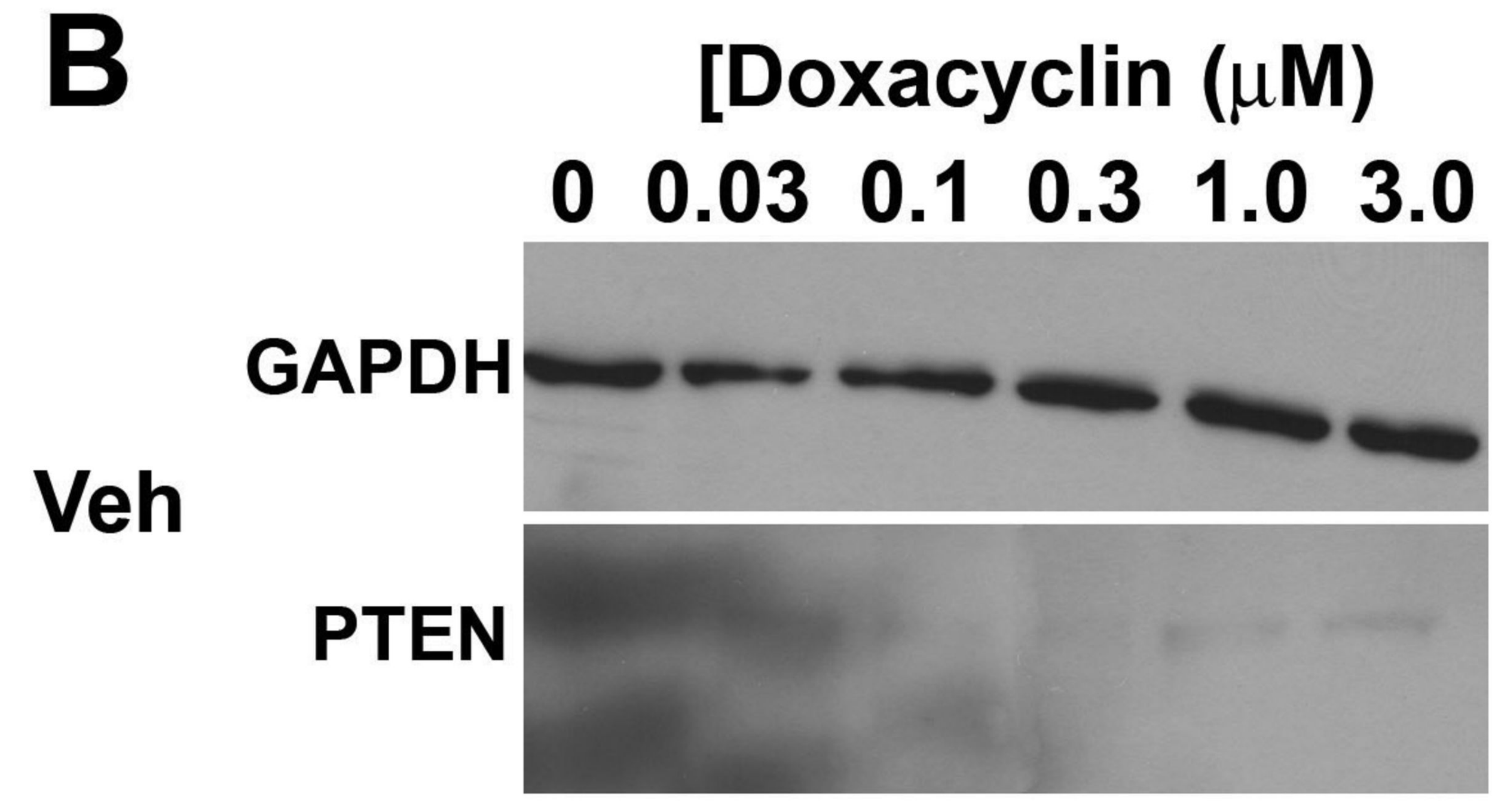
C


E6
BPH-1
1 2 3 4
5 6 7 8
GAPDH
P-SMAD 1,5,8
PTEN
P-Akt

A


2000
1800
1600
1400
1200
1000
800
600
400
200
0
Cells / 20 x Field
Vehicle
BMP-4
*
*
Control
0.03 ug/ml
0.1 ug/ml
0.3 ug/ml
1.0 ug/ml
3 ug/ml
[Dox]

B


[Doxacyclin (μM)
0 0.03 0.1 0.3 1.0 3.0
GAPDH
Veh
PTEN
GAPDH
BMP
PTEN

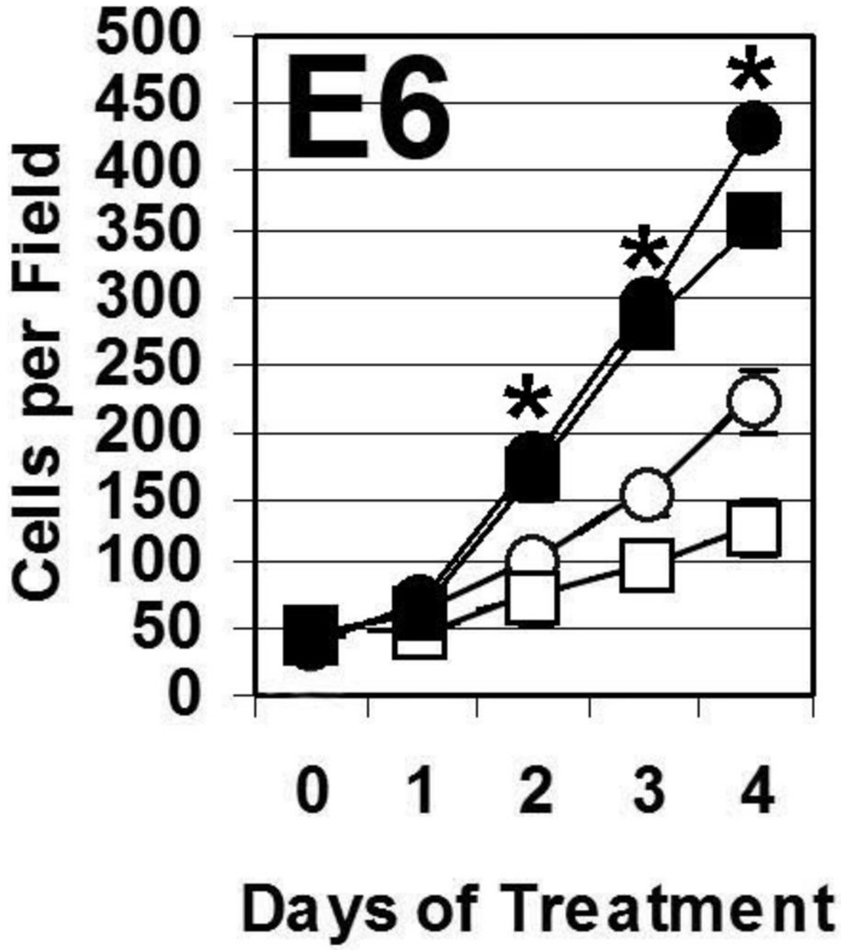
A

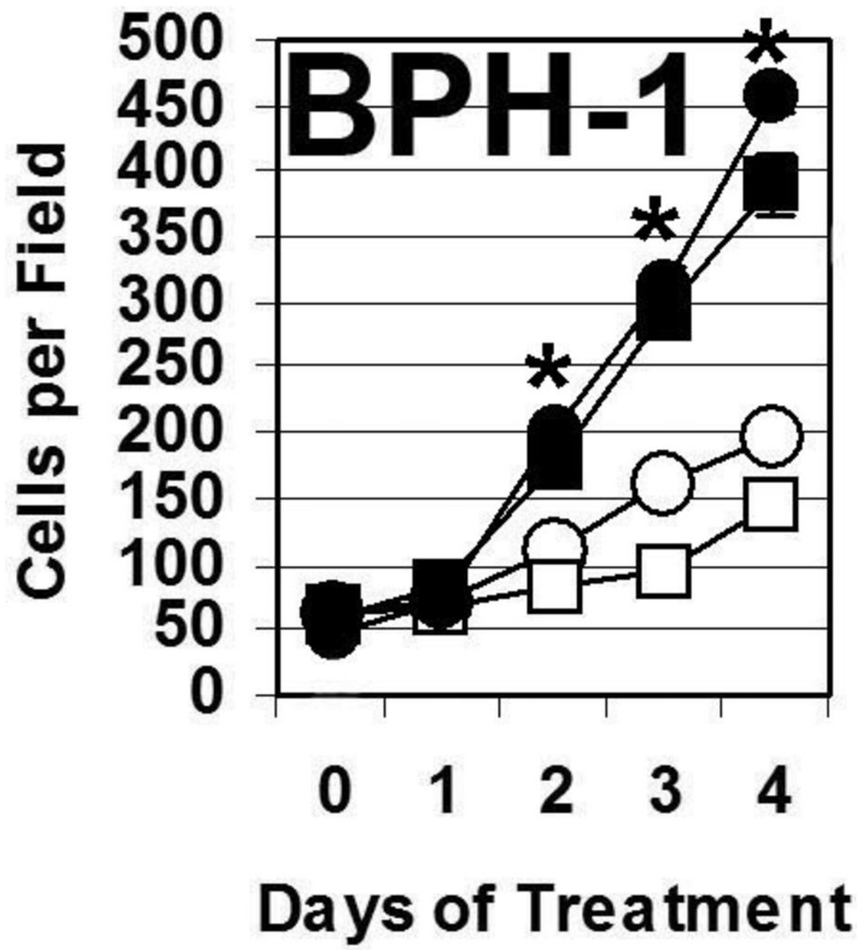
Nonsilencing Control

Nonsilencing 10 ng/ml BMP

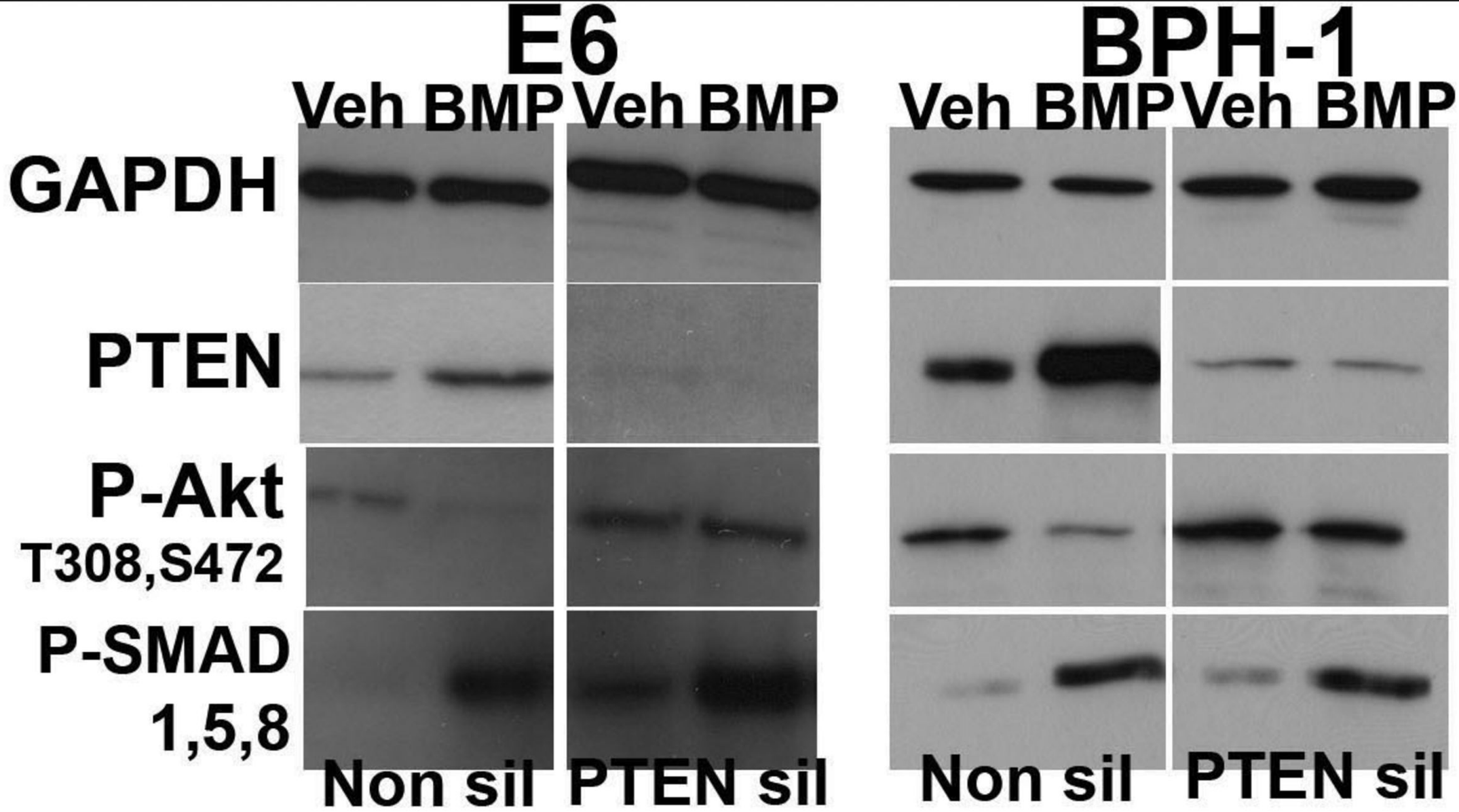
PTEN siRNA Control

PTEN siRNA 10 ng/ml BMP

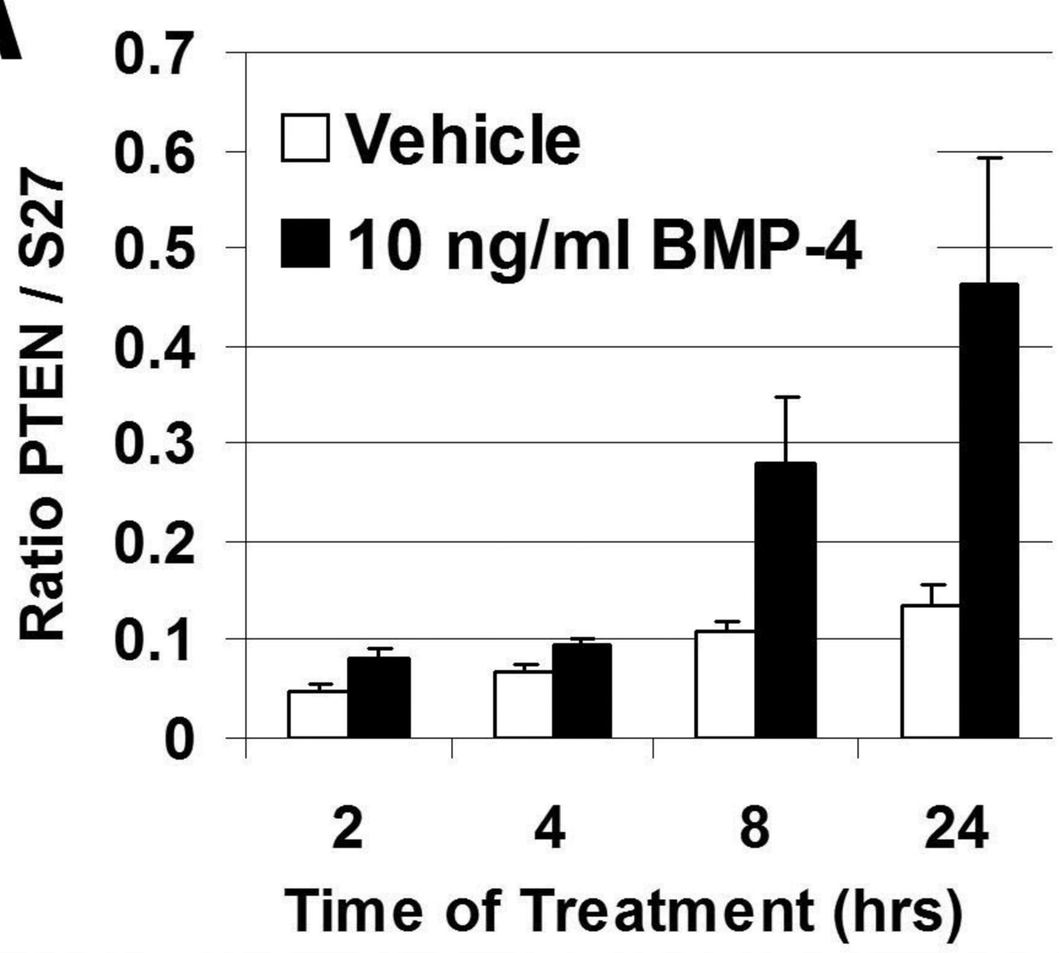




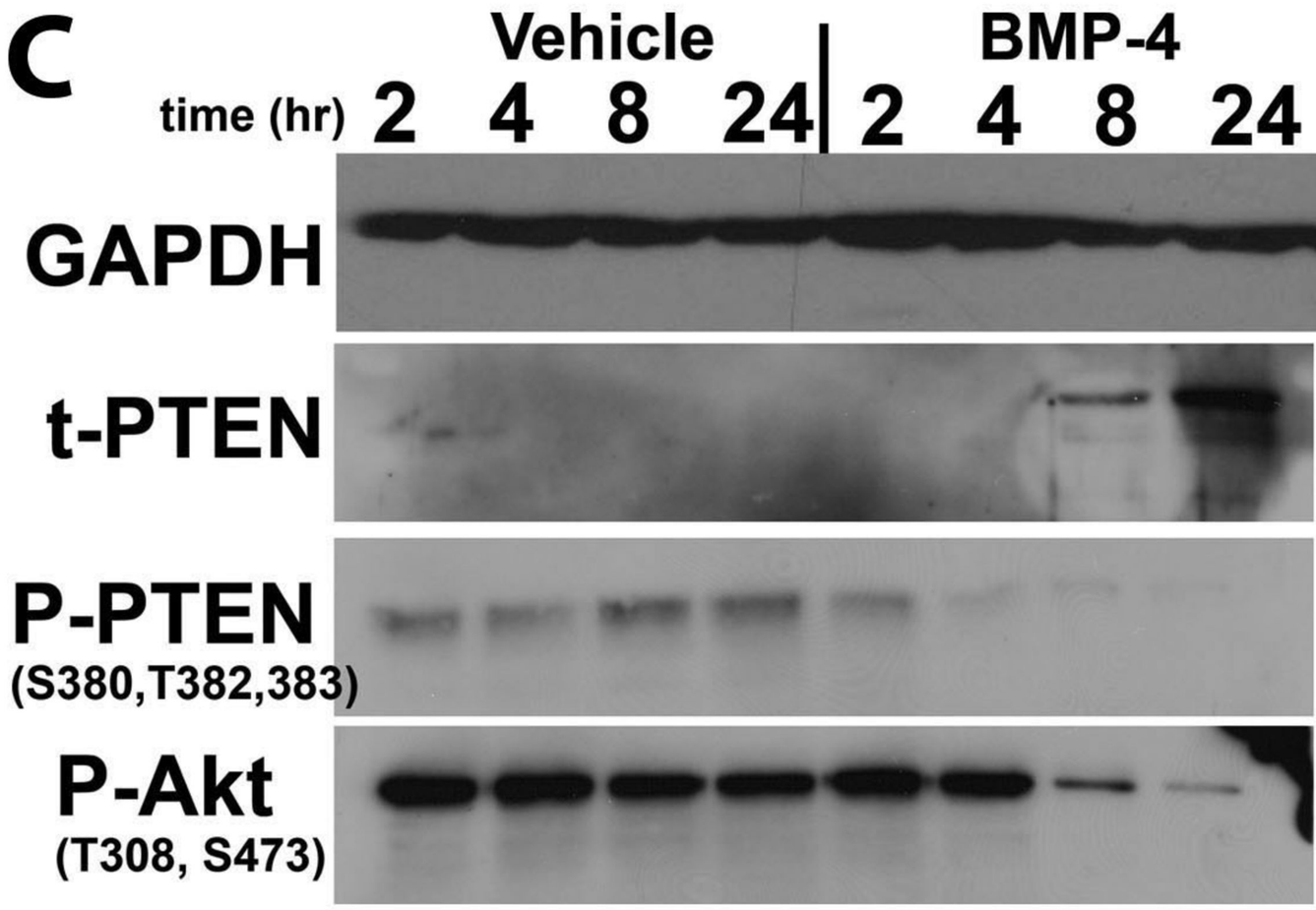
B



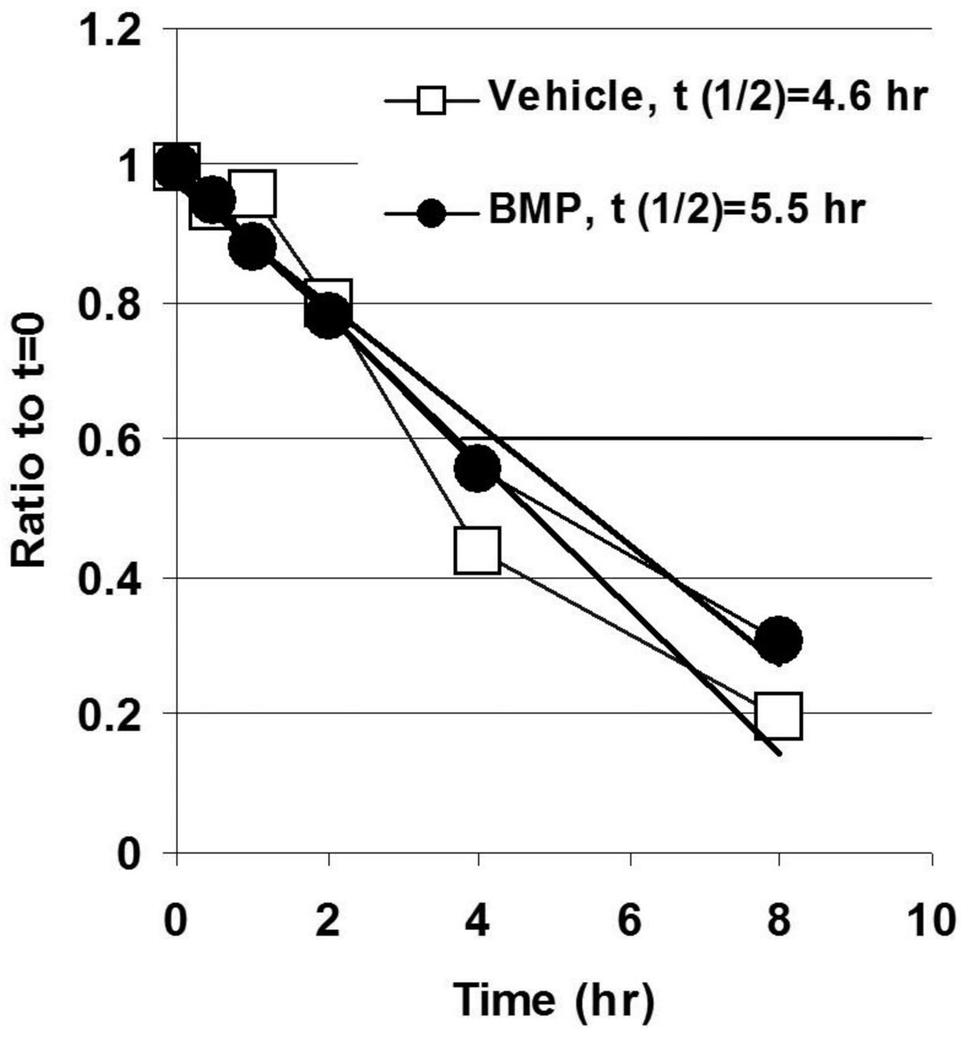
A


Ratio PTEN / S27
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Vehicle
10 ng/ml BMP-4
2
4
8
24
Time of Treatment (hrs)

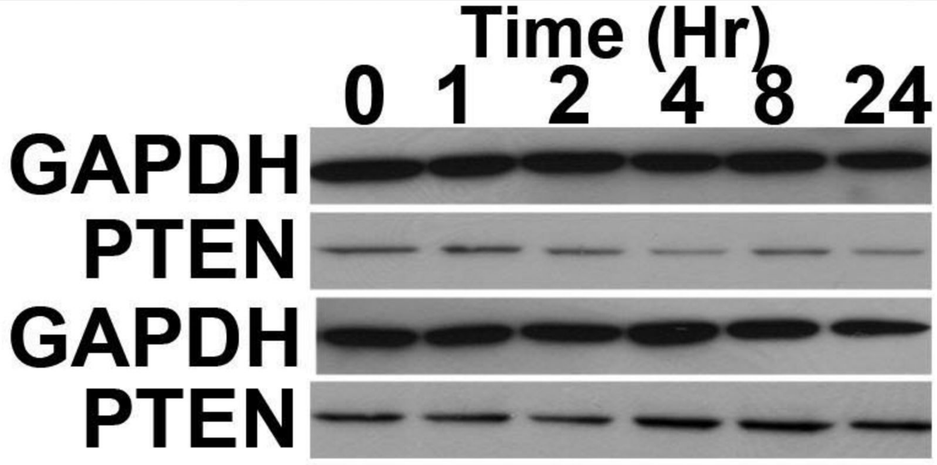
C

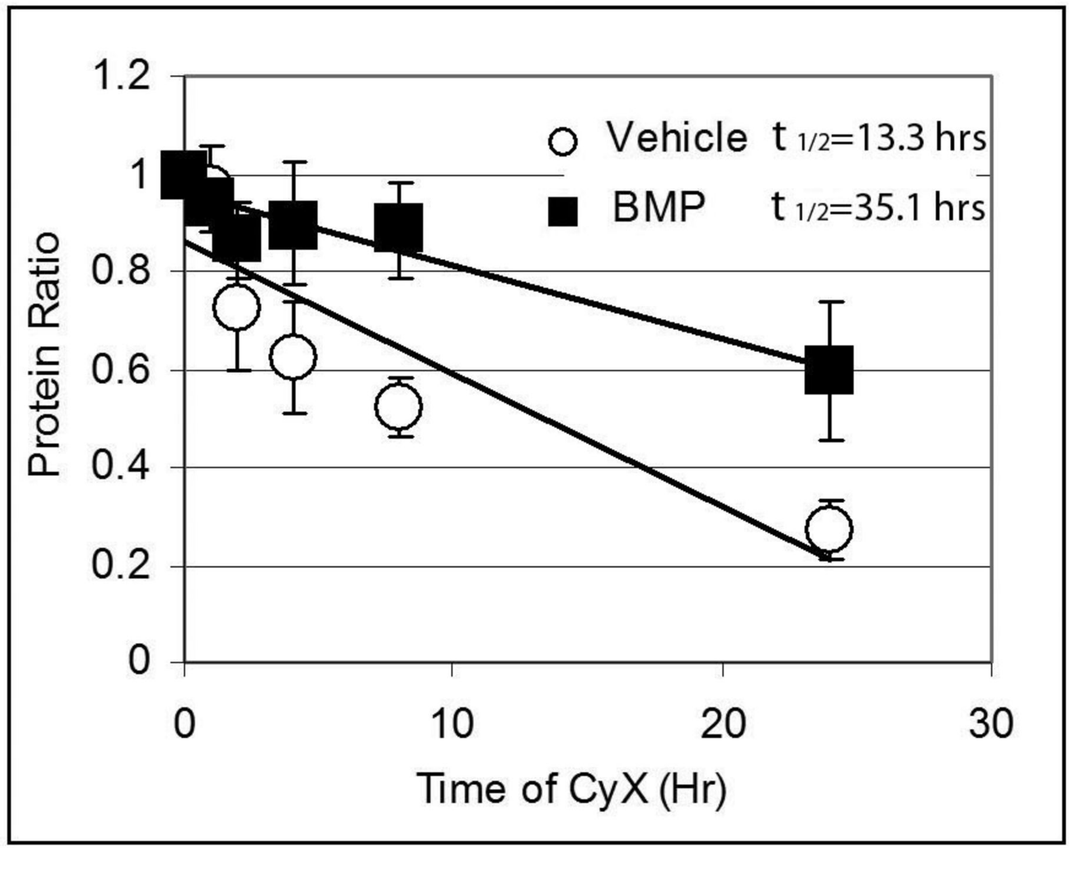

Vehicle
BMP-4
time (hr) 2 4 8 24 2 4 8 24
GAPDH
t-PTEN
P-PTEN
(S380,T382,383)
P-Akt
(T308, S473)

B


Ratio to t=0
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle, t (1/2)=4.6 hr
BMP, t (1/2)=5.5 hr
0
2
4
6
8
10
Time (hr)

D


Time (Hr)
0 1 2 4 8 24
GAPDH
PTEN
GAPDH
PTEN


Protein Ratio
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle t 1/2=13.3 hrs
BMP t 1/2=35.1 hrs
0
10
20
30
Time of CyX (Hr)