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**Research Article** **Oncology**

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# The polycomb group protein Bmi-1 represses the tumor suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells

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**The polycomb group protein B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1) is dysregulated in various cancers, and its upregulation strongly correlates with an invasive phenotype and poor prognosis in patients with nasopharyngeal carcinomas. However, the underlying mechanism of Bmi-1–mediated invasiveness remains unknown. In the current study, we found that upregulation of Bmi-1 induced epithelial-mesenchymal transition (EMT) and enhanced the motility and invasiveness of human nasopharyngeal epithelial cells, whereas silencing endogenous Bmi-1 expression reversed EMT and reduced motility. Furthermore, upregulation of Bmi-1 led to the stabilization of Snail, a transcriptional repressor associated with EMT, via modulation of PI3K/Akt/GSK-3β signaling. Chromatin immunoprecipitation assays revealed that Bmi-1 transcriptionally downregulated expression of the tumor suppressor PTEN in tumor cells through direct association with the *PTEN* locus. This in vitro analysis was consistent with the statistical inverse correlation detected between Bmi-1 and PTEN expression in a cohort of human nasopharyngeal carcinoma biopsies. Moreover, ablation of PTEN expression partially rescued the migratory/invasive phenotype of Bmi-1–silenced cells, indicating that PTEN might be a major mediator of Bmi-1–induced EMT. Our results provide functional and mechanistic links between the oncoprotein Bmi-1 and the tumor suppressor PTEN in the development and progression of cancer.**

## Introduction

Polycomb group (PcG) proteins are epigenetic gene-silencing proteins that have been implicated in embryonic development and oncogenesis (1–4). Numerous studies have shown that PcG proteins are frequently dysregulated in various cancer types and strongly correlate with an invasive or metastatic phenotype (5–10). PcG protein B lymphoma Mo-MLV insertion region 1 homolog (Bmi-1), the first functionally identified PcG member, was originally identified as an oncogene cooperating with c-Myc in a murine lymphomagenesis model (11, 12). In humans, Bmi-1 plays an essential role in maintaining the self-renewal of normal and malignant human mammary stem cells (13). Meanwhile, Bmi-1 is also dysregulated in various human malignant neoplasms (14–19). Previously, we reported that upregulation of Bmi-1 correlates with invasion of nasopharyngeal carcinomas (NPCs) and poor prognosis in patients (20). A possible mechanism of tumorigenesis involves repression of the *Ink4a-Arf* locus, which encodes the tumor suppressors INK4A and ARF (21), by direct binding of Bmi-1. However, Bmi-1 is also required for tumor development in an orthotopic transplantation model for glioma independent of Ink4a/Arf (22). Although the molecular mechanism of PcG in mammalian embryonic development and decisions of cell fate has been thoroughly elucidated by the identification of multiple potential PcG-targeted genes via genome-wide ChIP-on-chip analyses (23–25), the precise role of Bmi-1 in cancer invasion and metastasis remains largely unknown.

Epithelial-mesenchymal transition (EMT) is known to be a central mechanism responsible for invasiveness and metastasis of various cancers (26, 27). A key step in EMT is downregulation of E-cadherin (28, 29). Snail-related zinc-finger transcriptional repressors (Snail and Slug) (30, 31), the repressor SIP-1/ZEB-2 (32), ΔEF-1/ZEB-1 (33), as well as bHLH transcription factors Twist (34) and E12/E47 (29, 35) are the most prominent suppressors of E-cadherin transcription; these proteins act by binding to specific E-boxes in the E-cadherin promoter. Recently, modification of chromatin structure within the E-cadherin promoter was shown to correlate with EMT (36). It has been reported that EZH2 mediates transcriptional silencing of the tumor suppressor gene E-cadherin by trimethylation of the H3 lysine 27 (37).

Herein, we demonstrate that ectopic expression of Bmi-1 in normal nasopharyngeal epithelial cells (NPECs) is sufficient to cause

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EMT, while knockdown of Bmi-1 in NPC cells reduces cell invasion. Most importantly, we show that *PTEN* is a direct target of Bmi-1. Bmi-1 binds to the *PTEN* locus and downregulates PTEN expression, which consequently activates the PI3K/Akt pathway, stabilizes Snail, and downregulates E-cadherin, ultimately leading to enhanced invasiveness of epithelial cells. Taken together, our results provide an explanation for the aggressive nature of human tumors overexpressing Bmi-1 and the mechanism that links Bmi-1 to the key tumor suppressor PTEN.

## Results

*Ectopic expression of Bmi-1 induces EMT and enhances the invasiveness of NPECs in vitro*. Previously we reported that upregulation of Bmi-1 induces immortalization of NPECs (20). Meanwhile, a dramatic morphological change was observed in Bmi-1–induced immortalized NPECs, in which the typical cobblestone-like appearance of normal epithelium was replaced by a spindle-like, fibroblastic morphology (Figure 1A), which suggested that Bmi-1/NPECs might have undergone EMT. In order to determine whether Bmi-1 induces EMT, we probed the cells with epithelial and mesenchymal markers. As shown in Figure 1B, Bmi-1/NPECs exhibited the typical EMT phenotype, including downregulation of epithelial markers E-cadherin and α-catenin and upregulation of mesenchymal markers fibronectin and vimentin. The EMT phenotype was confirmed by immunofluorescent staining (Figure 1C). The same phenomena were observed in Madin-Darby canine kidney (MDCK) cells transduced with Bmi-1 (Supplemental Figures 1 and 2; supplemental material available online with this article; doi:10.1172/JCI39374DS1).

Cancer progression is aided when EMT augments tumor cell motility and consequent invasion into the basement membrane leading to advanced metastasis. Thus, we investigated the effect of Bmi-1 on cell invasion and motility by conducting assays for Matrigel-coated Boyden chamber invasion and wound healing. As shown in Figure 1D, Bmi-1/NPECs displayed significantly increased invasiveness in comparison with vector control cells. A scratch wound-healing assay also indicated that NPECs overexpressing Bmi-1 exhibited significantly enhanced mobility compared with vector control cells (Supplemental Figure 3). The invasive and migratory phenotype dependent on overexpression of Bmi-1 was also observed in MDCK cells (Supplemental Figure 4). Taken together, our results suggest that upregulation of Bmi-1 is sufficient to induce EMT and enhance invasiveness of NPECs in vitro.

*Silencing endogenous Bmi-1 represses the EMT phenotype and reduces transformation and metastatic potential of NPC cells*. To further investigate the impact of Bmi-1 on EMT, we silenced endogenous Bmi-1 in CNE2 and HONE-1 NPC cells using specific shRNAs and examined the resulting phenotype. Both shRNAs specifically knocked down endogenous Bmi-1 protein in both NPC cell lines. shRNA #2 was more efficient, and was therefore chosen for subsequent studies (Figure 2A). As shown in Figure 2, A and B, silencing endogenous Bmi-1 in NPC cells led to enhanced expression of epithelial markers and concomitant decreased expression of mesenchymal markers. Matrigel invasion chamber assays revealed that ablation of endogenous Bmi-1 markedly reduced invasiveness of NPC cell lines CNE2 and HONE-1 (Figure 2B). We next examined the effect of Bmi-1 on the tumorigenic activity of NPC cells using an anchorage-independent growth assay. As shown in Figure 2C, depletion of endogenous Bmi-1 in CNE2 and HONE-1 cells caused significant inhibition of their anchorage-independent growth, as indicated by reduction in colony number and colony size on soft agar ($P < 0.01$). Therefore, we determined that Bmi-1 is essential for malignant transformation of NPC cells.

We next evaluated in vivo effects of Bmi-1 depletion on invasion and metastasis using an experimental metastasis assay in which we injected CNE2 cells producing shRNA #2 against Bmi-1 or control shRNA into the lateral tail vein of 6-week-old nude mice and evaluated cell growth in the lung. Control shRNA cells formed 8 to 40 metastatic nodules per lung in all 7 mice analyzed, which were evaluated under a dissection microscope. In contrast, 7 nude mice injected with cells expressing shRNA #2 against Bmi-1 formed 0 to 6 nodules per lung (Figure 2D). H&E staining confirmed that both the number and the volume of micrometastatic lesions were markedly reduced in the lungs of mice injected with shRNA #2 (Figure 2E). Our data indicate that Bmi-1 is necessary for the aggressive metastatic phenotype of CNE2 cells.

*Upregulation of Bmi-1 activates the PI3K/Akt/GSK-3β pathway*. Our recent study suggested that Bmi-1 could modulate Akt activity in breast cancer cells in a $p16^{INK4A}$-independent manner (38). Because activation of the PI3K/Akt pathway is emerging as a central feature of EMT (26, 39–42), we asked whether Bmi-1 regulates Akt activity in NPC cells. As shown in Figure 3A, upregulation of Bmi-1 did indeed facilitate phosphorylation of Akt in both NPEC1 and NPEC2 cells, whereas Akt phosphorylation was inhibited in cells transduced with siRNA against Bmi-1. The increase in Akt phosphorylation was accompanied by a change in phosphorylation of GSK-3β, a downstream target protein of Akt, suggesting that upregulation of Bmi-1 activates the Akt/GSK-3β pathway in NPC cells.

Previously, GSK-3β activity was demonstrated to be necessary for the maintenance of epithelial architecture by dual regulation of Snail (43, 44). As shown in Figure 3A, Western blotting revealed that, compared with vector cells, expression of Snail significantly increased in Bmi-1/NPECs but decreased in shRNA/NPC cells (Figure 3A). However, real-time RT-PCR shows no significant change in *Snail* mRNA (L.-B. Song, unpublished observations), suggesting that modulation of Snail expression might be due to posttranslational modification. We next examined the half-life of Snail in Bmi-1/NPEC1 and vector/NPEC1 cells. The half-life of Snail was determined to be approximately 25 minutes in vector/NPEC1 cells, which is consistent with a previous report (44). However, its half-life dramatically increased to more than 90 minutes in Bmi-1/NPEC1 cells (Figure 3B). On the other hand, shRNA #2 expression reduced the half-life of Snail protein from approximately 120 minutes to less than 60 minutes in CNE2 cells (Figure 3B). Immunofluorescence staining confirmed the supposition that the extension of the half-life of Snail was primarily due to its subcellular localization, which is regulated by GSK-3β (44, 45). As shown in Figure 3C, Snail localized to both the nucleus and cytoplasm in Vector/NPECs, whereas it was mainly detected in the nucleus of Bmi-1/NPECs. In control CNE2 and HONE-1 cells, Snail was primarily nuclear, but its expression decreased markedly as it translocated to the cytoplasm following knockdown of Bmi-1 (Figure 3C).

In addition, several kinase inhibitors, including wortmannin (PI3K inhibitor), U0126 (MEK inhibitor), and PD98059 (MAPK inhibitor), were used to investigate whether the stabilization of Snail was caused by activation of PI3K/Akt/GSK-3β pathway. Supplemental Figure 5 shows that expression of phosphorylated Akt was reduced by wortmannin treatment, leading to further reduction of phosphorylated GSK-3β and Snail. However, no obvious alterations to phosphorylated GSK-3β or Snail were found in cells treated with MAPK (PD98059) or MEK (UO126) inhibitors.

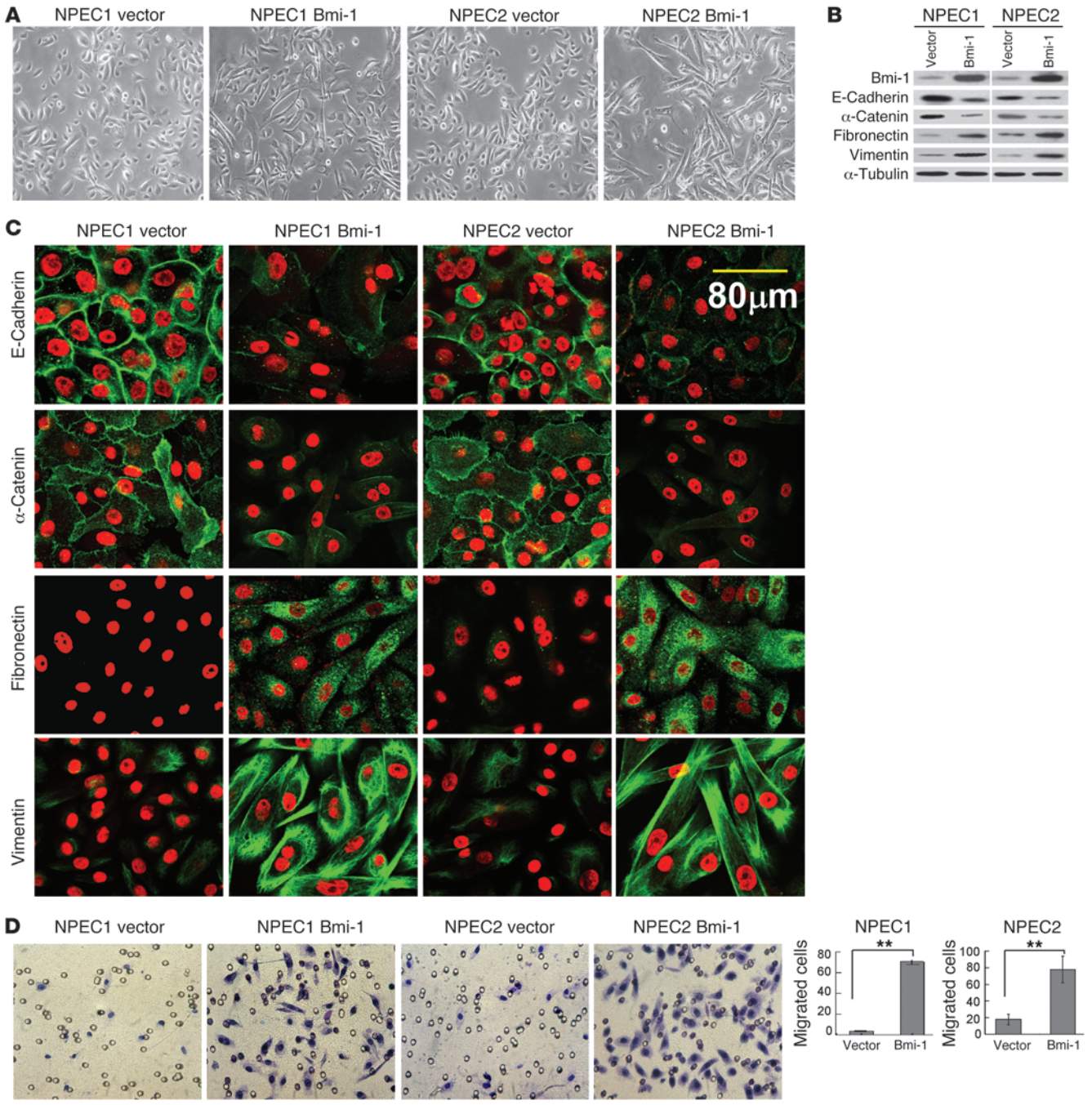



**Figure 1**

Overexpression of the Bmi-1 gene induces EMT in NPEC cells. (**A**) Morphology of NPEC1 and NPEC2 expressing either the control vector pMSCV or pMSCV/Bmi-1 are shown by phase contrast. Original magnification, ×400. (**B**) Expression of the epithelial proteins E-cadherin and α-catenin and the mesenchymal proteins fibronectin and vimentin in NPEC1 and NPEC2 cells expressing control vector pMSCV or pMSCV/Bmi-1 were detected by Western blot. α-Tubulin was used as a loading control. (**C**) NPEC1 and NPEC2 cells expressing control vector pMSCV or pMSCV/Bmi-1 were replated on 10% fetal bovine serum/RPMI-1640–precoated coverslips. After an additional 24 hours, cells were stained for E-cadherin, α-catenin, fibronectin, vimentin, and DAPI and analyzed by confocal microscopy. The green signal represents staining for the corresponding protein, while the red signal signifies nuclear DNA staining with rhodamine. Scale bar: 80 μm. (**D**) The invasive properties of the cells were analyzed in by an invasion assay using a Matrigel-coated Boyden chamber. Migrated cells were plotted as the average number of cells per field of view from 3 different experiments, as described in Methods. Original magnification, ×400. Error bars represent SEM. $^{**}P < 0.01$.

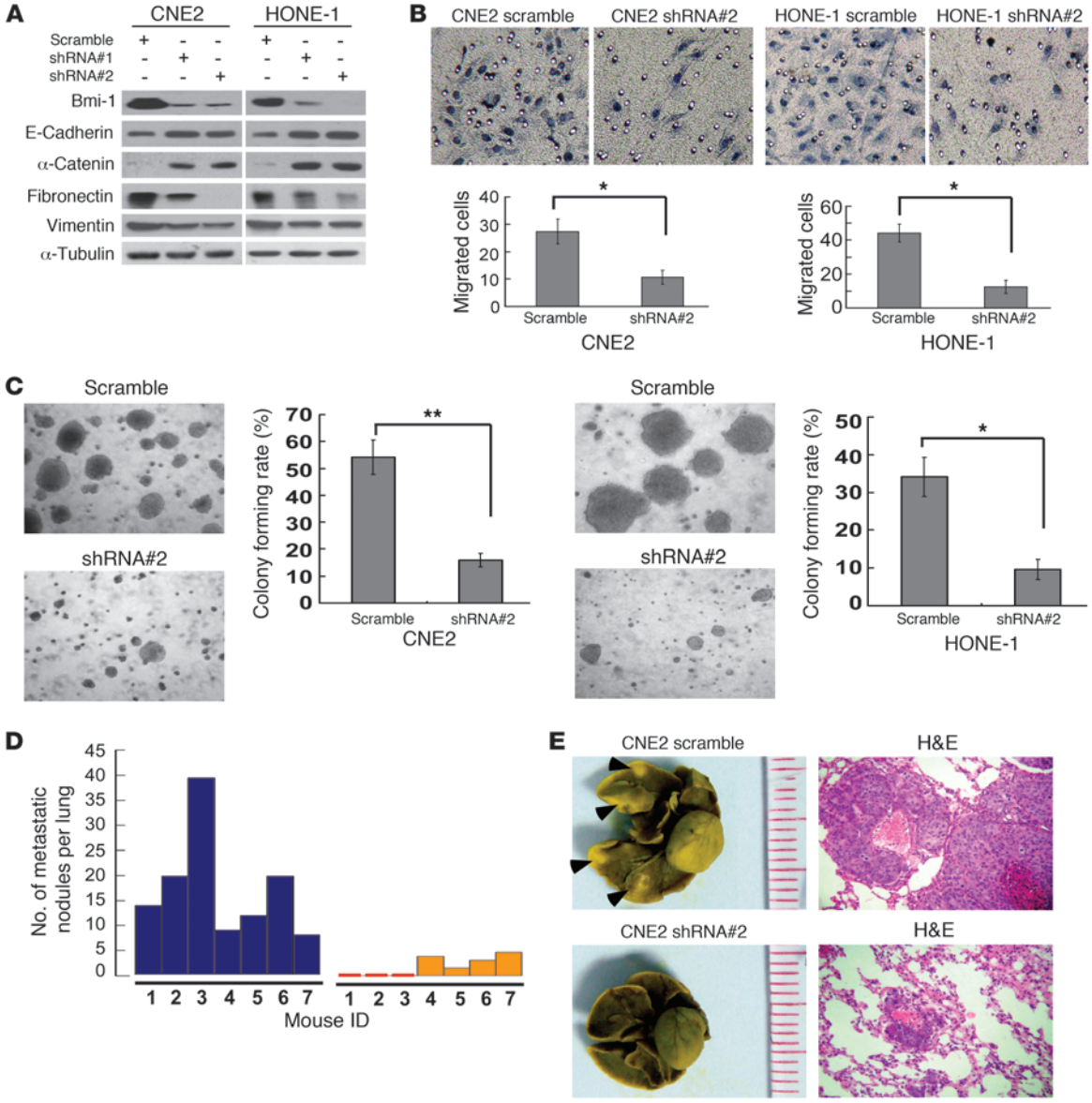
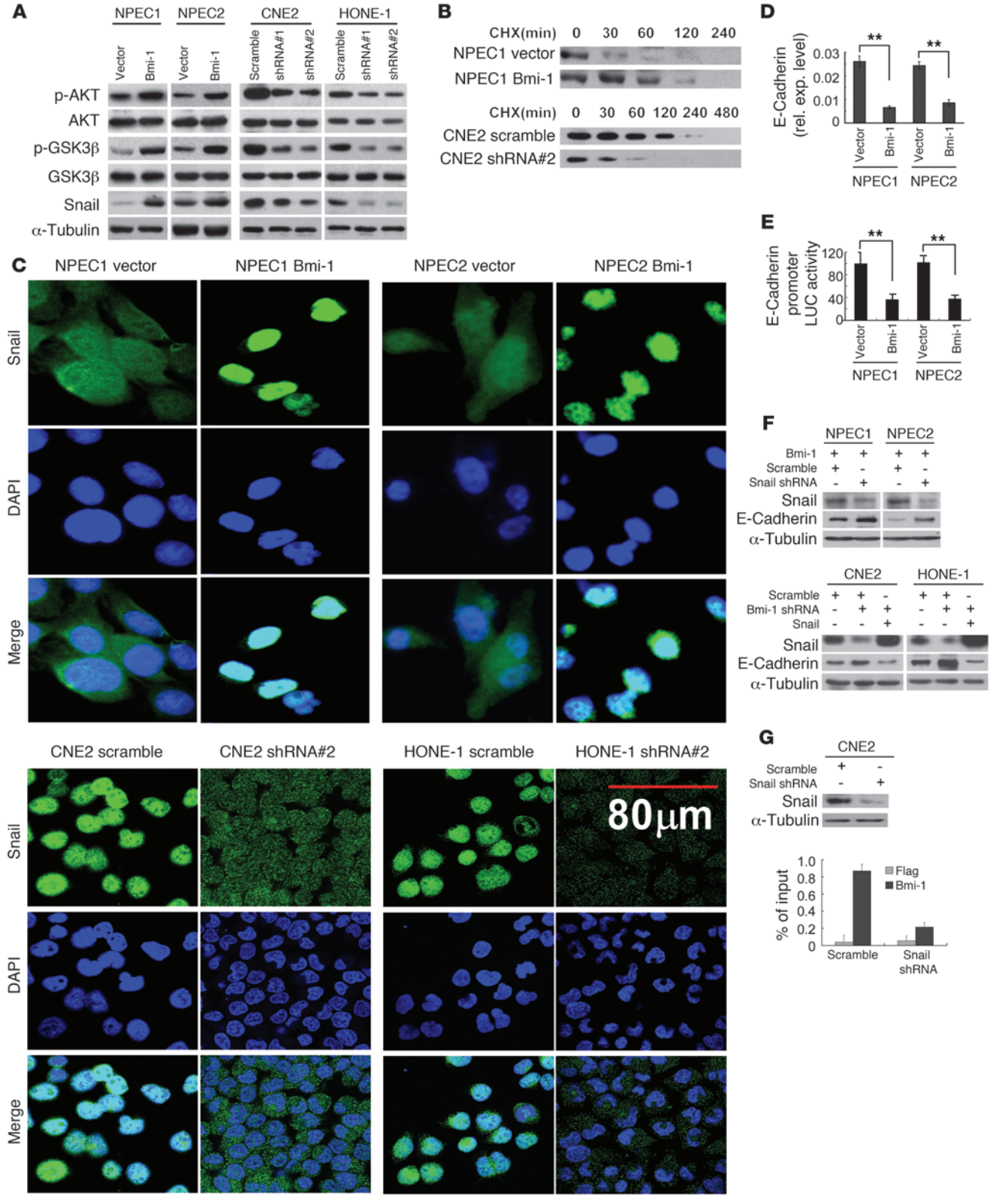



**Figure 2**

Suppression of endogenous Bmi-1 expression in NPC cells reverses EMT and transformation in vitro and reduces metastasis in vivo. (**A**) Expression of the indicated proteins was analyzed by Western blot. (**B**) The invasive properties of Bmi-1 shRNA– (shRNA #2) or scrambled shRNA–expressing NPC cell lines were analyzed in an invasion assay using a Matrigel-coated Boyden chamber as described in Figure 1D. Original magnification, ×400. Error bars represent SEM. *$P < 0.05$. (**C**) Anchorage-independent growth of Bmi-1 shRNA or scrambled shRNA cells in soft agar. Original magnification, ×400. Error bars represent SEM. *$P < 0.05$; **$P < 0.01$. (**D**) Number of metastases in lungs of mice ($n = 7$ per group) 8 weeks after tail vein injection of scrambled control shRNA (blue; mean ± SEM, 17.6 ± 11.0) and Bmi-1 shRNA CNE2 cells (yellow; mean ± SEM, 2.8 ± 2.5). The nodules were examined under an anatomical microscope. (**E**) Left: Representative lungs stained with trinitrophenol. Arrowheads indicate the metastatic nodes. Right: Representative H&E staining of lung metastatic tumors is shown. Original magnification, ×200.

*Snail is required for Bmi-1–mediated E-cadherin downregulation*. We utilized real-time PCR to investigate whether Snail is involved in Bmi-1–induced E-cadherin downregulation at the mRNA level. As shown in Figure 3D, the level of E-cadherin mRNA was significantly reduced in Bmi-1/NPECs compared with control cells (Figure 3D). Luciferase reporter assays indicated that downregulation of E-cadherin was achieved through suppression of E-cadherin promoter activity (Figure 3E). Moreover, E-cadherin expression could be rescued by silencing Snail in Bmi-1/NPECs, and ectopic expression of Snail blocked upregulation of E-cadherin in Bmi-1 shRNA/NPC cells (Figure 3F).

Because it has been reported that PRC2 represses E-cadherin transcription by binding to its promoter in a Snail-dependent manner (45), we examined whether Bmi-1 associates with the E-cadherin promoter. ChIP assays revealed that Bmi-1 was able to bind the E-cadherin promoter. Furthermore, the interaction was compromised when Snail was silenced (Figure 3G), suggesting that the transcriptional regulation by Bmi-1 of E-cadherin


A
NPEC1
NPEC2
CNE2
HONE-1
Vector
Bmi-1
Scramble
shRNA#1
shRNA#2
p-AKT
AKT
p-GSK3β
GSK3β
Snail
α-Tubulin
B
CHX(min) 0 30 60 120 240
NPEC1 vector
NPEC1 Bmi-1
CHX(min) 0 30 60 120 240 480
CNE2 scramble
CNE2 shRNA#2
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E-Cadherin (rel. exp. level)
0.03
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Vector
Bmi-1
NPEC1
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120
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NPEC2 vector
NPEC2 Bmi-1
Snail
DAPI
Merge
CNE2 scramble
CNE2 shRNA#2
HONE-1 scramble
HONE-1 shRNA#2
80μm
F
Bmi-1
Scramble
Snail shRNA
Bmi-1 shRNA
G
% of input
1.0
0.8
0.6
0.4
0.2
0
Flag
Bmi-1
Scramble
Snail shRNA

**Figure 3**
Bmi-1 induces PI3K/Akt activity and downregulates E-cadherin. (**A**) Cell extracts were analyzed by immunoblotting with antibodies against the indicated proteins. (**B**) Half-life analysis of the Snail protein. Cells were treated with 20 μM cycloheximide (CHX) for the indicated times and then analyzed by Western blotting. (**C**) The effect of Bmi-1 on the subcellular localization of Snail proteins (green) was examined under a fluorescent microscope. Scale bar: 80 μm. (**D**) E-cadherin mRNA expression was examined by real-time RT-PCR in triplicate. Error bars represent SEM ($n$ = 3). Rel. exp., relative expression. (**E**) Luciferase activity under the control of the human E-cadherin promoter construct was measured in the indicated cells in triplicate. Error bars represent SEM ($n$ = 3). $^{**}P < 0.01$. (**F**) Repression of E-cadherin by Bmi-1 is dependent on Snail. (**G**) Binding of Bmi-1 to the E-cadherin promoter at different Snail expression levels was analyzed by ChIP. The results display the means ± SEM for 2 experiments performed in triplicate.

requires Snail. Taken together, our results indicate that Snail plays an important role in Bmi-1 regulation of E-cadherin.

*Bmi-1 represses the tumor suppressor PTEN by associating at the PTEN locus*. As a transcriptional repressor, Bmi-1 is unable to activate the PI3K/Akt pathway directly. We speculated that Bmi-1–dependent activation of the PI3K/Akt pathway was due to transcriptional repression of an Akt-negative regulator by Bmi-1. It is well known that the tumor suppressor PTEN negatively regulates the PI3K/Akt pathway (46). Therefore, the expression of PTEN was examined in Bmi-1–infected as well as Bmi-1 shRNA–infected and control vector–infected cells. As shown in Figure 4A, expression of PTEN was lower in Bmi-1/NPECs than in vector control cells, whereas silencing Bmi-1 upregulated PTEN expression in CNE2 and HONE-1 NPC cells (Figure 4A). Real-time RT-PCR analysis revealed that overexpression of Bmi-1 resulted in downregulation of *PTEN* mRNA, while downregulation of Bmi-1 led to an increase in the *PTEN* mRNA level (Figure 4A). These results were confirmed by transiently transfecting Cos-7 cells with a Bmi-1 expression vector to reduce endogenous expression of PTEN in a dose-dependent manner (Figure 4B). ChIP assays were performed to investigate whether Bmi-1 associates with the *PTEN* locus. As shown in Figure 4, C and D, Bmi-1 binds to the region of the *PTEN* promoter from –0.9 kb to –2.3 kb and from 0.4 kb to 1.4 kb; the $p16^{INK4A}$ and GAPDH loci were used as positive and negative controls, respectively. In addition, Bmi-1 bound to the *PTEN* promoter much less efficiently in Bmi-1 shRNA/CNE2 cells (Figure 4E), indicating that the association was specific. All of the results mentioned above demonstrate that Bmi-1 can transcriptionally repress PTEN expression.

It has been reported that Bmi-1–containing complexes are recruited to H3K27 trimethylation through the Pc and HP1 chromodomains (47). Consistent with these observations (24), our data revealed that the $p16^{INK4}$ gene was co-occupied by PRC1 members (Bmi-1 and Ring2), PRC2 members (EZH2 and SUZ12), and H3K27 trimethylation. Moreover, using the same ChIP products, several other PRC members including Ring 2, EZH2, and SUZ12 as well as H3K27 trimethylation were found at the *PTEN* locus (Figure 4F). Taken together, our results show that *PTEN* is a direct target of PRC and is regulated by PRC in a similar manner to p16.

*Ablation of PTEN expression restores the migratory/invasive phenotype and tumorigenicity of Bmi-1–silenced cancer cells*. To analyze the functional correlation between PTEN and Bmi-1, we next tested whether knockdown of PTEN expression in Bmi-1–silenced cancer cells could reactivate PI3K/Akt/GSK-3β signaling and restore their migratory/invasive phenotype. As expected, silencing PTEN with both PTEN shRNAs in Bmi-1–silenced cells increased the expression of phosphorylated Akt and Snail (Figure 5A). Moreover, a Matrigel invasion chamber assay revealed that knockdown of PTEN partially restored the invasiveness of Bmi-1 shRNA/NPC cells. Partially restored motility and invasiveness of Bmi-1 knockdown cells expressing PTEN shRNAs was confirmed by a wound-healing assay and 3D Matrigel cultures (Supplemental Figure 6). To decipher the functional correlation between PTEN and Bmi-1 in vivo, xenograft tumor experiments were performed. As shown in Figure 5C, in contrast to scrambled shRNA cells, Bmi-1 shRNA cells exhibited greatly reduced tumor growth, while PTEN knockdown partially rescued the reduced tumorigenicity in Bmi-1–silenced cells. These results suggest that PTEN could be a major negative regulator of Bmi-1–induced invasiveness/migration and tumorigenicity in cancer cells.

*Bmi-1 expression negatively correlates with PTEN and E-cadherin in NPC*. We further analyzed the expression levels of Bmi-1, Snail, PTEN, and E-cadherin in NPC cell lines and in clinical NPC samples. Western blot and real-time RT-PCR analyses revealed that Bmi-1 and Snail were markedly upregulated at both the protein and mRNA levels when compared with NPECs. Protein and mRNA expression levels for PTEN and E-cadherin were downregulated in NPC cell lines compared with normal cells (Figure 6, A and B). Furthermore, we measured expression of Bmi-1, Snail, PTEN, and E-cadherin in 2 normal nasopharyngeal epithelium tissues and 11 clinical NPC samples using real-time RT-PCR. As shown in Figure 6C, the 11 NPC samples presented significantly higher levels of Bmi-1 (2- to 32-fold) and Snail (2- to 17-fold) as well as lower levels of PTEN (2- to 36-fold) and E-cadherin (2- to 14-fold) with respect to normal nasopharyngeal epithelium tissues. Statistical analysis revealed that Bmi-1 expression positively correlated with Snail ($r$ = 0.882, $P < 0.01$) but negatively correlated with PTEN ($r$ = –0.664, $P$ = 0.026) and E-cadherin ($r$ = –0.809, $P < 0.01$). Taken together, our results reveal an inverse relationship between high Bmi-1/Snail expression and low PTEN/E-cadherin expression in human cancers, further supporting a model of Bmi-1 association with the *PTEN* locus leading to transcriptional repression of PTEN expression, which results in activation of PI3K/Akt/GSK-3β/Snail signaling and E-cadherin downregulation (Figure 6D).

## Discussion

NPC, one of the most common cancers in southern China, has the propensity to invade adjacent regions and metastasize to regional lymph nodes and distant organs (48). Efforts to elucidate the molecular mechanism underlying tumorigenicity, invasion, and metastasis of NPC are therefore warranted in order to develop novel treatments and a possible cure. In the current study, we have demonstrated that overexpression of Bmi-1 confers EMT to NPECs, and knockdown of Bmi-1 in NPC cell lines represses EMT and reduces invasiveness. We further demonstrated that Bmi-1 can directly target the tumor suppressor PTEN, activate the PI3K/AKT/Snail pathway, and ultimately suppress E-cadherin expression.

Dysregulation of Bmi-1 alters cell proliferation, apoptosis, senescence, and stem cell self-renewal and correlates with the invasive and metastatic phenotype of several human cancer types (14, 16, 18, 20, 49, 50). However, the precise role and molecular mechanism underlying Bmi-1 involvement is ambiguous. EMT was originally identified as a critical factor in embryogenesis (51). During tumor progression, EMT is often involved in invasion and metastasis (52).

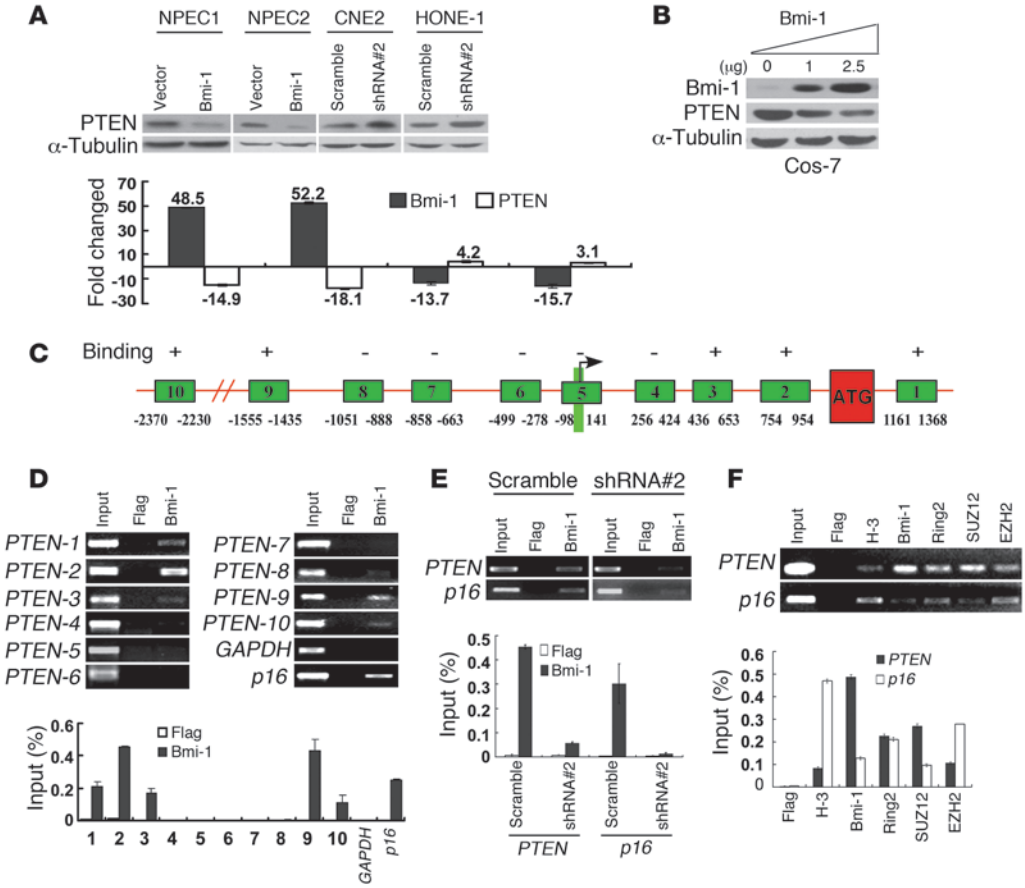


**Figure 4**

Bmi-1 represses PTEN by binding to the *PTEN* promoter locus. (**A**) Top: Immunoblots showing expression of PTEN in the indicated cells. Bottom: Fold change of *PTEN* mRNA was analyzed by real-time RT-PCR. A representative result from 3 different experiments is shown. Error bars indicate SEM ($n = 3$). (**B**) Cos-7 cells were transiently transfected with different amounts of pcDNA3/Bmi-1 and analyzed by Western blot. (**C**) Schematic representation of the PTEN promoter regions with or without binding affinity for Bmi-1. Precipitated DNA was amplified by PCR using primers specific for regions 1–10. The arrow indicates the transcriptional start site. ATG, translation start codon. (**D**) ChIP was performed by using anti–Bmi-1 antibody or anti-Flag antibody to identify Bmi-1 binding sites on the *PTEN* promoter in CNE2 cells. The *p16* promoter was used as a positive control, and *GAPDH* was used as a negative control. (**E**) ChIP analysis of Bmi-1 binding efficiency in CNE2 cells expressing the scrambled control shRNA or Bmi-1 shRNA #2. (**F**) Specific binding for H3K27 (H-3), Ring2, SUZ12, and EZH2 on *PTEN* and *p16* loci was analyzed. Enriched chromatin was analyzed by the Bio-Profile computer-assisted imaging system. In **D**–**F**, bar graphs show the means ± SEM performed in triplicate.

observations) are congruent with the prior observation; that is, Bmi-1 overexpression in primary mammary epithelial cells induces cell scattering and only moderately elevates migration/invasiveness without typical morphological changes in the monolayer culture. This cell type–specific function has been observed with other oncogenes, such as Ras, which alone induces only epithelial polarity modification in EpH4 cells but causes EMT in other epithelial cell lines (56). Overexpression of ILEI also induces cell type– or culture-specific EMT or cell scattering (57). Thus, Bmi-1–induced EMT may be a cell type–specific function.

EMT is a key developmental program that is often activated during embryonic development, tumor invasion, and metastasis. Evidence has shown that EMT can engender cells with properties of stem cells (58). Bmi-1 is required for proliferation of various differentiated cells and for self-renewal of stem cells (59–61). Thus, we examined whether Bmi-1 could induce a stem cell–like phenotype in NPC cell lines. We observed reduced ABCG2 levels, decreased side population cell proportions, and smaller and fewer spheres formed under nonadherent, serum-free culture conditions in Bmi-1–silenced cells compared with scrambled control cells (Supplemental Figure 7). These data suggest that a key role of Bmi-1 in EMT and tumor metastasis might be stem cell maintenance.

Herein, we demonstrate that Bmi-1 induces EMT in NPECs and suppression of Bmi-1 in NPC cells reverses EMT. However, the typical EMT morphological change observed in NPECs only occurs in cells passaged several times after introduction of Bmi-1 (often after passage 5). Unlike the well-established cell lines, primary NPECs require several passages under selection before reaching a uniform phenotype after introduction of Bmi-1. To determine whether EMT is directly related to Bmi-1 expression, we introduced Bmi-1 into MDCK cells, which have been widely used in EMT studies (34, 53, 54). We observed the typical EMT phenotypes 1 week after retroviral transduction of Bmi-1 (Supplemental Figures 1–3). Thus, Bmi-1 is sufficient to induce EMT in certain epithelial cells.

A recent report showed that Bmi-1 overexpression in MCF10A cells did not induce the EMT phenotype, but readily did so when co-expressed with RasG12V (55). Our data (Y. Feng, unpublished

Although a significant correlation between Bmi-1 expression and local invasion of the primary tumor was observed in our previous study, we failed to show a significant correlation between Bmi-1 expression and metastasis in NPC biopsies (20). This suggests that overexpression of Bmi-1 alone is not sufficient to induce metastasis in NPC patients, and it may play a more important role in inducing local invasion of NPC. In addition, the limited sensitivity of immunohistochemistry used in the previous study may have encumbered the results. In the current study, we observed Bmi-1 overexpression in all 11 NPC specimens by real-time PCR, although at differing levels. Thus, we believe it is important to perform

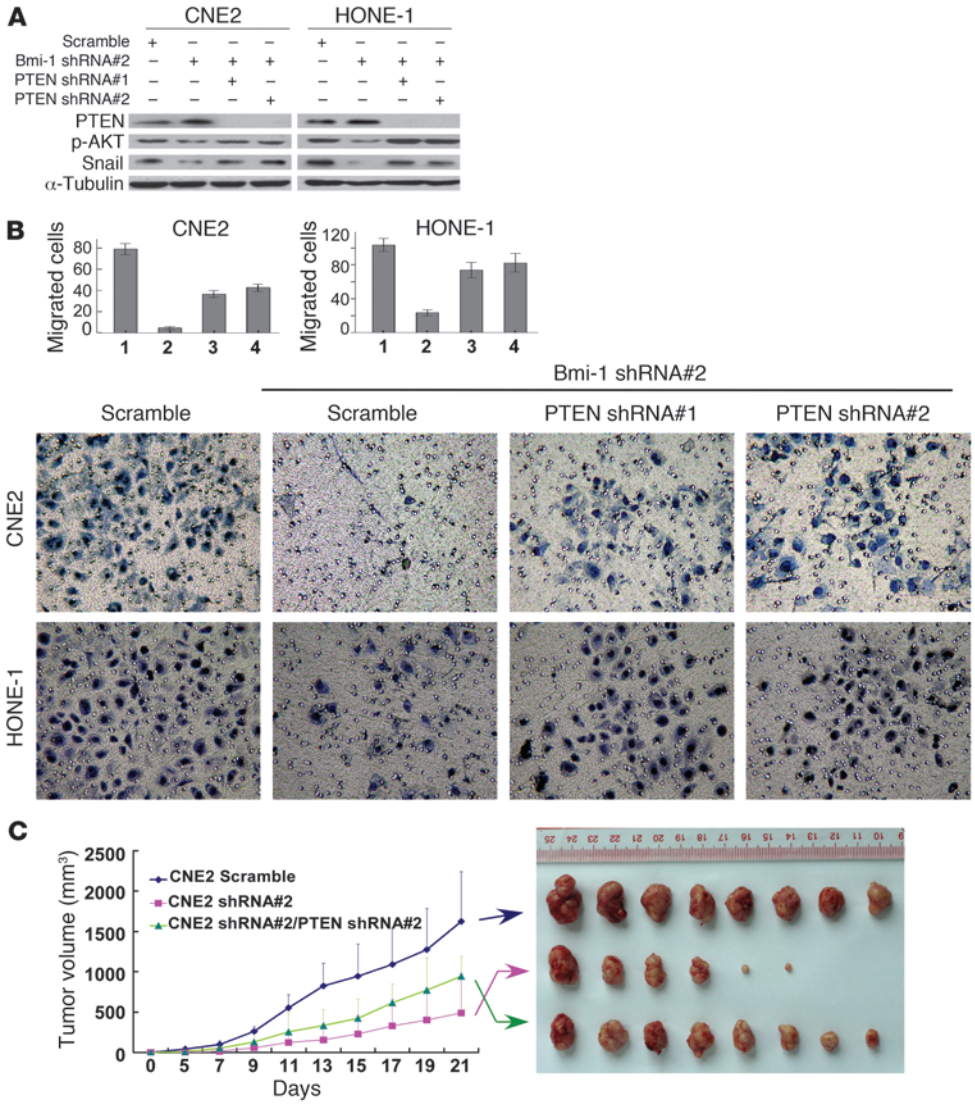


**Figure 5**
Inhibition of PTEN expression by shRNA rescues migration/invasiveness and tumorigenicity in Bmi-1–silenced cells. (**A**) Inhibiting PTEN expression by shRNAs results in activated Akt and rescue of Snail in Bmi-1–silenced CNE2 and HONE-1 cells, as determined by Western blotting. (**B**) The migration/invasiveness-inducing properties of PTEN shRNAs or a scrambled control shRNA in Bmi-1 knockdown cell lines (CNE2 and HONE-1) using a Matrigel-coated Boyden chamber as described in Figure 1D. Original magnification, ×400. Error bars represent SEM. Lane 1, scramble control shRNA; lane 2, Bmi-1 shRNA #2; lane 3, Bmi-1 shRNA #2 and PTEN shRNA #1; lane 4, Bmi-1 shRNA #2 and PTEN shRNA #2. (**C**) Mice were injected subcutaneously with cells ($10^5$ cells/mouse). Tumor volumes were measured as described in Methods and plotted as the mean ± SEM. The left panel displays tumor growth in nude mice injected with the 3 groups of cells (CNE2 scrambled control, CNE2 Bmi-1 shRNA #2, and CNE2 Bmi-1 shRNA #2/PTEN shRNA #2 cells).

another clinical study with a larger cohort of samples to clearly demonstrate a correlation between Bmi-1 expression and the metastatic status of NPC.

To our knowledge, this is the first study demonstrating an essential role for Bmi-1 in induction of EMT. Aside from Bmi-1, dysregulation of PcG genes is widespread in human tumors and correlates with the invasive or metastatic phenotype of certain carcinomas (6, 9, 10). A recent report described a novel role for an EZH2-associated methyltransferase complex in controlling actin polymerization in fibroblast cells (62). It suggested that EZH2 might contribute to the metastatic development of cancer cells via regulation of actin-dependent cell adhesion and migration. In addition, EZH2 has been implicated in EMT induction by directly targeting ADRB2 and E-cadherin (37, 63). Taken together, EZH2 may induce EMT and malignancy through multiple pathways. Thus, PcG proteins may play important roles in EMT induction.

An activated PI3K/Akt pathway is well documented for various human malignancies and sometimes correlates with an aggressive phenotype (reviewed in ref. 64). The PI3K/Akt pathway plays a central role in EMT (26, 39–42). In this study, we observed hyperactivation of PI3K/Akt in Bmi-1–induced EMT, which correlates with hyperphosphorylated GSK-3β, Snail stabilization, and E-cadherin downregulation. Snail triggers EMT by directly repressing E-cadherin transcription (30). Snail's transcriptional activity is regulated by GSK-3β, which is negatively regulated by PI3K/Akt, MAPK, and Erk (44). Thus, the PI3K/Akt pathway seems to play a criteria role in Bmi-1–induced EMT. However, another crucial pathway regulated by GSK-3β, the Wnt pathway, which is also involved in EMT and stem cell regulation, does not seem to be involved in EMT induced by Bmi-1 (Supplemental Figure 8).

Here we show, for what we believe is the first time, that the tumor suppressor PTEN is a downstream target of Bmi-1, which provides the molecular basis for activation of PI3K/Akt signaling in Bmi-1–overexpressing cells (38, 55), although there may be more Bmi-1 targets that likely contribute to EMT and invasion/metastasis. PTEN is a dual lipid and protein phosphatase whose activity is lost by mutation, deletion, or promoter methylation silencing at a high frequency in many primary and metastatic human cancers (46, 65, 66). PTEN functions as a negative regulator of the PI3K/Akt pathway via dephosphorylation of PI(3,4,5)P3, ultimately participating

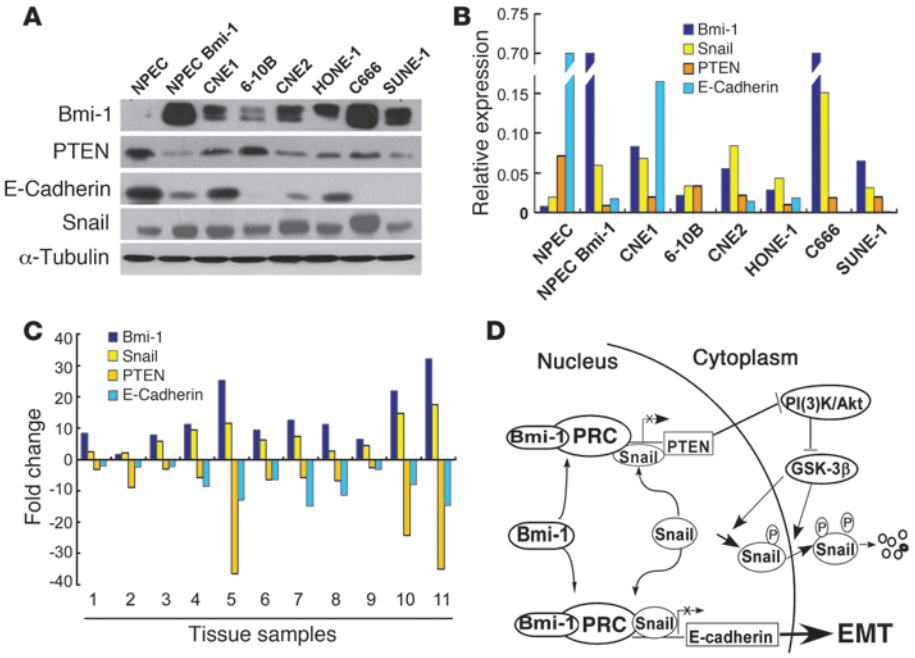


**Figure 6**
Endogenous expression of Bmi-1, Snail, PTEN, and E-cadherin in NPC cell lines and biopsies. (**A** and **B**) Endogenous expression of Bmi-1, Snail, PTEN, and E-cadherin in NPEC and NPC cancer cell lines were examined by Western blot (**A**) and real-time RT-PCR (**B**). mRNA expression levels for *Bmi-1*, *Snail*, *PTEN*, and E-cadherin were normalized with respect to GAPDH in real-time RT-PCR experiments. Columns represent the mean from 3 parallel experiments. (**C**) Real-time RT-PCR data of Bmi-1, Snail, PTEN, and E-cadherin in 11 cases of NPC biopsies are presented as the fold change from the average expression levels in 2 normal tissues. Columns are the average of 3 parallel experiments. (**D**) Schematic representation of the major molecular mechanism of Bmi-1–induced EMT.

in regulation of the cell cycle, proliferation, apoptosis, cell adhesion, and EMT during embryonic development and cancer progression (42, 67). Our results provide multiple lines of evidence to support PTEN as a target of Bmi-1. First, there is an inverse relationship between Bmi-1 and PTEN expression in cells and human tumors. Second, inhibition of PTEN expression by shRNA in Bmi-1–repressed cells rescues PI3K/AKT activity and migration/invasiveness. Most importantly, Bmi-1 is able to bind the *PTEN* locus, which was co-occupied by PRC1/2 members as well as H3K27 trimethylation. In addition, the binding efficiency of Bmi-1 to the *PTEN* promoter is partially reduced after knockdown of Snail by a specific shRNA in NPC cells as determined by a ChIP assay (Supplemental Figure 9). Moreover, we demonstrate that Snail associates with the *PTEN* promoter and that binding efficiency can be reduced by silencing its expression (Supplemental Figure 10). Thus, our data indicate that binding of Bmi-1 to the *PTEN* promoter could be partially mediated by Snail. Similar results were published recently demonstrating that PRC2 could bind to the *PTEN* promoter in a Snail-dependent manner (45). In addition, Snail could repress PTEN expression by binding to the *PTEN* promoter during irradiation (68). Our data indicate that there might be a positive feedback loop between suppression of PTEN and activation of Snail in NPC cells. Given that targets for the PRC2/3/4 member Suz12 are cell type–specific (69), it is unknown whether PTEN is a universal target for Bmi-1 across systems.

Interestingly, several independent genetic studies suggest that PTEN is a potential target of Bmi-1. First, both Bmi-1 transgenic and heterozygous mutant *PTEN* (mPTEN3–5) mice show an increased incidence of T cell lymphoma (70, 71). Second, deletion of Arf or Ink4a-Arf only partially rescues defects in cerebellum development in Bmi-1–deficient mice (21, 72), while knockout of *PTEN* induces enhanced neural stem/progenitor cell proliferation and self-renewal (73). Finally, PTEN plays a role in the prevention of leukemogenesis in mice (20, 74), while overexpression of Bmi-1 induces this process (59). However, demonstrating a direct link between Bmi-1 and PTEN in vivo may require generating double-transgenic or double-knockout mice.

In summary, Bmi-1 plays an important role in the pathogenesis of NPC by inducing EMT partially by targeting the tumor suppressor *PTEN*, thus activating the PI3K/Akt pathway. Uncovering a novel function and molecular mechanism for Bmi-1 overexpression in cancer provides important insight into understanding tumor progression and metastasis.

## Methods

*Cell culture, plasmids, and antibodies*. Primary NPEC cultures and immortalized NPECs induced by Bmi-1 were established as described previously (20) and grown in keratinocyte/serum-free medium (Invitrogen). All NPC cell lines were maintained in RPMI supplemented with 10% fetal bovine serum. Bmi-1 shRNA constructs were described previously (75). Other plasmids and antibodies are described in Supplemental Methods. Retroviruses were produced by transient transfection as described previously (76).

*Tissue samples*. Tissue specimens from 11 NPC tissues and 2 normal controls were obtained from the archives of the Department of Sample Resource of the Sun Yat-sen University Cancer Center. Patients' consent and approval from the Sun Yat-sen University Cancer Center Institute Research Ethics Committee were obtained in order to use these clinical materials for research purposes.

*Immunofluorescence analysis*. Cells were stained for immunofluorescence on coverslips as described previously (20). Briefly, the cells were incubated with primary antibodies against E-cadherin, α-catenin, fibronectin, or vimentin and then incubated with rhodamine-conjugated or FITC-conjugated goat antibodies against rabbit or mouse IgG (Jackson ImmunoResearch Laboratories). The coverslips were counterstained with DAPI and imaged with a confocal laser-scanning microscope (Olympus FV1000). Data were processed with Adobe Photoshop 7.0 software.

*Migration assay*. See Supplemental Methods for details.

*Wound-healing assay*. See Supplemental Methods for details.

*3D morphogenesis assay*. Twenty-four–well dishes were coated with Growth Factor Reduced Matrigel (BD Biosciences) and covered with growth medi-

um supplemented with 2% Matrigel as previously described (2). Cells were trypsinized and seeded at a density of $10^4$ cells/well, and medium was replaced with 2% Matrigel for 3 to 4 days. Microscopic images were captured at 2-day intervals for 2–3 weeks.

*Anchorage-independent growth assays*. Six-well plates were covered with a layer of 0.6% agar in medium supplemented with 20% fetal bovine serum. Cells were prepared in 0.3% agar and seeded in triplicate at 3 different dilutions ranging from $1 \times 10^3$ to $5 \times 10^5$. The plates were incubated at 37°C in a humid atmosphere of 5% $CO_2$ for 4 weeks. Each experiment was repeated at least 3 times. Colonies were photographed between 18 and 24 days at an original magnification of ×200 under phase contrast.

*Experimental tumor and metastasis assay*. Tumor formation and metastasis of Bmi-1 shRNA cancer cells were determined by tail vein intravenous injection into 4- to 6-week-old Balb/C athymic nude mice (nu/nu) (see Supplemental Methods). The modified transplantation through subcutaneous injection is referred to in the report of Quintana et al. (77). Briefly, mice were subcutaneously injected with cells ($10^5$/mouse) mixed with 25% Matrigel (BD), and tumor sizes were measured every other day in order to calculate tumor volumes. All mice were sacrificed 3 weeks after injection.

*Dual luciferase reporter assay*. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Cells at 50% confluence in 12-well dishes were transfected using Fugene 6 (Roche). All experiments were performed in triplicate. Expression of firefly and renilla luciferases were analyzed 48 hours after transfection, according to the manufacturer's instructions.

*Real-time RT-PCR analysis*. Real-time RT-PCR was carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) (see Supplemental Methods). The primer sequences are provided in Supplemental Table 2.

*ChIP assays*. ChIP was performed as previously described (78). See Supplemental Methods for details.

*Statistics*. Statistical analyses were performed using SPSS 10.0. Differences among variables were assessed by $\chi^2$ analysis or 2-tailed Student's *t* tests. Data were presented as the mean ± SEM unless otherwise indicated. A *P* value less than 0.05 was considered statistically significant.

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