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The Role of Polycomb Group Gene Bmi-1 in the Development of Prostate Cancer

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14. ABSTRACT
We proposed to investigate the role of Bmi-1 (a member of polycomb gene family) in human prostate cancer (CaP) development. Here, we present the work accomplished during the first year of the project. Immunohistochemical analysis of prostatic specimens of 125 human CaP patients showed that Bmi-1 protein levels are highly elevated in patients with advanced disease. To understand the mechanism of action of Bmi-1, we employed two-prong strategy. Firstly, Bmi-1 was knocked down in CaP cells (LNCaP, DU145 and PC-3) by employing siRNA technique. Bmi-1-silenced CaP cells exhibited decreased proliferative and clonogenic potential. Secondly, Bmi-1 was over-expressed in CaP cells by transfecting Bmi-1 overexpressing plasmid (pbabe-Bmi-1) in CaP cells. Bmi-1-overexpressing cells exhibited increased clonogenicity and rate of proliferation. Based on the outcome of micro-array analysis, we analyzed CaP cells for Cyclin D1 (the cell cycle regulatory protein) and Bcl-2 (pro-survival protein). Silencing of Bmi-1 caused a decrease in the Cyclin D1 and Bcl-2, however an increase in p16 was observed. On the contrary, overexpression of Bmi-1 caused an increase in the levels of Cyclin D1, Bcl2- and a decrease in p16 levels. Since Cyclin D1 is a the target of Wnt signaling and Bcl-2 is the major target of Sonic Hedgehog (SHH) signaling, we hypothesize that the Bmi-1 regulates the expression of Cyclin D1 and Bcl-2 by interacting with Wnt /SHH signaling in CaP cells. We have taken steps to understand this mechanism in CaP cells and in this regard further experiments are underway. The successful outcome of these studies will provide deep-insight into the mechanism of CaP cell proliferation and would identify novel molecular targets for CaP chemotherapy.

15. SUBJECT TERMS
Bmi-1, Wnt Signaling, Bcl-2, TCF, Prostate Cancer

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Introduction:

Prostate cancer (CaP) is the most common visceral cancer diagnosed in men; it is the second leading cause of cancer related deaths in males in the United States and the Western world (1). The lack of effective therapies for advanced CaP reflects to a large extent, the paucity of knowledge about the molecular pathways involved in CaP development. Thus, the identification of new predictive biomarkers will be important for improving clinical management, leading to improved survival of patients with CaP. Such molecular targets, especially those that are indicative of proliferation, invasiveness of the disease and survival of cancerous cells (even after chemotherapy) will also be excellent candidate targets for staging the disease and establishing effectiveness of therapeutic and chemopreventive intervention of CaP (2).

The critical pathological processes that occur during the development and progression of human CaP and are known to confer aggressiveness to cancer cells are (i) abolishment of senescence of normal prostate epithelial cells (ii) self-renewablity of CaP cells even after chemotherapy and radiation and (iii) dysregulated cell cycle resulting in unchecked proliferation of cancer cells (3-4). Polycomb group (PcG) family of proteins (which form multimeric gene-repressing complexes) have been reported to be involve in self-renewablity, cell cycle regulation, and senescence (5-7). Bmi-1 is a transcription repressor originally identified as a c-myc cooperating oncogene in murine lymphoma and has emerged as an important member of PcG family (8). It has been shown to determine the proliferative potential of normal and cancer cells and is reported to be required for the self-renewal of hematopoietic and neural stem cells (9). The human Bmi-1 is located on the short arm of chromosome 10p13, a region known to be involved in translocations in various leukemias and rearrangements in malignant T-cell lymphomas (9). Bmi-1 has been shown to be overexpressed in lymphomas, non–small cell lung cancer, B-cell non-Hodgkin's lymphoma, breast cancer, colorectal cancer and nasopharyngeal carcinoma (10). Bmi-1 was has been showed to be a useful molecular marker for predicting occurrence of myelodysplastic

syndrome and prognosis of the patients (11). Cellular target genes of Bmi-1 have been identified and include *ink4a* and *ink4b* loci, encoding $p16^{INK4A}$, $p19^{ARF}$, and $p15^{INK4B}$ (12). Recently, Glinsky *et al* have shown that the activation of Bmi-1 might be associated with the malignant behavior of CaP cells (13). In the current study, we provide evidence about the over-expression of Bmi-1 in human CaP cells (in particular in highly aggressive and androgen-independent cell types) and tissue specimens and show that this correlates with the clinical stages of human CaP. We also show that the over expression of Bmi-1 breaks the senescence of normal prostate epithelial cells as well as drives proliferation of CaP cells by regulating the expression of pro-survival and proliferation-associated genes such as Cyclin D1 and Bcl-2. We propose a role for Bmi-1 protein in CaP development and suggest its potential use as a biomarker in the clinical management of CaP.

Body

Under this section we provide information about the experimental design for tasks # 1-2 and materials and methods used to accomplish our objectives as stated in the proposal.

Experimental Design for Specific Aim #1.

We conducted the experiments to define the effect of overexpression and silencing of Bmi-1 gene in CaP cells. For this purpose, we (a) knockdown the Bmi-1 gene by transfection of siRNA and (b) overexpressed the Bmi-1 gene by transfecting Bmi-1 construct (pbabe-Bmi-1 plasmid provided by Professor Chi Van Dang, Professor of Cell Biology, School of Medicine, The Johns Hopkins University, Baltimore, MD) in PC3 (androgen-insensitive), LNCaP (androgen-sensitive), CWR22Rv1 (androgen-sensitive) and normal prostate epithelial cells (PrEC) cells. We then studied the growth and viability of transfected cells *in vitro* by employing the MTT assay. To investigate the effect of Bmi-1 gene on the rate of proliferation of CaP cells, we employed ^{3}[H]thymidine uptake assay. This assays measures the amount of ^{3}[H]thymidine taken up by dividing cells (for DNA synthesis) thus gives a measure of the rate of division or proliferation of cells. Bmi-1 silenced and Bmi-1 overexpressing CaP cells were cultured in presence of ^{3}[H]thymidine and ^{3}[H] thymidine uptake was measured by Liquid scintillation counter. These cells were also measured for DNA content. Since Bmi-1 was observed to increase the proliferative potential of CaP cells and to establish that Bmi-1 indeed was a driving force for proliferating cells, we investigated whether Bmi-1 has to potential to drive proliferation of normal prostate epithelial cells.

For this purpose, Bmi-1 was overexpressed in normal prostate epithelial cells (PrEC). We chose PrEC cells because under normal culture conditions, PrEC cells are known to replicate between 3-4 cycles and after 4 cycles, these cells enter into a mode of senescence. The break of senescence in normal epithelial cells is a hall mark of progression towards proliferation. As a control to study, another set of PrEC cells were transfected alone vector (pbabe). Further a microarray was performed with Bmi-1 silenced LNCaP cells to understand the mechanism of action of Bmi-1 in CaP cells. Experiments conducted under this aim provided information whether genes involved in proliferation are regulated by Bmi-1 gene. These data were validated by western blot analysis. We analyzed the expression level of Cyclin D1, p16 and Bcl-2 protein in CaP cells. Next we investigated whether the overexpression generates the data contrary to what was observed in Bmi-1 silenced cells. For this purpose Bmi-1 was overexpressed in LNCaP, PC-3 and DU145 cells by transfecting pbabe-Bmi-1 plasmid. Cell lysates prepared from these cells were analyzed for Cyclin D1, Bcl-2 and p16 proteins by employing western blot analysis. To understand the mechanism through which Bmi-1 regulates Cyclin D1, we carried out experiments on critical pathways which are already know to be associated with Cyclin D1 expression. This includes Wnt/β-catenin signaling pathway. We asked whether Bmi-1 has any association with Wnt/β-catenin signaling (which is itself reported to control Cyclin D1). Interestingly, we found that Bmi-1 overexpression causes an increase in the transcriptional activation of *TCF*-responsive element (a bio-marker of Wnt signaling) in CaP cells. Since Bcl-2 was observed to be modulated by Bmi-1, we investigated if Bmi-1 has any association with sonic hedgehog (SHH) pathway that is very well know to regulate Bcl-2. For this purpose we determined the expression level of Bcl-2 in Bmi-1-overexpressing and Bmi-1-silenced CaP cells in presence of Cyclopamine, a SHH pathway inhibitor. We also tested if re-introduction of Bmi-1 would restore the Bcl-2 levels in CaP cells pre-treated with cyclopamine (SHH inhibitor). Further, we investigated an association of tcf and Bcl-2 in CaP cells. We investigated the mechanism through which Bmi-1 drives the Tcf/Bcl-2 signaling in CaP cells.

Material and Methods:

Cell Lines: Normal prostate epithelial cell (PrEC) was procured from Cambrex. Virally transformed prostate epithelia cells (RWPE-1), PC-3, CWR22Rv1, DU-145 and LNCaP cancer cells were obtained from ATCC

(Manassas, VA). Cells were cultured in appropriate media and were kept in a humidified atmosphere of 95% air and 5% CO_2 in an incubator at 37 ^oC.

Plasmids and siRNA: The pbabe-Bmi-1 plasmid was a kind gift from Dr. Chi V. Dang (The John Hopkins University, Baltimore, MD). pbabe vector was purchased from Addgene Inc. (Cambridge, MA). Bmi-1-siRNA and scrambled siRNA were commercially purchased from Dharmacon (Lafayette, CO). Vector-based shRNA plasmid pGeneCLIP and pGeneCLIP-Bmi-1-shRNA were procured from SA Biosciences Corporation (Fredrick, MD)

Transfections. For siRNA transfection studies, CaP cells were plated at a density of 1 x 10^6 cells per well in 6-well plates and incubated for 24, 36 and 48 h in complete medium. Using Amaxa nucleofactor kit (Gaithersburg, MD), cells were transfected with siRNAs i.e., non silencing siRNA (100 nM) and Bmi-1 siRNA (100 nM). Cells were harvested after 24, 36 and 48 h and analyzed for expression of Bmi-1. For pbabe-Bmi-1 plasmid transfection studies, CaP cells (1 x 10^6 cells per well) were transfected with 1-2 μg of the Bmi-1 construct. For controls, the same amount of empty vector, pbabe and GFP vector (as positive control for transfection) were also transfected. Cells were harvested after 24, 36 and 48 h and analyzed for expression of Bmi-1. For overexpressing Bmi-1 stably, retroviral transfection of CaP cells was performed.

Transcriptional activity of TCF and Bcl-2: pGL3-Bcl-2 was procured from Dr. Vladmier Speiglaman (Department of Dermatology, University of Wisconsin, Madison, WI) pTK-TCF-Luc (TopFlash & FopFlash) was procured from Upstate Laboratories (Lake Placid, NY). Cells were transfected with the plasmids (200 ng/well) for 24 h. *Renilla* luciferase (20 ng/well, pRL-TK; Promega, Madison, WI) was used as an internal control. In addition, for controls, the same amount of empty vectors, were transfected in cells. The cells were then harvested and transcriptional activity was measured in terms of luciferase activity in quadruplicates by using dual-luciferase reporter assay system (Promega, Madison, WI).

Western blot analysis. Cell lysates were prepared in cold lysis buffer [(0.05 mmol/L Tris-HCl, 0.15 mmol/L NaCl, 1 mole/L EGTA, 1 mol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO4, 0.5% NP-40, 1% Triton X-100, 1 mol/L phenyl methylsulfonyl flouride (pH 7.4)] with freshly added protease inhibitor cocktail (Protease

Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA). The lysate was collected, cleared by centrifugation, supernatant aliquoted and stored at -80 °C. The protein content in the lysates was measured by BCA protein assay (Pierce, Rockford, IL), as per the manufacturers' protocol. For Western blot analysis, 40 μg protein was resolved over 12% Tris-glycine polyacrylamide gels (Novex, Carlsbad, CA) under non-reduced conditions, transferred onto nitrocellulose membranes and subsequently incubated in blocking buffer (5% nonfat dry milk/1% Tween 20; in 20mmol/L TBS, pH 7.6) for 2 hours. The blots were incubated with appropriate primary (human reactive Bmi-1, Cyclin D1, Bcl-2 and p16), washed and incubated with appropriate secondary HRP-conjugated antibody (Amersham Biosciences, Piscataway, NJ). The blots were detected with chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ) and autoradiography, using XAR-5 film (Eastman Kodak, Rochester, NY). Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin (Sigma, St.Louis, MO).

Statistical analysis. All measures were summarized as means ± SE. Measures were examined for the appropriateness of a normality assumption by density estimation (data not shown). Associations of categoric variables were evaluated using the Fisher's exact test. Sample correlations were estimated using Spearman's rank correlation. All tests were two-sided and conducted at the alpha = 0.05 significance level. All statistical analyses were performed with the S-plus, Professional Version 6.2 (Insightful Corp., Seattle, WA) software.

Key Research Accomplishments:

We proposed three sub-aims under specific aim # 1. Following is the list of work completed under 1-17 months proposed time as described in the Statement of Work:

Proposed Task 1. To establish the involvement of Bmi-1 in the proliferation of human CaP cells.
Status : Submitted as 1st annual report

Proposed Task 2. To evaluate the effect of Bmi-1 overexpression and silencing on SHH and Wnt/β-catenin signaling pathways and to define the underlying mechanism of SHH-Bmi-1-β-catenin interaction at transcriptional and translational level in human CaP cells.

Sub-aim: *To determine the effect of Bmi-1 gene overexpression and silencing on the molecules associated with SHH signaling and on the transcriptional activities of Gli-1-Luc and Bcl-2-luc reporter (SHH pathway) in human CaP cells.*

Status: ***This project was transferred from University of Wisconsin to University of Minnesota. The wok on this project was suspended at least for 3 months. This was due to the process of transfer of funding from***

PI's earlier work place (University of Wisconsin) to PI's new work place (University of Minnesota). The award was relinquished by University of Wisconsin on 01/31/2010. The University of Minnesota set up a pre-award fund (for this project) on 4/13/2010. This caused a delay in executing and completing the some of the work as proposed under Task-2 of the proposal.

Experiments are underway and following tasks have been completed:

(i). **Stable Bmi-1-overexpressing CaP Cell Generation**: Stable CaP cells lines overexpressing Bmi-1 have been generated. For this purpose LNCaP cells were stably transfected with pbabe-Bmi-1. The transfections were performed by viral mode. LNCaP cells were selected in presence of puromycin. The selection of Bmi-1-overexpressing LNCaP cells under puromycin continued for 8 weeks. Cells were tested for Bmi-1 overexpression. Among 24 clones generated, we selected 3 clones those exhibited the highest degree of Bmi-1 expression level.

(ii). **Stable Bmi-1-silenced CaP Cell Generation by vector-based shRNA**: Stable CaP cells lines exhibiting least or no Bmi-1 expression levels have been generated. For this purpose LNCaP cells were stably transfected with vector-based shRNA plasmid, pGeneCLIP-Bmi-1-shRNA. The shRNA plasmids are designed using an experimentally validated algorithm. These constructs specifically knock down the expression of specific genes by RNA interference and allow for enrichment or selection of transfected cells. Each vector expresses a short hairpin RNA, or shRNA, under control of the U1 promoter and neomycin gene. Neomycin resistance permits selection of stably transfected cells. The ability to select or track and enrich shRNA-expressing cells brings RNA interference to cell lines with lower transfection efficiencies. Unlike siRNA, plasmid-based shRNA also provide a renewable source of RNA interference reagent. The transfections were performed by Lipofactamine method. LNCaP cells were selected in presence of neomycin analogue G418 (300 μg/ml). The selection of Bmi-1-overexpressing LNCaP cells under G418 continued for 8 weeks. Cells were tested for Bmi-1 expression. Among 20 clones generated, we selected 3 clones those exhibited the least or no Bmi-1 expression level.

(iii). **Use of Stable CaP Cells for Tumor Studies**: We plan to use Bmi-1-overexpressing LNCaP clones and Bmi-1-silenced LNCaP clones for tumor studies in xenograft mouse models. We plan to test the tumorigenic potential of Bmi-1-overexpressing and Bmi-1-silenced LNCaP clones in athymic nude mice. To achieve this

objective, we have very recently procured athymic nude mice from Harlen Tek laboratories. The animals are under acclimatization phase since their arrival in our animal facility.

Reportable Outcome

Bmi-1 protein expression in normal and CaP cells: As an attempt towards identifying the expression of Bmi-1 in CaP progression, we first measured protein expression levels by immunoblot analysis in several human prostate carcinoma cell lines, LNCaP, DU-145 and PC-3, and compared them to NHPE and RWPE-1 cells. Among three cell lines used, LNCaP is androgen-sensitive whereas DU-145 and PC-3 are androgen-independent. The choice of these cells was based on the fact that 80% CaP patients present with androgen-dependent disease at the time of diagnosis which later transforms into more aggressive, androgen-independent disease (14-15 and references therein). As shown in Figure 1A, all CaP cell lines exhibited a higher expression of Bmi-1 protein than in normal prostate epithelial cells. When the protein expression of Bmi-1 was compared among three cancer lines, based on the densitometric analysis of the immunoblots, highly aggressive PC-3 cells and DU145 exhibited 2.5-fold ($p<0.001$) higher expression than in LNCaP cells. These data suggest a possibility that expression of Bmi-1 protein may be correlated with disease progression and may play a role in aggressiveness of human CaP.

***Immunohistochemical analysis of Bmi-1 protein in normal and CaP specimens*:** In the next series of experiments, we used immunoperoxidase to determine Bmi-1 protein expression in specimens of age-matched normal and CaP representing all tumor stages. In the first experiment, a total of 80 samples were obtained. The staining intensity in tissue specimens were scored on a scale of 0-3. The staining pattern of Bmi-1 protein was compared in grade 1, grade 2 and grade 3 CaP specimens. The mean Bmi-1 expression was 1.5 ± 0.15 (mean ± S.E; n = 25) in grade 1 specimens, 2.5 ± 0.20 (mean ± S.E; n = 25) in grade 2 specimens, and 2.9 ± 0.15 (mean ± S.E; n = 30) in grade 3 specimens (Fig. 1B). These data show a progressive increase of Bmi-1 protein expression corresponding with increasing tumor grade in human CaP (Figure 1B).

Bmi-1 knockdown and Bmi-1 overexpression: Bmi-1 was knocked-down in CaP cells by employing siRNA technique. Cells were analyzed for Bmi-1 protein level at 24 and 36 h post-transfection. Bmi-1 protein levels was observed to be highly reduced at 36 h post-transfection (Figure 2A). This time point was selected for

further experiments and biochemical assay utilizing Bmi-1 siRNA transfections. Overexpression of Bmi-1 was achieved in CaP cells by employing transfecting pbabe-Bmi-1 plasmid in CaP cells. Cells were analyzed for Bmi-1 protein level at 24 and 36 h post-transfection. Bmi-1 protein level was observed to be highly increased at 36 h post-transfection in CaP cells. The data representing Bmi-1 protein at 36h is presented in Figure 2B. This time point was selected for further experiments and biochemical assay utilizing Bmi-1 siRNA transfections.

Effect of Bmi-1 knockdown and Bmi-1-overexpression on the growth of CaP cells: Next we investigated effect of Bmi-1 on the growth and viability of CaP cells by employing MTT assay. LNCaP cells are known to duplicate under culture conditions from 48-72 h. Similarly DU145 and PC-3 cells duplication takes 24 h under culture conditions. Culture dishes containing LNCaP, DU145 and PC-3 cells become confluent between 48-72 h. It is noteworthy that Bmi-1-silenced CaP cells did only grow between 50-65% even after 72 h post-transfection (Figure 3A). On the contrary, Bmi-1-overexpressing CaP cells exhibited significantly increased growth (Figure 3B). At 36 h post transfection control LNCaP cells displayed 35% growth while as Bmi-1 overexpressing LNCaP cells displayed 60 % growth. Similarly Bmi-1-overexpressing DU145 and PC-3 cells exhibited 75-100 % cell confluency at 36 h post-transfection as compared to control which exhibited 50% confluency (Figure 3B). These data suggest the importance of Bmi-1 in the growth of CaP cells.

Effect of Bmi-1 knockdown and Bmi-1-overexpression on of proliferation of CaP cells. We investigated whether Bmi-1 regulates the proliferation process of CaP cells. We employed a two-way approach where Bmi-1 was either knocked down or overexpressed in CaP cells and such CaP cells were later assessed for their proliferative and clonogenic potential by employing ^{3}[H]thymidine uptake assay. Firstly, LNCaP, PC-3 and DU145 cells were transfected with Bmi1-siRNA (100 nM). To investigate the effect of Bmi-1 gene suppression on the rate of proliferation of LNCaP, DU145 and PC-3 cells, we performed ^{3}[H]thymidine uptake assay. Suppression of Bmi1-1 expression resulted in the decreased rate of proliferation of CaP cells (Figure 4A). Bmi-1-overexpressing CaP cells displayed significantly increased rate of proliferation (Figure 4B).

Colony formation assay is used as a marker for proliferation of cells under *ex-vivo* conditions. Next, we investigated the effect of suppression of Bmi-1 on the clonogenic potential of CaP cells in *ex-vivo* conditions. For this purpose we employed a soft agar colony assay and assessed the clonogenic potential of CaP cells

transfected with Bmi-1 siRNA and pbabe-Bmi-1 plasmid. Bmi-1 knockdown resulted in a significant reduction in the number of colonies formed by CaP cells as compared to control siRNA-treated cells (Figure 5A). However, Bmi-1-overexpressing CaP cells exhibited increased clonogenicity potential as is evident from an increase in the average number of colonies formed by Bmi-1-overexpressing cells (Figure 5B). These data suggested that Bmi-1 confers proliferative attributes to the CaP cells.

Effect of Bmi-1 gene knockdown on CaP-associated genes: Next, we investigated the mechanism through which Bmi-1 controls the proliferation and survival of CaP cells. We performed a focused microarray analysis of 288 well-characterized proliferation and survival-associated genes in Bmi-1 suppressed-CaP cells. The list of genes that were observed to exhibit changes in their expression pattern in response to Bmi-1 knockdown in CaP cells is presented in Table 1. Most notably, we observed that suppression of Bmi-1 gene in CaP cells caused a significant reduction (> 95%) in the expression of Cyclin D1, Bcl-2, urokinase plasminogen activator (uPA), matrix metalloproteinase (MMP)-9 and nuclear factor kappa B (NFκB) (Table 1). We also observed an increased expression of p16, p15 and TIMP-3 (Table 1). Since Cyclin D1 and Bcl-2 were highly responsive to Bmi-1 gene suppression; we selected these genes for further biochemical studies.

Effect of Bmi-1 gene knockdown and Bmi-1-overexpression on Cyclin-D1 and Bcl-2 Levels: Increased Cyclin-D1 activity and Bcl-2 are considered important for the increased proliferation and survival of cancerous cells (16-21). Next, we analyzed the effect of Bmi-1 gene-suppression on the expression of cyclin D1 and Bcl-2 in LNCaP, DU145 and PC-3 cells. Bmi-1 knockdown caused a decrease in the expression level of Cyclin D1 and Bcl-2 protein in CaP cells (Figure 6A). Next we determined the effect of Bmi-1 overexpression on the expression levels of cyclin D1 and Bcl-2. CaP cells transfected with Bmi-1 construct exhibited a significant increase in the expression level of cyclin D1 and Bcl-2 protein (Figure 6B). These data were consistent with microarray data suggesting a possible association between the Bmi-1, Cyclin D1 and Bcl-2 during progression of human CaP.

Effect of Bmi-1-overexpression on transcriptional activation of TCF-responsive element: Cyclin D1 expression has been reported to be regulated by Wnt signaling (22). Since we observed that Bmi-1 also regulate

Cyclin D1 expression, we asked whether there is any association between Bmi-1 and Wnt signaling. Next we investigated effect of Bmi-1 overexpression on Wnt signaling by evaluating the transcriptional activation of *Tcf-responsive* element (marker of Wnt signaling) by employing luciferase reporter assay. It is noteworthy that Bmi-1 over-expression caused an increase in the transcriptional activation of TCF-responsive element suggesting that Bmi-1-induced Cyclin D1 expression might be through the activation of Wnt signaling (Figure 7). This is the first report where Bmi-1 is shown to regulate Wnt signaling. To fully understand the association between Bmi-1 and Wnt signaling, the work is underway.

Effect of Bmi-1 overexpression on the replicative life of normal prostate epithelial cells (PrEC): Transfection of pbabe-Bmi-1 plasmid significantly increased the replicative life of PrEC cells upto 8 passages which generally enters into a stage of senescence after 4-5 divisions (Figure 8).These data suggest that Bmi-1 possess the potential to drive normal cells towards proliferation.

Effect of Sonic hedgehog (SHH) signaling inhibition on Bmi-1-silenced and Bmi-1 overexpressing CaP cells: Keeping in view that (1) modulations in Bmi-1 expression cause modulations in the expression levels of anti-apoptotic protein Bcl-2 as shown in Figure 6, and (2) the transcriptional activation of Bcl-2 is known to be regulated by sonic hedgehog signaling, we next determined the effect of cyclopamine (SHH signaling inhibitor) treatment on the growth and viability of LNCaP and PC-3 cells exhibiting varied expression levels of Bmi-1. To achieve our objective, Bmi-1-suppressed and Bmi-1 overexpressing CaPs were treated with Cyclopamine for 12 h. As is shown in Figure 9A, Cyclopamine treatment was observed to cause 35% reduction of viability of control CaP cells (transfected with scrambled siRNA alone). Bmi-1-deficient CaP cells were significantly responsive to cyclopamine treatment and 90% reduction in the viability of Bmi-1-suppressed cells was observed (Figure 9A). On the contrary, Bmi-1-overexpressing CaP cells were non-responsive to Cyclopamine treatment (Figure 9B). Bmi-1 over-expression was observed to abrogate the effect of Cyclopamine treatment in CaP cells (Figure 9B). When tested for Bcl-2 protein, Bmi-1 overexpressing CaP cells were observed to exhibit elevated levels and the reverse was observed in Bmi-1 deficient LNCaP cells when were treated with cyclopamine (Figure 10A-B). These data suggest that Bmi-1 confers the survivability characteristics to CaP cells by inducing

the expression levels Bcl-2 (the anti-apoptotic protein) post chemotherapeutic treatment (Figures 9 & 10). These data provide evidence that in chemoresistant CaP cells, Bcl-2 activation is SHH-independent.

***Analysis of Promoter Region of Bcl-2 gene for TCF- transcriptional factor binding sites*:** Since we observed that Bmi-1 induces the activation of (1) TCF, transcriptional factor and (2) Bcl-2, the anti-apoptotic factor, we investigated the possibility of interaction among Bmi-1, Bcl-2 and TCF. We investigated possible binding sites on the promoter region of Bcl-2 gene by employing web-based TESS analysis. Interestingly, Bcl-2 promoter region exhibited multiple sites where TCF transcriptional factor possess the affinity to bind (Figure 11). These data suggested that Bcl-2 is a target of Wnt signaling.

Effect of TCF-knockdown on the transcriptional activation of Bcl-2 in CaP cells: On the basis of our observations that (1) Bmi-1 induces Bcl-2-independent of SHH signaling activation ,(2) Bmi-1 induces TCF transcriptional activation, and (3) that Bcl-2 promoter region has multiple sites for TCF binding, we next asked whether Bcl-2 is itself a target of Bmi-1-induced Wnt signaling. For this reason, TCF expression was knocked-down in LNCaP and PC-3 cells by employing TCF-specific shRNA. As compared with control CaP cells (transfected with scrambled shRNA alone), the transcriptional activation of Bcl-2 was significantly reduced in CaP cells-deficient of TCF (Figure 12). These data suggest that Bcl-2 expression is regulated by Wnt signaling in CaP cells.

Effect of Bmi-1 introduction on Bcl-2 promoter activity in TCF-silenced LNCaP cells: We asked if Bmi-1 introduction in *Tcf*-silenced cells could restore Bcl-2 transcriptional activation in cells. For this purpose LNCaP cells were transfected with *Tcf*-dominant negative vector to downregulate the expression of Bmi-1. These cells were transfected with Bcl-2-luc construct and Bcl-2 promoter activity was measured by reporter assay. As expected, *Tcf*-dn LNCaP cells exhibited reduced Bcl-2 promoter activity. However, when a set of LNCaP cells (cultured under similar conditions) exhibiting dominant negative Tcf were transfected again with Bmi-1-overexpressing plasmid, Bcl-2 promoter activity was observed to be significantly restored. These data suggest

that Bmi-1 has the potential to induce Bcl-2 expression in CaP cells. Further, when tested, these cells also exhibit restored *Tcf*-transcriptional activation.

Effect of Bmi-1 introduction on Bcl-2 promoter activity in LNCaP cells pretreated with SHH inhibitor: We asked if Bmi-1 introduction in LNCaP cells pretreated with SHH inhibitor, Cyclopamine (that reduces Bcl-2 levels) could restore Bcl-2 transcriptional activation in these cells. For this purpose LNCaP cells were treated with Cyclopamine for 24h to inhibit SHH pathway activation thus downregulating the levels of the target protein Bcl-2 (SHH target). These cells were transfected with Bcl-2-luc construct and Bcl-2 promoter activity was measured by reporter assay. As expected, Cyclopamine-treated LNCaP cells exhibited reduced Bcl-2 promoter activity. However, when a set of cyclopamine-treated LNCaP cells (cultured under similar conditions) were transfected again with Bmi-1-overexpressing plasmid, Bcl-2 promoter activity was observed to be significantly restored. These data suggest that Bmi-1 has the potential to induce Bcl-2 expression in CaP cells even under extreme conditions (Bcl-2 ablation in this case). We hypothesize that this might be one of the potential mechanisms through Bmi-1 confers survivability to cancerous cells undergoing chemotherapy. To summarize, we show that Bmi-1 confers the survivability to chemoresistant CaP cells and identified Bcl-2 as a target of Wnt signaling in CaP cells. To understand the mechanism through which CaP cells survive even after chemotherapy, and the role of Bmi-1 as a potential target for chemotherapy for CaP, further studies are underway in my laboratory.

Conclusion

Recent experimental observations documented an increased Bmi-1 expression in human non-small-cell lung cancer, human breast carcinomas, and established breast cancer cell lines, suggesting that an oncogenic role of Bmi-1 activation may be extended beyond leukemia and, perhaps, may affect progression of the epithelial malignancies as well (8-13, 23-24). Over expression of Bmi-1 is reported to confer invasive potential to glioma and breast cancer cells, and cause malignant phenotype in rat and human cancer cells, however, the role of Bmi-1 in human CaP metastasis is yet to be elucidated (8-13, 23-24). In the present study, we observed that Bmi-1

gene controls the invasiveness and growth of human CaP cells under *in vitro* and *in vivo* conditions through the regulation of Cyclin D1 and Bcl-2. To our knowledge, this report is the first demonstration that Bmi-1 regulates Cyclin D1 and Bcl-2 in human CaP cells.

In our preliminary studies (data generated in the first year of the project), we show that Bmi-1 protein levels are elevated in CaP patients if high grade tumor. Further, we also show that overexpression of Bmi-1 gene increases the rate of proliferation and invasion of CaP cells and its suppression reverses this effect. We provide evidence that suppression of Bmi-1 gene reduces the proliferative and clonogenic potential of human CaP cells. On the contrary, overexpression of Bmi-1 was observed to increase the clonogenic potential of human CaP cells.

Cyclin D1 is a critical protein that is required for cell division and proliferation. Cyclin D1 levels have been reported to be increased during several cancer types (25-33). CaP patients have been shown to exhibit increased Cyclin D1 levels (34-35), however the mechanism through which Cyclin D1 levels are altered during cancer development is not fully understood. In this study, we provide evidence that Cyclin D1 is regulated by Bmi-1 in CaP cells. It is noteworthy that CaP cells deficient in Bmi-1 exhibited decreased Cyclin D1 levels. However, Bmi-1-overexpressing CaP cells displayed an increase in Cyclin D1 protein levels suggesting significance of Bmi-1 protein for Cyclin D1 expression in CaP cells.

To understand the mechanism through which Bmi-1 regulates Cyclin D1, we studied critical pathways which are known to be associated with Cyclin D1 expression. This includes Wnt/β-catenin signaling pathway. We asked whether Bmi-1 has any association with Wnt/β-catenin signaling (which is itself reported to control Cyclin D1). Interestingly, we found that Bmi-1 overexpression causes an increase in the transcriptional activation of TCF-responsive element (a bio-marker of Wnt signaling) in CaP cells. These data are highly significant. This finding is novel and is the first report showing that Bmi-1 regulates Wnt signaling in CaP cells. Wnt signaling is report to be involved in the proliferation and chemoresistance of CaP cells. Further work to understand the mechanistic role in Wnt signaling in CaP cells is underway and will be completed by the end of 2nd year of the proposed project.

Bcl-2 protein is known to play an important role in the survival of cancer cells by conferring anti-apoptotic potential to cells (18-21). Bcl-2 levels are reported to be high in cancer cells including CaP cells (36). In the current study, we provide evidence that Bmi-1 is associated with expression of Bcl-2 in CaP cells. Bmi-1-deficient CaP cells exhibited decreased Bcl-2 levels while as Bmi-1-overexpressing CaP cells exhibited increased Bcl-2 levels. As evident from reports which suggest that Bmi-1 confers renewability or stemness characteristics to cancer cells and Bcl-2 confers survivability characteristics to cancer cells, our data showing association between Bmi-1 and Bcl-2 is highly significant. However, it would be important to fully understand the mechanism through which Bmi-1 regulates Bcl-2 in CaP cells. To understand the mechanism through which (1) Bmi-1-induces Wnt signaling, and (2) Wnt-signaling regulate Bcl-2 expression in CaP cells, we have planned experiments and some of experiments are in progress.

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Legends to Figures

Figure 1. Bmi-1 protein levels in (A) normal prostate epithelial cells, CaP cells and (B) human prostatic tumor tissues. (A) Expression of Bmi-1 protein in NHPE, RWPE1 and prostate cancer cells LNCaP, DU145 and PC-3 by western blotting. Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin antibody. The histogram indicates the relative density of the bands normalized to β-actin. Representative data for five experiments are shown here. Each bar represents mean of relative densities ± S.E. NS, non-significant. (**B)** Immunostaining for matriptase in representative specimens of CaP specimens of tumor stages I-III and non-neoplastic regions of prostatic specimens of CaP patients. CaP specimens were assigned tumor grades on the basis of Gleason pattern and Gleason score as described in Materials and Methods. Immunoreactive Bmi-1 protein was observed in a coarsely granular pattern in cell cytoplasms of epithelial cells of grade 1, grade 2 and grade 3 prostatic adenocarcinoma. There was minimal staining of occasional stromal cells in non-neoplastic regions. Bmi-1 expression was weak in normal and moderate to strong in advanced CaP specimens. ➡ Arrows indicate staining for Bmi-1 in cancer regions. Magnification X 40.

Figure. 2. Effect of Bmi-1-siRNA and pbabe-Bmi-1 plasmid transfection on the expression levels of Bmi-1 protein in CaP cells: (A) Immunoblots represent the effect of Bmi-1-siRNA transfection on the expression level of Bmi-1 protein in LNCaP, DU145 and PC-3 cells. Cells were transfected with 100 nM of siRNA directed against Bmi-1 and scrambled siRNA (100 nM). Cells were harvested at 24 and 36 h post transfection. Cell lysates were prepared as described under Materials and methods. The expression level of Bmi-1 was determined by western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for β-actin. **(B)** Immunoblots represent the effect of pbabe-Bmi-1 plasmid transfection on the expression level of Bmi-1 protein in LNCaP, DU145 and PC-3 cells at 36 h post transfection. Cells were transfected with 2 mg of pbabe-Bmi-1 plasmid and equal amount of vector (pbabe-puro). Cells were harvested at 24 and 36 h post transfection. Cell lysates were prepared as described under Materials and methods. The expression level of Bmi-1 was determined by western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for β-actin.

Figure 3. Effect of Bmi-1-knockdown and Bmi-1 over-expression on the growth and viability of CaP cells: **(A)** The histogram represents the % viability of human CaP cells LNCaP, DU145 and PC-3 at 72 h post-transfection as measured by MTT assay. **(B)** The histogram represents the % viability of human CaP cells LNCaP, DU145 and PC-3 at 36 h post-transfection as measured by MTT assay.

Figure 4. Effect of Bmi-1-knockdown and Bmi-1 over-expression on the rate of proliferation of CaP cells: **(A)**Histogram showing rate of ^{3}[H]thymidine uptake in Bmi-1-silenced CaP cells, LNCaP, DU145 and PC-3. Cells were transfected with Bmi-1-siRNA (100nM). Control cells were transfected with scrambled siRNA (100nM). Cells were incubated for 36h, the last 16 h of which were in the presence of [^{3}H]thymidine (0.5 μCi/ml). Each bar represents mean ± SE of three independent experiments. *indicates $p<0.05$. **(B)** Histogram showing rate of ^{3}[H]thymidine uptake in Bmi-1-overexpressed CaP cells, LNCaP, DU145 and PC-3. Cells were transfected with pbabe-Bmi-1(2 μg). Control cells were transfected with pbabe vector alone (2 μg). Cells were incubated for 36h, the last 16 h of which were in the presence of [^{3}H]thymidine (0.5 μCi/ml). Each bar represents mean ± SE of three independent experiments. *indicates $p<0.05$.

Figure 5. Effect of Bmi-1-knockdown and Bmi-1 over-expression on the clonogenic potential of CaP cells. **(A)**Histogram showing number of colonies formed by Bmi-1-silenced LNCaP, DU145 and PC-3 cells. Bmi-1-silenced cells were seeded in agarose and incubated at 37°C as described under Materials and methods. After 10 days of incubation, the cells were stained with crystal violet/methanol and colonies were counted. Each bar in the histogram represents mean ± S.E., * indicates $p<0.05$. All experiments were repeated three times with similar results. **(B)**Histogram showing number of colonies formed by Bmi-1-overexpressing LNCaP, DU145 and PC-3 cells. Bmi-1-overexpressing CaP cells were seeded in agarose and incubated at 37°C as described under Materials and methods. After 10 days of incubation, the cells were stained with crystal violet/methanol and colonies were counted. Each bar in the histogram represents mean ± S.E., * indicates $p<0.05$. All experiments were repeated three times with similar results.

Figure 6. Effect of Bmi-1-knockdown and Bmi-1 over-expression on the expression level of prominent proliferation-associated proteins in CaP cells. (A) Immunoblots represent the effect of Bmi-1-knockdown on

the expression level of Cyclin D1, Bcl-2 and p16 proteins in LNCaP, DU145 and PC-3 cells. Cells were transfected with 100 nM of siRNA directed against Bmi-1 and scrambled siRNA (100 nM). Cells were harvested at 36 h post transfection. Cell lysates were prepared as described under Materials and methods. The expression level of Cyclin D1, Bcl-2 and p16 were determined by western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for β-actin. **(B)** Immunoblots represent the effect of Bmi-1-overexpression on the expression level of Cyclin D1, Bcl-2 and p16 proteins in LNCaP, DU145 and PC-3 cells. Cells were transfected with 2 mg of pbabe-Bmi-1 plasmid and equal amount of vector (pbabe-puro). The expression levels of proteins were determined by western blot analysis. Equal loading was confirmed by probing the immunoblots for β-actin. The immunoblots shown here are representative of three independent experiments with similar results.

Figure 7. Effect of Bmi-1-knockdown and Bmi-1 over-expression on the transcriptional activation TCF-responsive element in CaP cells. Histogram represents the effect of Bmi-1 over-expression on the transcriptional activation of TCF responsive element (marker of Wnt/β-catenin signaling) in LNCaP, DU145 and PC-3 cells. CaP cells were transfected with pTK-TCF-Luc (pTopFlash)-constructs. pFopFlash and *Renilla* luciferase were used as negative and internal control respectively. For controls, the same amount of empty vectors, were transfected in cells. The transcriptional activity was measured in terms of luciferase activity as described under Materials and methods. Relative luciferase activity was calculated with the values from vector alone group.

Figure 8. Effect of Bmi-1 over-expression on the rate of replication or proliferation of normal prostate epithelial cells (PrEC): Micrographs showing the morphology of Bmi-1 overexpressing PrEc cells. PrEC cells were transfected with 2 mg of pbabe-Bmi-1 plasmid and equal amount of vector (pbabe-puro). Confluent dishes containing Bmi-1-overexpressing cells and vector-transfected PrEC cells were split or seeded after every 36 h. (upper Panel) Cell splitting or seeding continued for 4 passages or replication cycles in pbabe-transfected PrEC cells and did not duplicate after 4 passages and entered into senescence phase.(Lower Panel) Cell splitting or seeding continued for 8 passages or replication cycles in pbabe-transfected PrEC cells and cell replication

continued upto 4 passages. Since this was a transient transfection and the overexpression effect of Bmi-1 lasted upto 8^{th} passage only. Cells entered into senescence phase at 9^{th} passage. Inset regions (400X) showing cells with senescent morphology features of live cells such as globular shape.

Figure 9. Effect of Sonic Hedgehog signaling inhibition on the growth of CaP cells exhibiting varied Bmi-1 expression levels. Histogram represents the effect of Cyclopamine (SHH inhibitor) treatment for 12 h on the viability of Bmi-1-suppressed and Bmi-1-overexpressing LNCaP cells. **(A)** To achieve Bmi-1 knockdown, cells were transfected with 100 nM of siRNA directed against Bmi-1 and scrambled siRNA (100 nM). For controls, the same amount of scrambled siRNA were transfected in cells. At 24 h post transfection, cells were treated with cyclopamine in fresh media. Control cells were treated with DMSO (vehicle alone). After 12 h incubation of cells with cyclopamine or vehicle alone, cell viability was measured by employing MTT assay. **(B)** To achieve Bmi-1 overexpression, cells were transfected with 2 mg of pbabe-Bmi-1 plasmid and equal amount of vector (pbabe-puro). For controls, the same amount of empty vectors, were transfected in cells. At 24 h post transfection, cells were treated with cyclopamine in fresh media. Control cells were treated with DMSO (vehicle alone). After 12 h incubation of cells with cyclopamine or vehicle alone, cell viability was measured by employing MTT assay.

Figure 10. Effect of Cyclopamine treatment on the expression level of Bcl-2 in Bmi-1-suppressed and Bmi-1-overexpressing CaP cells. (A) Immunoblots represent the effect of cyclopamine treatment on the expression level of Bcl-2 protein in LNCaP cells. Cells were transfected with 100 nM of siRNA directed against Bmi-1 and scrambled siRNA (100 nM). For controls, the same amount of scrambled siRNA was transfected in cells. At 24 h post transfection, cells were treated with cyclopamine in fresh media. Control cells were treated with DMSO (vehicle alone). After 12h incubation with either cyclopamine or vehicle alone (DMSO), cells were harvested. Cell lysates were prepared as described under materials and methods. The expression level of Bcl-2 protein was determined by western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for β-actin. (**B)** Immunoblots represent the effect of cyclopamine treatment on the expression level of Bcl-2 protein in Bmi-1 overexpressing LNCaP cells. To achieve Bmi-1 over-expression, cells were

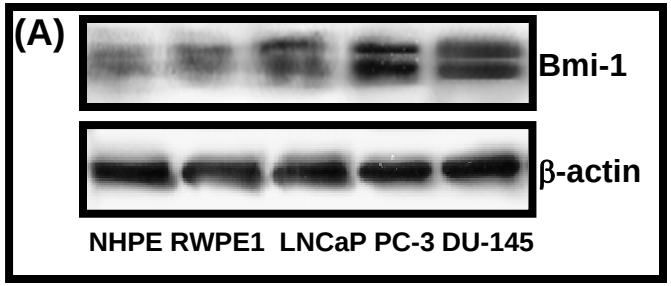
transfected with 2 mg of pbabe-Bmi-1 plasmid. For controls, the same amount of empty vector (pbabe-puro) were transfected in cells. At 24 h post transfection, cells were treated with cyclopamine in fresh media. Control cells were treated with DMSO (vehicle alone). After 12 h incubation of cells with cyclopamine or vehicle alone cells were harvested. Cell lysates were prepared as described under materials and methods. The expression level of Bcl-2 protein level was determined by western blot analysis. Equal loading was confirmed by stripping immunoblots and reprobing them for β-actin.

Figure 11. Analysis of Bcl-2 promoter region for TCF transcriptional binding sites. Bcl-2 promoter region was analyzed for binding sites for transcriptional factors by employing a web-based TESS-analysis program. Multiple binding sites for TCF transcriptional factor (key component of Wnt signaling) on promoter region of Bcl-2 gene were identified and are presented in the figure.

Figure 12. Effect of TCF-knockdown on the transcriptional activation of Bcl-2. Histogram represents the effect of TCF-knockdown on the transcriptional activation of Bcl-2 in LNCaP and PC-3 cells. CaP cells were transfected with pGL3-Bcl-2-Luc construct. For controls, the same amount of empty vector (pGL3) were transfected in cells. *Renilla* luciferase was used as internal control. The transcriptional activity was measured in terms of luciferase activity as described under materials and methods. Relative luciferase activity was calculated with the values from vector alone group. The data is presented as relative luciferase units (RLU).

Figure 13. Effect of Bmi-1-introduction on Bcl-2 promoter activity in *TCF*-dominant negative LNCaP cells. Histogram represents the effect of re-introduction of Bmi-1 on the transcriptional activation of Bcl-2 in *tcf*-dominant negative (dn) LNCaP cells. CaP cells were co-transfected with *Tcf*-dominant negative vector and pGL3-Bcl-2-Luc construct. Bcl-2 transcriptional activation was measured in these cells. *Tcf*-dn LNCaP cells were co-transfected with pbabe-Bmi-1 and pGL3-Bcl-2-luc plasmid. Bcl-2 transcriptional activation was measured in these cells. For controls, the same amount of empty vector (pGL3) were transfected in cells. *Renilla* luciferase was used as internal control. Relative luciferase activity was calculated with the values from vector alone group. The data is presented as relative luciferase units (RLU).

Figure 14. Effect of Bmi-1-introduction on Bcl-2 promoter activity in LNCaP cells pre-treated with SHH inhibitor Cyclopamine. Histogram represents the effect of Bmi-1 introduction on the transcriptional activation of Bcl-2 in LNCaP cells pretreated with SHH inhibitor Cyclopamine. LNCaP cells were transfected with pGL3-Bcl-2-Luc construct. 24h after transfection, LNCaP cells were treated with Cyclopamine for 24 h. After 24 h of cyclopamine treatment, Bcl-2 promoter activity in cells was measured. LNCaP cells were transfected with pGL3-Bcl-2-Luc construct. For controls, the same amount of empty vector (pGL3) and were transfected in cells treated with DMSO (vehicle control). *Renilla* luciferase was used as internal control. In another set of experiment (under similar culture conditions) LNCaP cells were treated with Cyclopamine for 24 h. After 24 h, cyclopamine treated cells were washed with PBS and were co-transfected with pbabe-Bmi-1 and pGL3-Bcl2-luc. *Renilla* luciferase was used as internal control. The Bcl-2 transcriptional activity was measured. Relative luciferase activity was calculated with the values from vector alone group. The data is presented as relative luciferase units (RLU).


(A)
Bmi-1
β-actin
NHPE RWPE1 LNCaP PC-3 DU-145

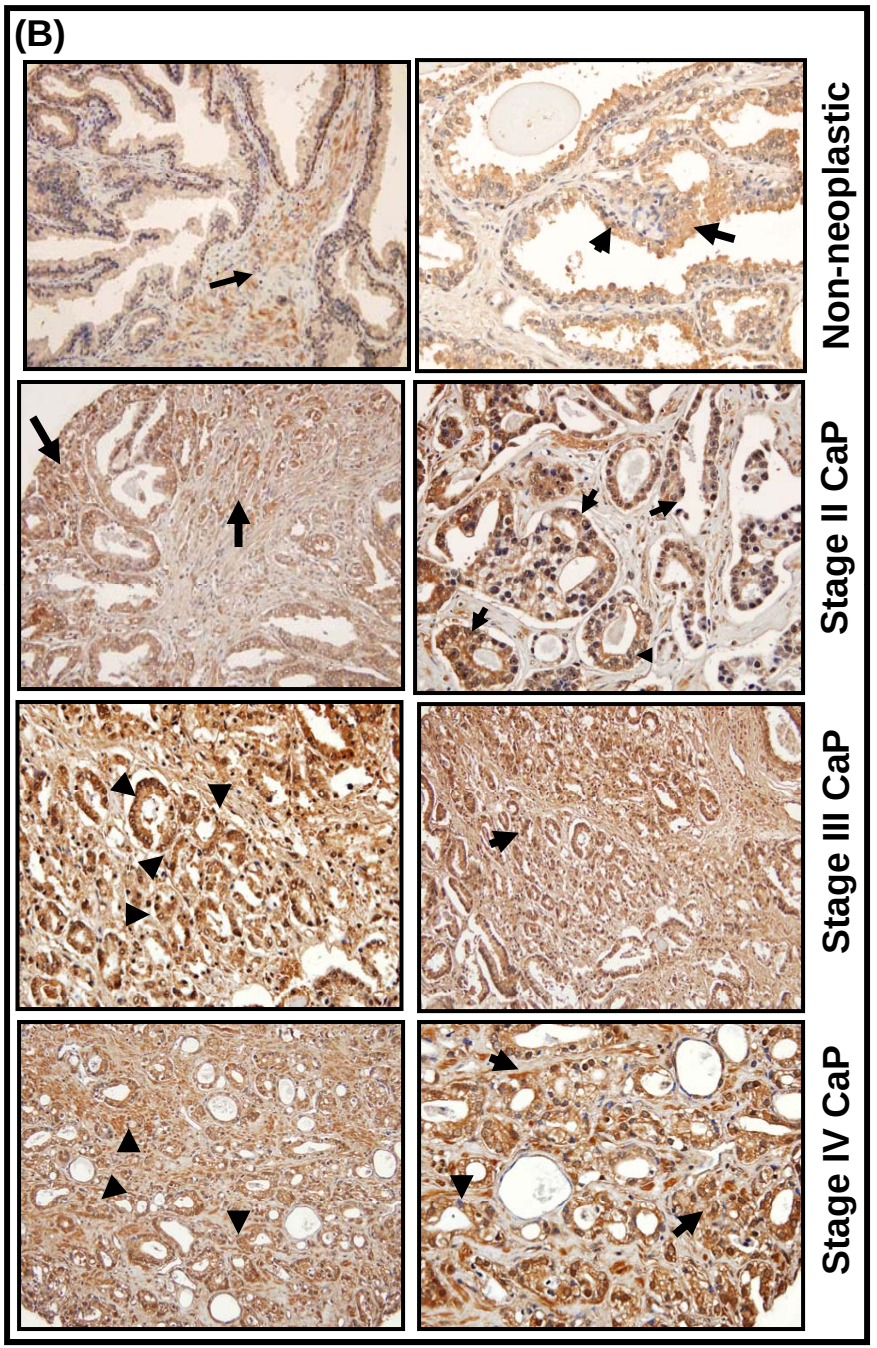

(B)
Non-neoplastic
Stage II CaP
Stage III CaP
Stage IV CaP

Figure 1

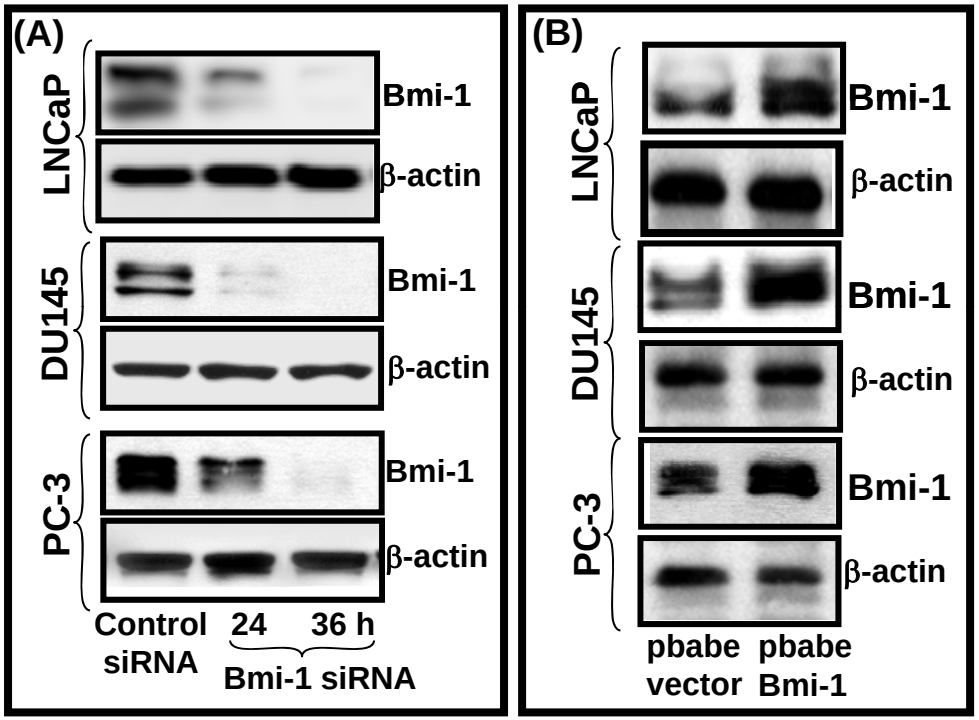

(A)
LNCaP
Bmi-1
β-actin
DU145
Bmi-1
β-actin
PC-3
Bmi-1
β-actin
Control siRNA
24
36 h
Bmi-1 siRNA
(B)
LNCaP
Bmi-1
β-actin
DU145
Bmi-1
β-actin
PC-3
Bmi-1
β-actin
pbabe vector
pbabe Bmi-1

Figure 2

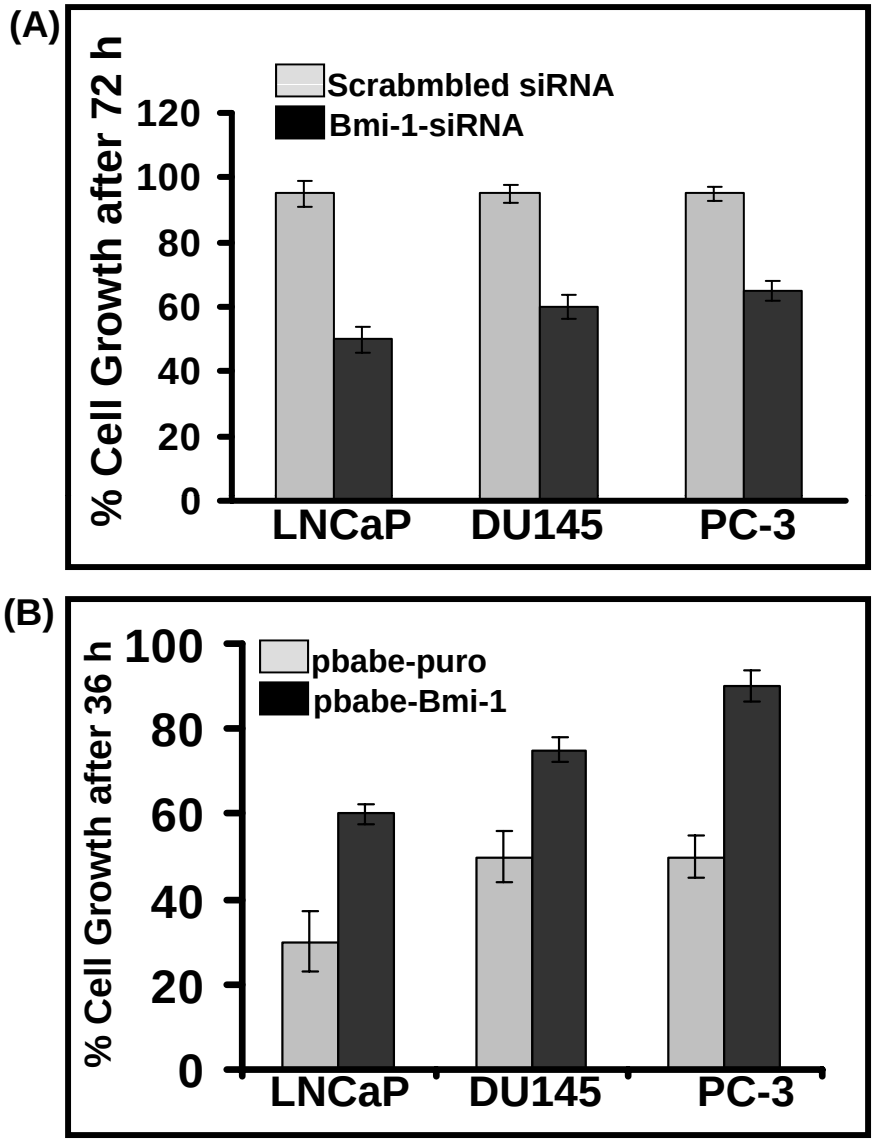

(A)
% Cell Growth after 72 h
Scrabmbled siRNA
Bmi-1-siRNA
120
100
80
60
40
20
0
LNCaP
DU145
PC-3
(B)
% Cell Growth after 36 h
pbabe-puro
pbabe-Bmi-1
100
80
60
40
20
0
LNCaP
DU145
PC-3

Figure 3

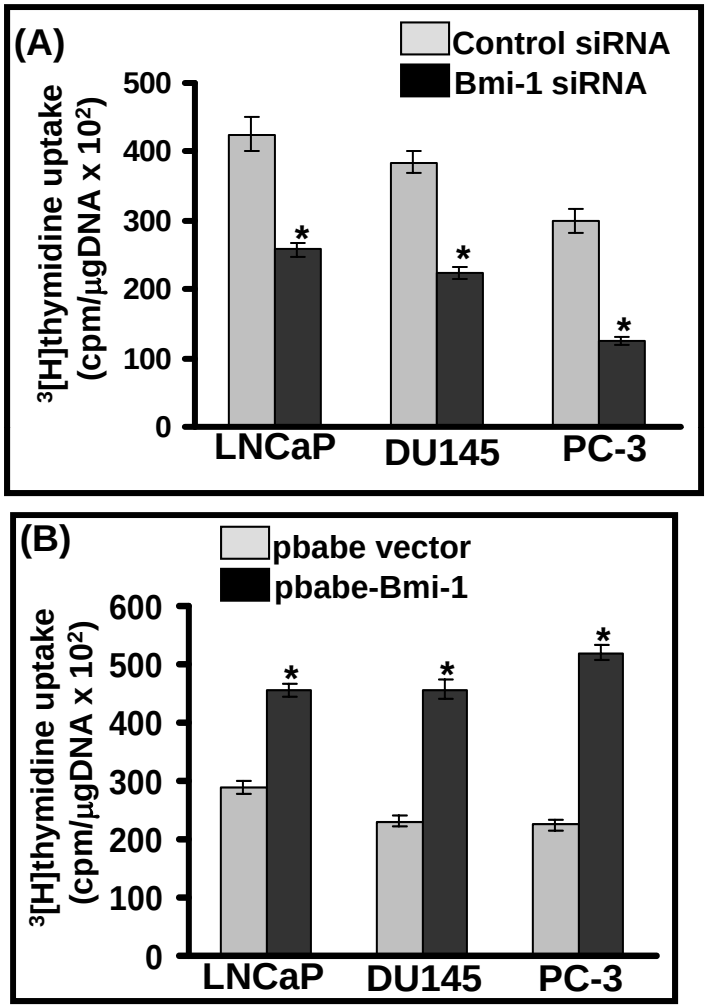

(A)
Control siRNA
Bmi-1 siRNA
^{3}[H]thymidine uptake (cpm/μgDNA x 10^2)
500
400
300
200
100
0
*
*
*
LNCaP
DU145
PC-3
(B)
pbabe vector
pbabe-Bmi-1
^{3}[H]thymidine uptake (cpm/μgDNA x 10^2)
600
500
400
300
200
100
0
*
*
*
LNCaP
DU145
PC-3

Figure 4

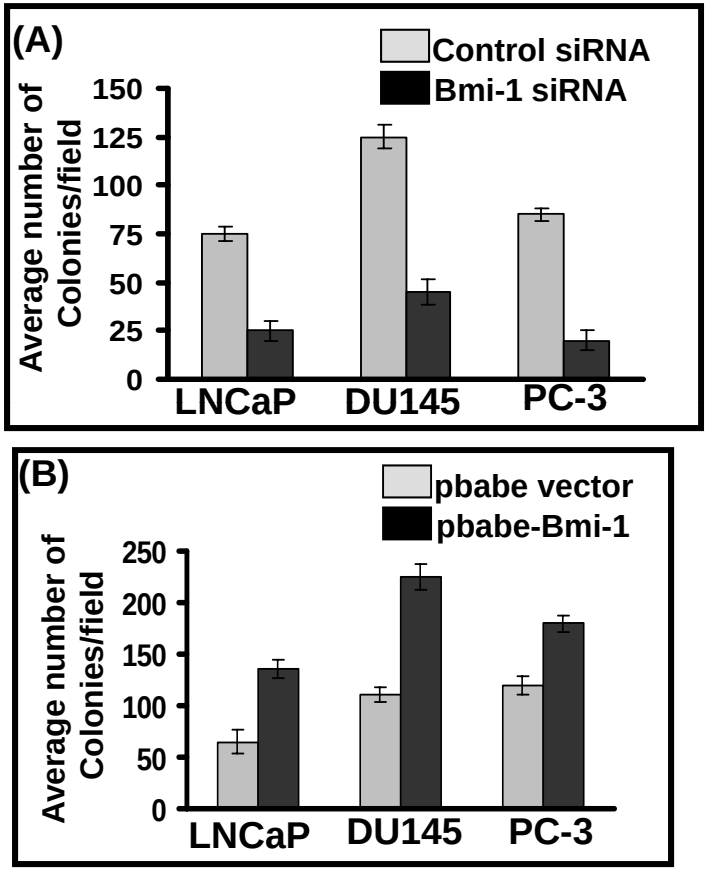

(A)
Control siRNA
Bmi-1 siRNA
Average number of Colonies/field
150
125
100
75
50
25
0
LNCaP
DU145
PC-3
(B)
pbabe vector
pbabe-Bmi-1
Average number of Colonies/field
250
200
150
100
50
0
LNCaP
DU145
PC-3

Figure 5

Table 1. Effect of Bmi-1 knockdown on Expression of Proliferation-Associated Genes in CaP Cells

Gene Bank Accession Number	Gene Description	Gene status after Bmi-1-siRNA transfection
NM_053-56	Cyclin D1	Down-regulated **
NM_000075	Cyclin-dependent kinase 4 (Cdk4)	Down-regulated **
NM_000657	Bcl-2	Down-regulated **
NM_005163	Akt1	Down-regulated *
NM_004570	PI3K (catalytic gamma peptide)	Down-regulated *
NM_002658	Urokinase plasminogen activator	Down-regulated **
NM_001005862	Erbb2	Down-regulated **
NM_005417	Src	Down-regulated *
NM_002467	c-myc	Down-regulated **
NM_002228	c-Jun	Down-regulated *
NM_005343	H-Ras	Down-regulated *
NM_001033756	Vascular endothelial growth factor (VEGF)	Down-regulated **
NM_0004994	Matrix metalloproteinase -9 (MMP-9)	Down-regulated **
NM_003998	Nuclear factor kappa B 1(NFκB1)	Down-regulated *
NM_001530	HIF-1	Down-regulated *
NM_000875	Insulin growth factor 1(IGF1)	Down-regulated **
NM_0008756	Insulin growth factor 1(IGF2)	Down-regulated **
NM_000586	Interlukin 2(IL-2)	Down-regulated **
NM_013430	GGT2	Down-regulated *
NM_006254	Protein kinase C delta (PKC δ)	Down-regulated *
NM_005400	Protein kinase C epsilon (PKC ε)	Down-regulated*
NM_058195	p16/INK4	Up-regulated **
NM_004936	p15	Up-regulated **
NM_000076	p57	Up-regulated **
NM_006016	CD164	Up-regulated *
NM_016279	Cadherin-9	Up-regulated *
NM_000362	TIMP-3	Up-regulated *
NM_173206	PIAS2	Up-regulated **

* Represents 2- 5 fold and ** represents more than 5 fold.

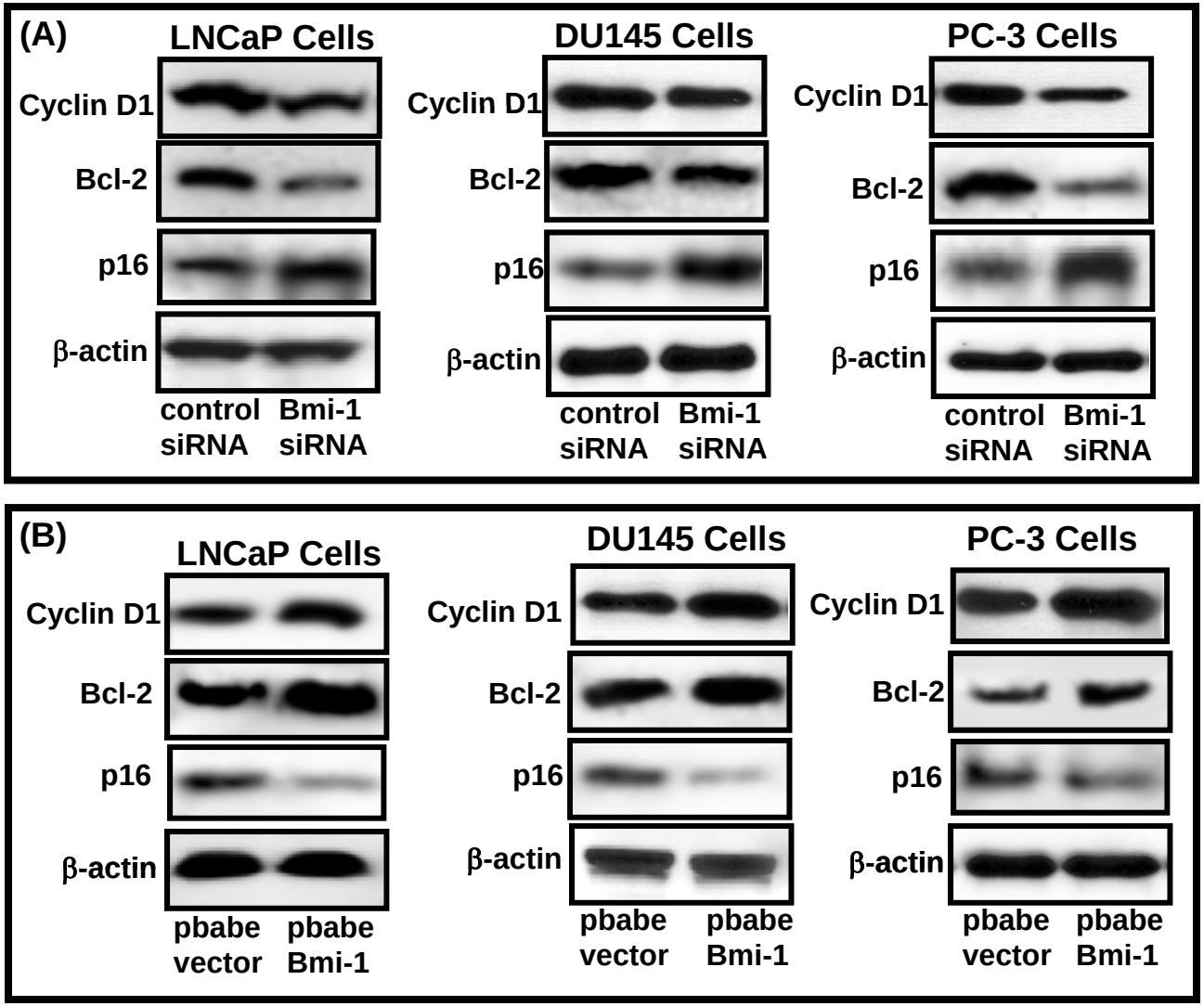

(A)
LNCaP Cells
DU145 Cells
PC-3 Cells
Cyclin D1
Bcl-2
p16
β-actin
control siRNA
Bmi-1 siRNA
(B)
LNCaP Cells
DU145 Cells
PC-3 Cells
Cyclin D1
Bcl-2
p16
β-actin
pbabe vector
pbabe Bmi-1

Figure 6

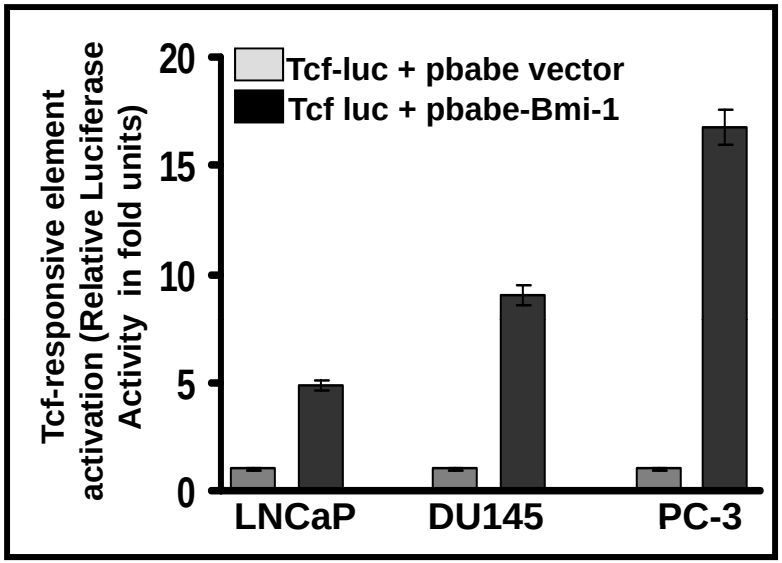

Tcf-responsive element activation (Relative Luciferase Activity in fold units)
20
15
10
5
0
Tcf-luc + pbabe vector
Tcf luc + pbabe-Bmi-1
LNCaP
DU145
PC-3

Figure 7

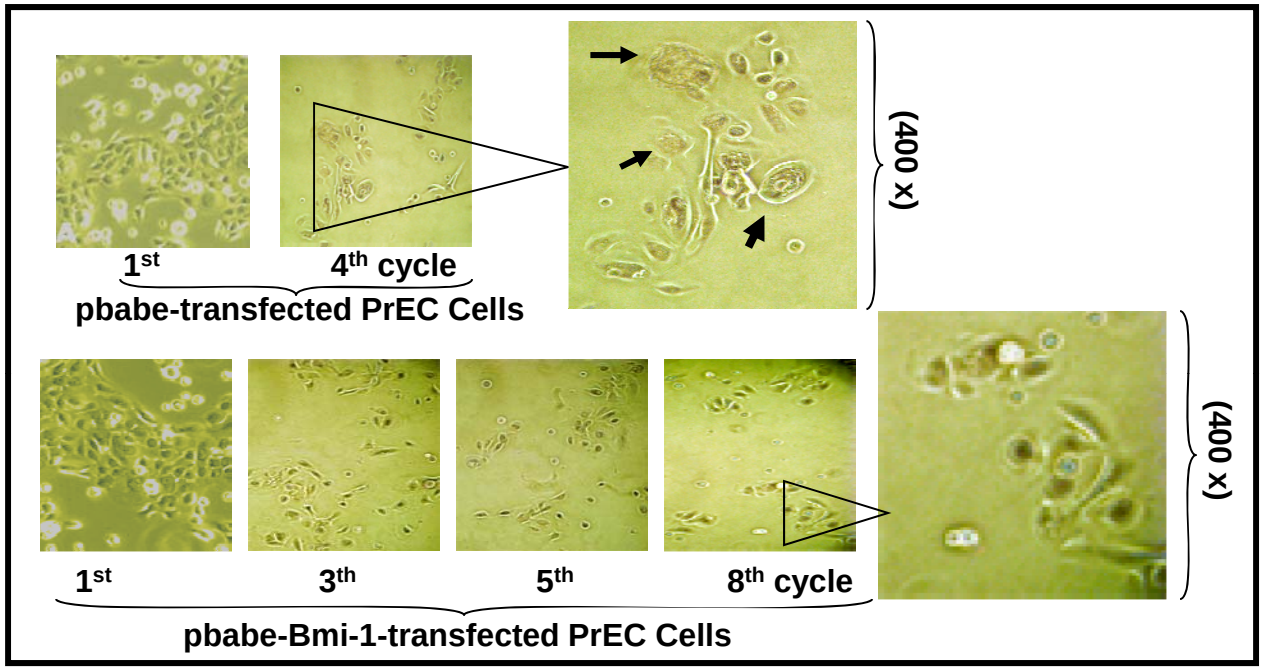

1st
4th cycle
pbabe-transfected PrEC Cells
(400 x)
1st
3th
5th
8th cycle
pbabe-Bmi-1-transfected PrEC Cells
(400 x)

Figure 8

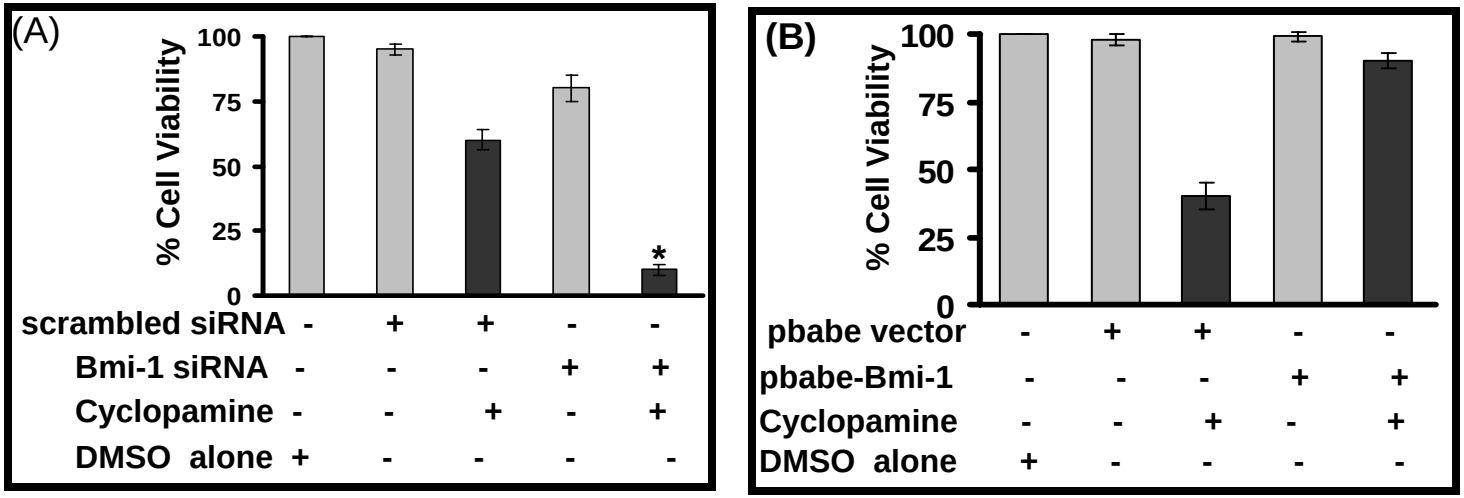

(A)
% Cell Viability
100
75
50
25
0
*
scrambled siRNA - + + - -
Bmi-1 siRNA - - - + +
Cyclopamine - - + - +
DMSO alone + - - - -
(B)
% Cell Viability
100
75
50
25
0
pbabe vector - + + - -
pbabe-Bmi-1 - - - + +
Cyclopamine - - + - +
DMSO alone + - - - -

Figure 9

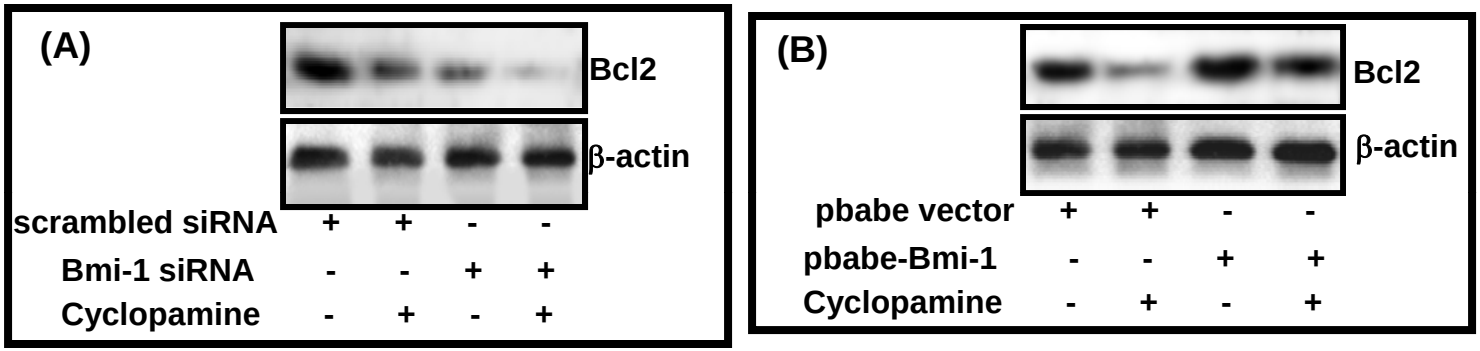

(A)
Bcl2
β-actin
scrambled siRNA + + - -
Bmi-1 siRNA - - + +
Cyclopamine - + - +
(B)
Bcl2
β-actin
pbabe vector + + - -
pbabe-Bmi-1 - - + +
Cyclopamine - + - +

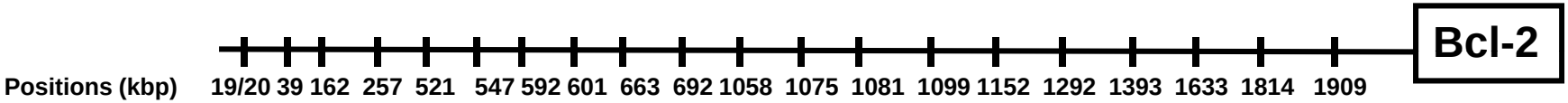
Figure 10

(TESS Analysis)

Analysis of Promoter Region of Bcl-2 Gene

Length of Sequence for TCF binding on Bcl-2 promoter : 5

Binding sites for
transcriptional factor TCF
(T00999-T1001)



1.98 kbp

Figure 11

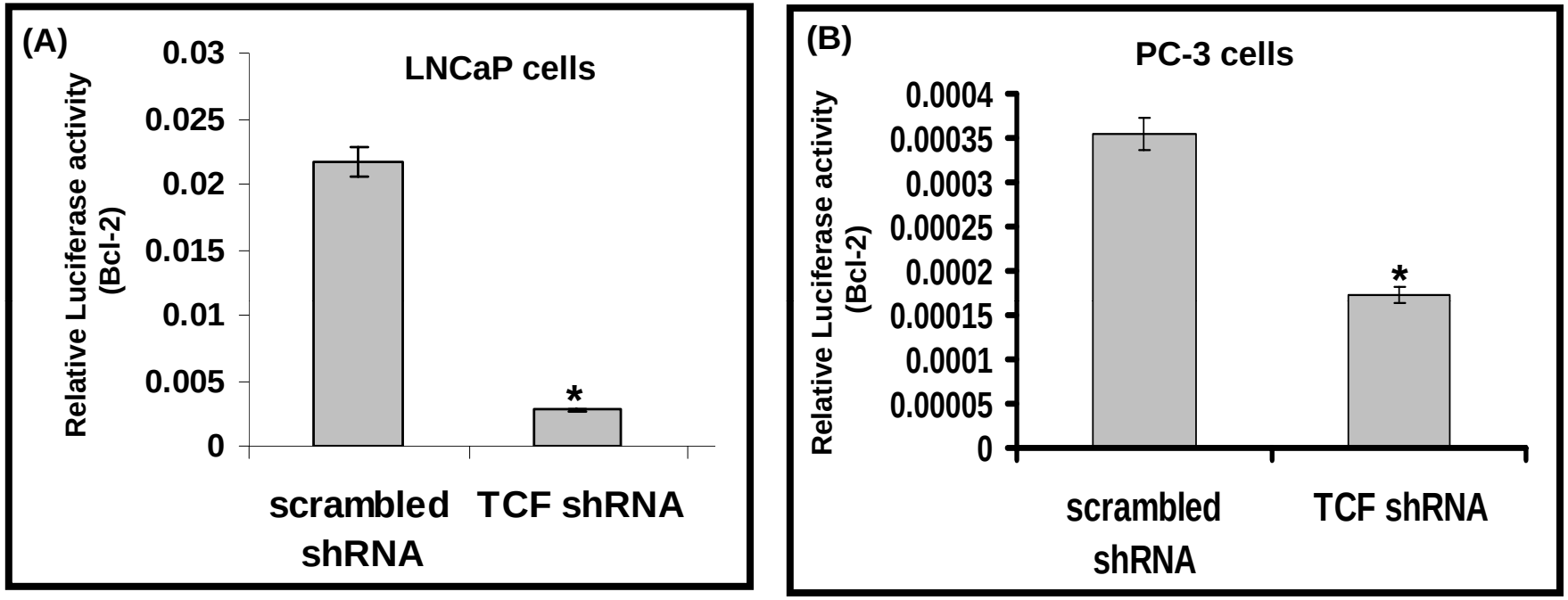

(A)
LNCaP cells
Relative Luciferase activity (Bcl-2)
0.03
0.025
0.02
0.015
0.01
0.005
0
*
scrambled shRNA
TCF shRNA
(B)
PC-3 cells
Relative Luciferase activity (Bcl-2)
0.0004
0.00035
0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
*
scrambled shRNA
TCF shRNA

Figure 12

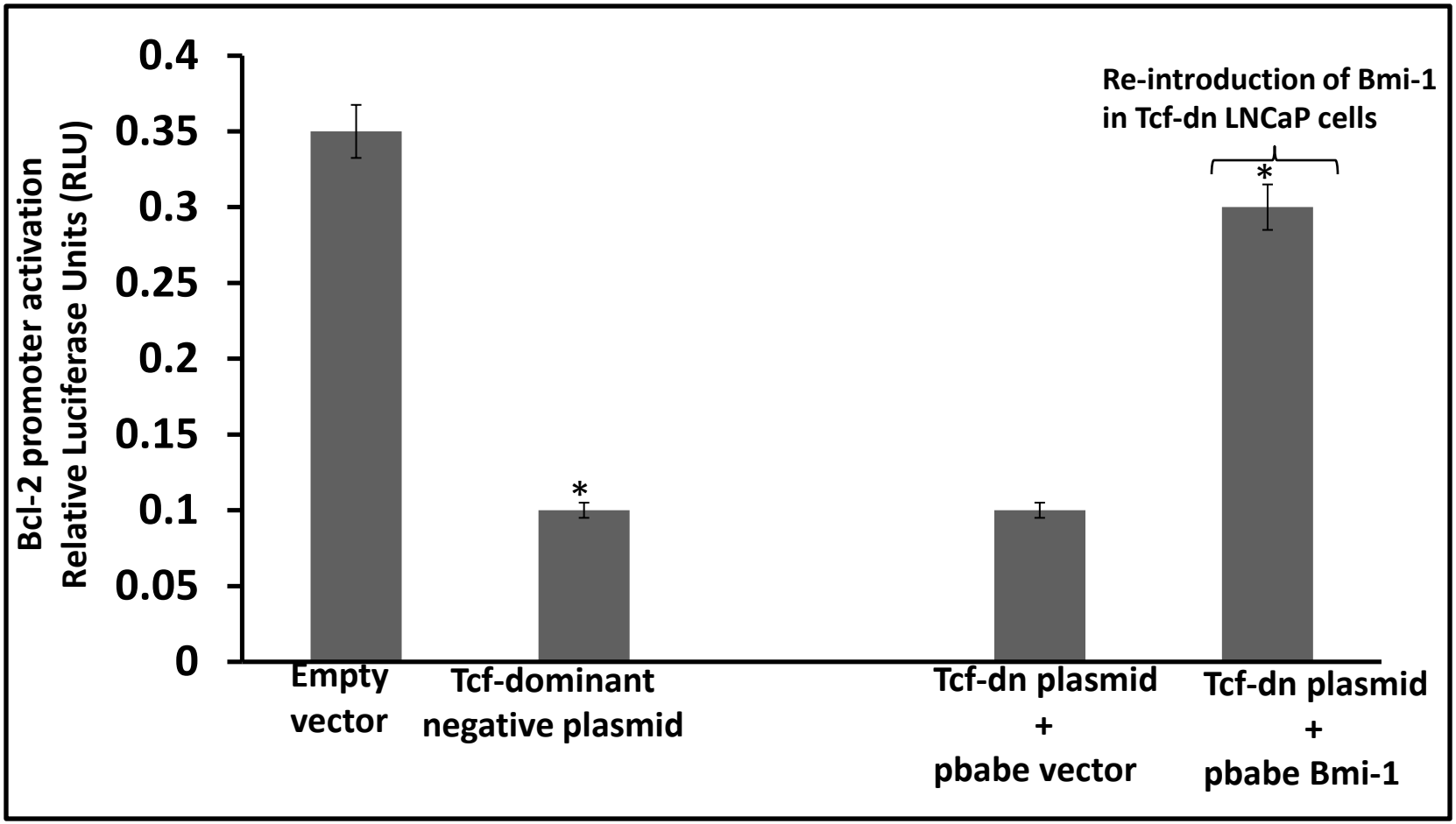

Bcl-2 promoter activation
Relative Luciferase Units (RLU)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
Re-introduction of Bmi-1
in Tcf-dn LNCaP cells
Empty
vector
Tcf-dominant
negative plasmid
Tcf-dn plasmid
+
pbabe vector
Tcf-dn plasmid
+
pbabe Bmi-1

Figure 13

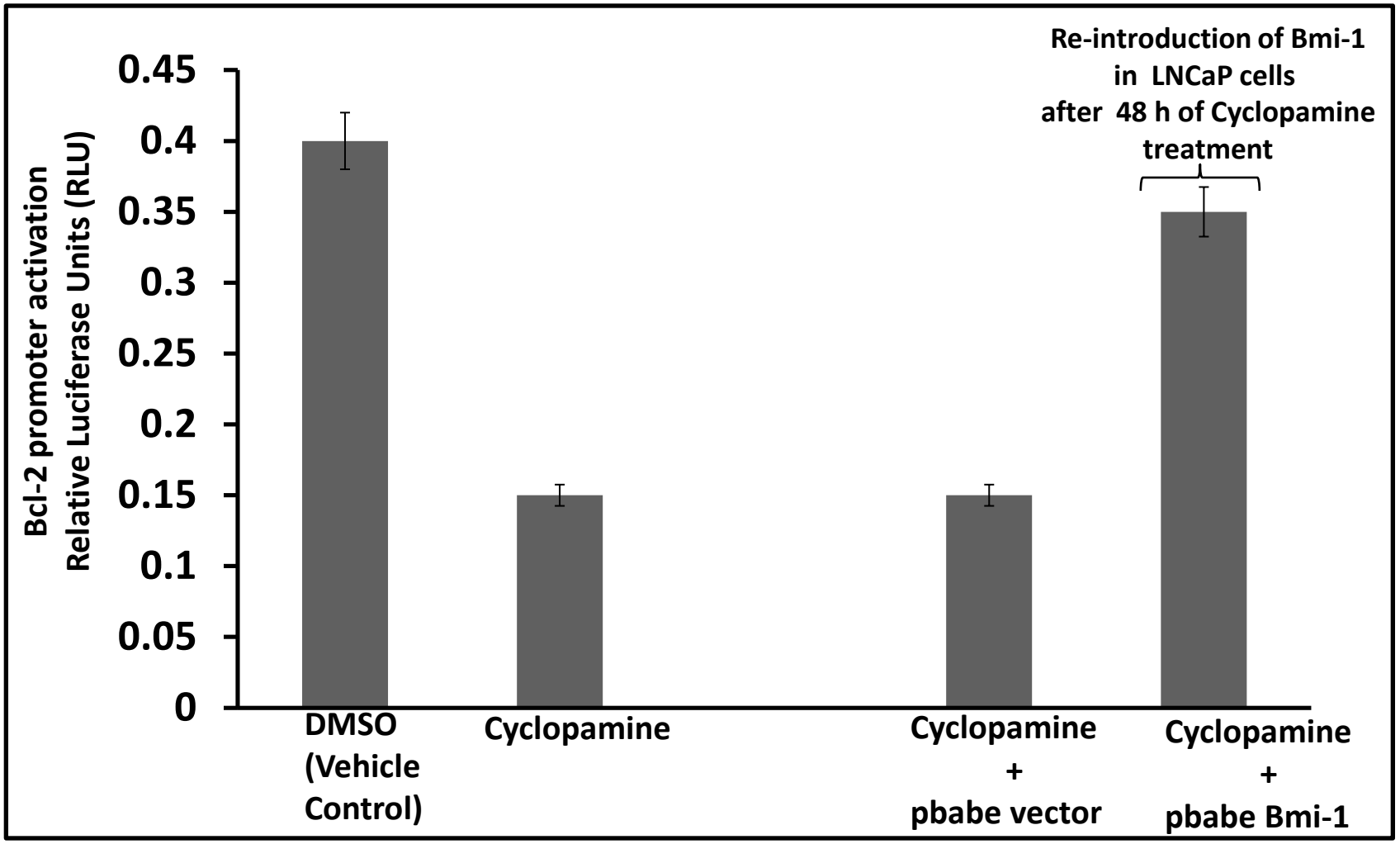

Re-introduction of Bmi-1
in LNCaP cells
after 48 h of Cyclopamine
treatment
Bcl-2 promoter activation
Relative Luciferase Units (RLU)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
DMSO
(Vehicle
Control)
Cyclopamine
Cyclopamine
+
pbabe vector
Cyclopamine
+
pbabe Bmi-1

Figure 14