Elevated Bmi-1 expression is associated with dysplastic cell transformation during oral carcinogenesis and is required for cancer cell replication and survival

Bmi-1 is a polycomb group protein that was identified as a c-myc cooperating oncogene in murine lymphomas (van Lohuizen et al., 1991; Pirrotta, 1998). Proliferative capacity of leukemic and normal haemopoietic stem cells derived from Bmi-1−/− mice was compromised, suggesting the possible role of Bmi-1 in the maintenance of tumour stem cell phenotype (Lessard and Sauvageau, 2003). Bmi-1 is one of the core subunits of the polycomb repressive complex 1, which regulates the diverse biological processes, including X chromosome inactivation, carcinogenesis, and stem cell renewal (Li et al., 2006; Sparmann and van Lohuizen, 2006). Cellular target genes of Bmi-1 have been identified and include inka4a and inka4b loci, encoding p16INK4A, p19ARF, and p15INK4B (Jacobs et al., 1999). Bmi-1 is believed to promote cellular proliferation by repressing the expression of the inka4a locus, which plays pivotal roles in the onset of cellular senescence in many different types of human somatic cells (reviewed in Itahana et al., 2004). During normal replication of primary human cells, Bmi-1 expression level is decreased notably in senescent cells (Itahana et al., 2003). When overexpressed, Bmi-1 is able to extend the replicative life span of human diploid fibroblasts (Itahana et al., 2003) and immortalize the post-selection population of human mammary epithelial cells by activation of endogenous telomerase gene, hTERT (Dimri et al., 2002). Therefore, in normal human cells, downregulation of Bmi-1 expression may be important for the establishment of the senescence program.

Cellular senescence is physiologically important because it is a potent tumor suppressor mechanism that must be overcome for cells to be immortalized and transformed (Campisi, 2005; Dimri, 2005). The molecular mechanisms of senescence have therefore been investigated extensively and yielded several important regulators of senescence (Itahana et al., 2004). The intrinsic, telomere-dependent senescence is triggered by accumulation of shortened and dysfunctional telomeres with altered telomeric state (Harley et al., 1990; Karseder et al., 2002). The extrinsic senescence is telomere-independent and triggered in cells after exposure to environmental factors, such as genotoxic stress, in vitro culture shock, and oncogenic stimuli (Serrano et al., 1997; Ramirez et al., 2001; Itahana et al., 2004; Chen et al., 2005; Dimri, 2005). The onset of extrinsic senescence is frequently associated with induction of p16INK4A (Itahana et al., 2004). The mechanisms resulting in p16INK4A upregulation during senescence are not fully understood, although recent studies found a positive control by ETS1 and negative regulation of its expression by Id-1, Bmi-1, and CBX7 (Ohtani et al., 2001; Itahana et al., 2003; Gil et al., 2004). In particular, Bmi-1 overexpression is detected in several types of human cancers (Bea et al., 2001; Kim et al., 2004a, b; Breuer et al., 2005).
2007 Cancer Research UK indicate that Bmi-1 may act through p16 INK4A-independent pathway in these cells. Thus, our results ga, Madison, WI, USA), and 125 et al SCC15, FaDu, Tu-139, 1483, HEp-2, HeLa, and RKO cells were can be found elsewhere (Kang et al, 2000). BaP-T, SCC4, SCC9, SCC15, FaDu, Tu-139, 1483, HEp-2, HeLa, and RKO cells were cultured as previously described (Rey et al, 2000).

Reverse transcription (RT) – PCR
Total RNA was isolated from the cultured cells using Trizol™ reagent (Invitrogen, Carlsbad, CA, USA) and was subjected to RNase-free DNase I digestion at 37°C for 2 h to eliminate any contaminating genomic DNA. Five µg of DNA-free total RNA was dissolved in 15 µl H2O, and RT reaction was performed in the first strand buffer (Invitrogen) containing 300 U Superscript II (Invitrogen), 10 mM dithiotretiol, 0.5 µg random hexamer (Promega, Madison, WI, USA), and 125 µM dNTP. The annealing reaction was carried out for 5 min at 65°C, and cDNA synthesis was performed for 2 h at 37°C, followed by incubation for 15 min at 70°C to stop the enzyme reaction. The RT product was diluted with 70 µl H2O.

To amplify Bmi-1 cDNA, PCR reaction was performed with 2 µl RT product using the following primers: 5’-AGCAGAATTCTGATCATC GAAACAA-3’ (forward) and 5’-CCTAAACCCG ATGAAGTTGCTGA-3’ (reverse). The PCR amplification was allowed for 30 cycles at 94°C (1 min)/53°C (1 min)/72°C (1 min), followed by 7 min incubation at 72°C. This PCR condition with 1 µl RT product was used for exponential amplification of the starting cDNA. To control for the contamination with genomic DNA, we also performed PCR reactions using the samples without prior RT reaction. No visible amplification was obtained without RT. Polymerase chain reaction amplifications of GAPDH, hTERT, MMP-1, MMP-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed as described previously (Kang et al, 2003).

Western blotting
Whole-cell extracts (WCE) were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon protein membrane (Millipore, Billerica, MA, USA). The antibodies for p16INK4A (Ab-1) were purchased from Calbiochem (San Diego, CA, USA), and those for Bmi-1 (H99), β-actin (H-196), and CDK4 (C22) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Bmi-1 gene amplification study
Genomic DNA was isolated from five strains of NHOK, two normal human oral fibroblasts (NHOF), and eight cancer cell lines, including six OSCC cells. The DNAs were digested with EcoRI and HindIII to completion, electrophoresed in 0.8% agarose gel, and transferred to nylon membrane. Bmi-1 genomic sequences were probed by Southern hybridisation using radiolabelled probe synthesised from Bmi-1 cDNA or GAPDH cDNA, according to the methods described elsewhere (Kang et al, 1998). The Bmi-1 radioactive signal was detected by PhosphorImager and quantitated against that of GAPDH. The normalised quantitated values were used for statistical test (one way ANOVA) to compare the mean Bmi-1 radioactive signals in the NHOK cultures and in the OSCC cell lines.

Immunohistochemical staining of Bmi-1 in paraffin-embedded tissues

In situ Bmi-1 expression was determined in oral mucosal tissue samples with normal (n=8), dysplastic (n=9), or cancerous (n=10) histopathology obtained from the Oral Pathology diagnostic laboratory at the University of California at Los Angeles School of Dentistry. The specimens were collected and processed according to the guidelines of the University of California at Los Angeles institutional review board. Immunohistochemistry was carried out with anti-Bmi-1 antibody (H-99, Santa Cruz, CA, USA) on 4-µm-thick sections according to the methods described elsewhere (Kim et al, 2004a). The samples were counterstained with haematoxylin. Bmi-1 staining intensity per each sample was arbitrarily scored by an oral pathologist as negative (−), barely detectable (++−), weak (++), moderate (+++), or strong (+++; see 2). In situ Bmi-1 staining was also performed in cultured NHOK and SCC4 cells according to the previously described method (Kang et al, 2000).

In situ staining for cellular senescence
Normal human oral keratinocytes cultures infected with the lentiviral vectors (LV-GFP and LV-Bmi-1i) were fixed in 2% formaldehyde/0.2% glutaraldehyde for 3–5 min at room temperature. The cells were then stained for senescence-associated β-galactosidase (SA β-Gal) activity in freshly prepared staining solution (Dimri et al, 1995). The presence of SA β-Gal activity was evidenced by a dark-green colour in the perinuclear cytoplasmic region.

Lentiviral vector construction and use of short-hairpin RNA (shRNA)
We utilised the lentivirus-based shRNA expression plasmid pLL3.7 to knockdown the expression of endogenous Bmi-1. The detailed method of using pLL3.7 to construct the lentivirus expressing shRNA is described elsewhere (Rubinson et al, 2003). We constructed pLL3.7-Bmi-1i using double-stranded oligonucleotide cassette containing the Bmi-1 target sequence (5’-AAG-GATTTGCTCCAGACATT-3’) as described previously (Bracken et al, 2003). The lentiviral vectors, LV-GFP and LV-Bmi-1i were prepared by transfecting 293 T cells with the RNA interference (RNAi) plasmids, respectively, pLL3.7 (insertless plasmid) and pLL3.7-Bmi-1i, using calcium phosphate transfection method in the presence of the packaging plasmid pCMV-AR8.2Vpx, and the envelope plasmid pCMV-VSV-G (Naldini et al, 1996). We constructed a lentivirus vector targeting the expression of hTERT (LV-hTERTi) using the plasmid pLL3.7-hTERTi, which expresses the shRNA containing the hTERT target sequence (5’-GCGGATTTGTGAAATGGA-3’; nucleotides 1947–1965 of the hTERT mRNA sequence, see GenBank Accession NM 198253). As a
negative control, we constructed another lentiviral vector (LV-Cont.), which expresses nonfunctional shRNA containing the hTERT mRNAs sequences (5′- GAACGTGCTGGCCTCGGC-3′; nucleotides 337–357) that fails to inhibit the endogenous expression of hTERT. The viral supernatant was collected at 24–36 h after transfection and concentrated by ultracentrifugation, as previously described (Stewart et al., 1999). Ultracentrifugation led to the concentration of the original viral supernatant by at least 12.5 fold. Rapidly proliferating NHOK, SCC4, and BaP-T cells were infected with 1 ml of the concentrated virus (LV-GFP, LV-Cont., LV-Bmi-1, and/or LV-hTERT) in the presence of 6 μg/mL polybrene for 3 h. This infection scheme invariably resulted in > 90% infection efficiency determined by the percentage cells labelled with green fluorescence. The infected cells were photographed using an inverted epifluorescence microscope (Nikon, Melvill, NJ, USA).

RESULTS

Bmi-1 is overexpressed in OSCC cells and tissues

To determine the association between Bmi-1 overexpression and oral carcinogenesis, we compared the expression levels of Bmi-1 in two independent cultures of NHOK and seven OSCC cells by semi-quantitative RT–PCR (Figure 1A and Table 1). RKO and HeLa cells, representing the colorectal and cervical cancer cells, respectively, were also included for comparison. All tested cancer cell lines expressed significantly higher level of Bmi-1 compared with those of NHOK. Replicating NHOK (strain 01-4, population doubling (PD) 16) expressed higher level of Bmi-1 than the senescing cells (strain 01-4, PD 18 and 05-1, PD 20). The Bmi-1 expression level was also determined by Western blotting in NHOK and the OSCC cell lines (Figure 1B). Bmi-1 protein was weakly detected in NHOK cultured in serum-free KGM and was not altered by the culture conditions (data not shown). As Bmi-1 is known to negatively regulate the expression of p16INK4A in some cells (Jacobs et al., 1999), we checked for the correlation between the expression levels of Bmi-1 and p16INK4A in our experimental system. Bmi-1 protein expression level was notably higher in most of the OSCC cell lines compared with that of NHOK, but did not correlate with the expression level of p16INK4A, which correlated more closely with the human papillomavirus (HPV) infection status (Table 1).

Bmi-1 protein expression in situ was determined by immunohistochemical staining of paraffin-embedded oral mucosal tissues with varying degrees of histopathology that covers the entire spectrum of oral carcinogenesis (Figure 1C). Bmi-1 protein

Figure 1  Bmi-1 is overexpressed in the cancer cells derived from OSCC. (A) Endogenous Bmi-1 expression level was compared by semi-quantitative RT–PCR in NHOK (01–4 and 05–1) and nine cancer cell lines including seven OSCC cell lines. Glyceraldehyde-3-phosphate dehydrogenase was amplified as a loading control. (B) Western blotting was performed to compare the Bmi-1 protein expression levels in NHOK and OSCC cells. HOK/Bmi-1, overexpressing exogenous Bmi-1 (Kim et al., 2006), was included as a positive control for Bmi-1 expression. In the same samples, we probed for the level of p16INK4A mRNA, to determine whether aberrant Bmi-1 expression correlated with downregulation of p16INK4A. β-actin was used as a loading control. (C) In situ immunohistochemistry was performed with oral mucosal tissue specimens showing the histological features of normal (n = 8), dysplasia (n = 9), and OSCC (n = 10). The representative examples of histology (left panel, haematoxylin–eosin staining) and the Bmi-1 staining (right panel) are shown in this figure. Bmi-1 expression was detected by DAB staining (brown color). As negative controls, we included normal and OSCC specimens subjected to the staining procedure in the absence of specific Bmi-1 antibody (–Bmi-1 Ab). The images were captured with original magnification of × 100. (D) NHOK (a–c) and SCC4 (d–f) cells were fixed, permeabilised, and stained for Bmi-1 by indirect immunoperoxidase method. Bar = 100 μm.
expression, revealed by brown 3,3′-diaminobenzidine hydrochloride (DAB) staining, was weakly detectable and limited to the basal cell layer of normal stratified epithelium (n = 8) and was not found in the upper spinosum and corneum layers. In contrast, all preneoplastic oral epithelial tissues (n = 9) displaying mild, moderate, and severe dysplasia as well as malignant oral lesions (n = 10) showed elevated Bmi-1 staining compared with those of the normal tissues (Table 2). The Bmi-1 staining in these aberrant tissues was detected in most of the epithelial layers containing viable cells, including stratum basale, spinosum, and granulosum.

Table 1 The OSCC cell lines and the status of Bmi-1, p16INK4A, p53, and HPV infection

| Cells          | Tissue origin | Bmi-1* | p16INK4Ab | p53 | HPV status |
|----------------|---------------|--------|-----------|-----|------------|
| BaP-TG         | gingiva       | –      | +         | –   | Targeted by E6 +/type 16 |
| SCC4          | tongue        | +      | +         | –   | Mutant     |
| SCC9          | tongue        | –      | –         | –   | –          |
| SCC15        | tongue        | –      | –         | –   | –          |
| HEP-2        | larynx        | +      | +         | Targeted by E6 +/type 18 |
| I-RB3        | oral          | +      | +         | Targeted by E6 +/type 18 |
| FaDu4       | pharynx       | +      | +         | Mutant    |
| Tu139       | larynx        | +      | –         | –   | Mutant     |
| RKO          | colorectum    | +      | –         | –   | Wild-type  |
| Hela6       | cervix        | +      | +         | Targeted by E6 +/type 18 |

* Bmi-1 overexpression was determined based on the RT–PCR and Western blotting results (Figure 1). Positive indicates enhanced expression compared with the baseline (negative) expression level found in the NHOK cultures. p16INK4A expression status is based on the Western blotting results (Figure 1B). cTumourigenic counterpart of NHOK (Park et al. 1995). dPurchased from American Type Culture Collection. The protein expression patterns for these cell lines were based on Munro et al. 1999. eGift of P. Sadis (Univ. Texas, Houston, TX, USA). fGift of G. Clayman (Univ. Texas, Houston, TX, USA). gGift of M. Kastan (Johns Hopkins, Baltimore, MD, USA).

Stratum corneum and lucidum did not show Bmi-1 staining owing to the lack of viable cells in these layers. We also compared the levels of Bmi-1 staining in situ in cultured normal and cancer cells. Normal human oral keratinocytes and SCC4 were subjected to indirect immunoperoxidase staining for Bmi-1 using anti-Bmi-1 antibody. Diffuse cytoplasmic Bmi-1 staining was noted in NHOK, whereas SCC4 cells showed strong intranuclear and faint cytoplasmic Bmi-1 staining (Figure 1D). These data are in keeping with the Western blotting result, which showed notable increase in the Bmi-1 expression level in SCC4 cells compared with that of NHOK. The above results indicate that the elevated Bmi-1 expression is associated with preneoplastic oral lesions and is sustained in oral cancer.

Table 2 Bmi-1 immunoreactivity in situ is elevated in the oral epithelium with dysplastic and cancerous histopathology

| Pathologic category | Specimen no. | Histopathologic finding                                      | Bmi-1* |
|---------------------|--------------|-------------------------------------------------------------|--------|
| Normal (n = 8)      | 1            | Normal oral epithelium                                      | +      |
|                     | 2            | Normal oral epithelium                                      | +/-    |
|                     | 3            | Normal oral epithelium                                      | +/-    |
|                     | 4            | Normal oral epithelium                                      |       |
|                     | 5            | Normal oral epithelium                                      |       |
|                     | 6            | Normal oral epithelium                                      | +/-    |
|                     | 7            | Normal oral epithelium                                      | +/-    |
|                     | 8            | Normal oral epithelium                                      |       |
| Dysplasia (n = 9)   | 1            | Moderate-severe epithelial dysplasia                         | +++    |
|                     | 2            | Focal keratosis and mild epithelial dysplasia                | ++     |
|                     | 3            | Mild epithelial dysplasia                                    | +      |
|                     | 4            | Moderate-severe epithelial dysplasia                         | +++    |
|                     | 5            | Moderate-severe epithelial dysplasia                         | ++     |
|                     | 6            | Hyperkeratosis and mild epithelial dysplasia                 | +++    |
|                     | 7            | Severe epithelial dysplasia                                  | +++    |
|                     | 8            | Mild epithelial dysplasia                                    | +++    |
|                     | 9            | Severe epithelial dysplia                                    | +++    |
| HNSCC (n = 10)      | 1            | Moderately differentiated squamous cell carcinoma            | ++     |
|                     | 2            | Moderately well differentiated squamous cell carcinoma       | ++     |
|                     | 3            | Superficial moderately differentiated squamous cell carcinoma| +++    |
|                     | 4            | Poorly differentiated squamous cell carcinoma                | +++    |
|                     | 5            | Moderately differentiated squamous cell carcinoma            | +      |
|                     | 6            | Well-differentiated squamous cell carcinoma                  | +      |
|                     | 7            | Well-differentiated squamous cell carcinoma                  | +      |
|                     | 8            | Moderately differentiated squamous cell carcinoma            | ++     |
|                     | 9            | Well-differentiated squamous cell carcinoma                  | +++    |
|                     | 10           | Moderately differentiated squamous cell carcinoma            | +++    |

The level of Bmi-1 immunostaining per each specimen was scored as negative (−), barely detectable (+/−), weak (+), moderate (+++), or strong (++++) by an oral pathologist, noting the level of chromogenic development after addition of the DAB substrate. The scoring was confirmed blindly by an individual without prior knowledge or understanding of the nature of the tissue specimens. Bmi-1 in normal oral epithelium was limited to the basal cell layer (Figure 1C). Bmi-1 staining in the dysplastic and the OSCC samples was homogenously detected in most of the epithelial layers including stratum basale, spinosum, and granulosum (Figure 1C).

Bmi-1 gene is not amplified in the OSCC cell lines

To determine whether the Bmi-1 overexpression in the OSCC cells resulted from gene amplification, we compared the amount of Bmi-1 genomic sequences by Southern blotting in normal and cancer cells. We included five strains of NHOK, two NHOF strains, and seven cancer cell lines including six OSCC cell lines. The Bmi-1-specific signals were obtained at 4.3 kb, 3.1 kb, and 2.4 kb fragments, and were compared among the tested samples as its ratio to that of GAPDH (Figure 2). We found that there was no statistically significant difference in the relative abundance of the Bmi-1 genomic sequences between the normal and the cancer cells. The Bmi-1 overexpression in OSCC may result from the mechanisms not involving gene amplification, such as promoter activation.

Bmi-1 knockdown inhibits cellular proliferation of normal and cancer cells

To investigate the biological role of Bmi-1, we knocked down the expression of endogenous Bmi-1 by RNAi in SCC4 cells and...
Bmi-1 gene is not amplified in OSCC. Genomic DNAs from five different NHOK strains, two NHOF strains, and seven cancer cell lines were digested with EcoRI I and Hind III and transferred for probing. Radiolabelled probes synthesised from Bmi-1 or GAPDH cDNA were hybridised sequentially onto the membrane. The phosphometric intensities were plotted as the ratio of Bmi-1 to GAPDH. The lack of statistical difference (P > 0.05) in the levels of Bmi-1-radioactive signals was determined by unpaired T-test (one-ways ANOVA) between the NHOK and the cancer groups.

exponentially replicating NHOK. For this purpose, we constructed a lentiviral vector (LV-Bmi-1i) capable of expressing shRNA targeting Bmi-1 using the pLL3.7 plasmid, which also contains the green fluorescent protein (GFP) expression cassette under a heterologous promoter (Rubinson et al, 2003). SCC4 cells and replicating NHOK (strain 05–10, PD 10) were infected with LV-Bmi-1i and the control lentiviral vector (LV-GFP) expressing GFP alone. At 3 days post-infection, more than 95% of the cultures infected with LV-GFP or LV-Bmi-1i demonstrated green fluorescence (Figure 3A), indicating efficient infectivity. Also, at 3 days post-infection, the endogenous Bmi-1 expression level was decreased by 83 and 85%, respectively, in NHOK and SCC4 cells infected with LV-Bmi-1i if compared with the cells infected with LV-GFP (Figure 3B). The SCC4 cells were maintained in culture for longer than 10 days post-infection, during which time the cells infected with LV-Bmi-1i showed a marked reduction in proliferative capacity (Figure 3A). The LV-GFP-infected SCC4 cells continued to replicate beyond the 10-day period, whereas the SCC4 culture exposed to LV-Bmi-1i contained sparse, flattened cells with GFP expression.

At 3 days post-infection, the majority of NHOK also demonstrated the fluorescence signal (Figure 4A). Normal human oral keratinocytes infected with LV-GFP underwent 16 PDs before the onset of senescence, whereas those infected with LV-Bmi-1i expressed increased SA-β-Gal activity and showed notable retardation of proliferation following the infection, completing only 12 cumulative PDs (Figure 4B). To determine whether the retarded proliferation in NHOK infected with LV-Bmi-1i represented the cellular senescence response, we compared the expression levels of GPRI, MMP-1, and MMP-3, which are the molecular markers of keratinocyte senescence (Kang et al, 2003). Semi-quantitative RT–PCR was performed with NHOK after 10 days post-infection (Figure 4C). Expression levels of GPRI, MMP-1, and MMP-3 were induced in NHOK infected with LV-Bmi-1i compared with those infected with LV-GFP. Also, the cells with Bmi-1i-induced OSCC replication arrest and death.
In the present study, we examined the possible involvement of the PcG protein Bmi-1 in oral carcinogenesis by (1) comparison of the Bmi-1 protein and RNA expression levels in normal and OSCC cells and tissues and (2) knockdown of endogenous Bmi-1 in normal and OSCC cells. The first part of the study revealed that Bmi-1 overexpression was consistently observed in the OSCC cells and tissues compared with the normal controls. The level of Bmi-1 expression in the OSCC cells is comparable to that of the cancer cells, that is, RKO and HeLa, derived from other cancer types. Importantly, Bmi-1 overexpression was also observed in 100% (9/9) of the preneoplastic oral mucosal tissues which included those with mild, moderate, or severe epithelial dysplasia. This finding indicates that Bmi-1 overexpression occurs very early during oral carcinogenesis and may be used as a biomarker of preneoplastic oral lesions.

Using the Bmi-1 promoter-luciferase construct, we found that the promoter activity was strongly induced in the OSCC cells compared with that of NHOK (unpublished observation), indicating that Bmi-1 overexpression results from promoter activation. Recent studies showed that Bmi-1 is a direct transcriptional target of c-Myc in human fibroblasts (Guney et al, 2006) and of E2F-1 in neuroblastomas (Nowak et al, 2006). c-Myc was found to be frequently amplified in OSCCs by comparative genomic hybridisation (Chen et al, 2004). Thus, c-Myc-dependent Bmi-1 promoter activation may account for the enhanced gene expression during dysplastic cell transformation in oral carcinogenesis, although the extent to which c-Myc regulates the Bmi-1 promoter activity in oral carcinogenesis needs further investigation.

Bmi-1 knockdown led to inhibition of cellular replication in both NHOK and OSCC cells. However, the cellular response seems to be different between the normal and cancer cells. In NHOK, Bmi-1 knockdown did not elicit an immediate response of replication arrest and/or loss of viable cells, although the cellular replication rate was notably reduced and the senescence prematurely triggered. In contrast, SCC4 and BaP-T cells underwent rapid arrest of replication upon infection with LV-Bmi-1i and showed drastic decrease in the viable cell number. After 8–10 days post-infection, the majority of the OSCC cells detached from the culture dish (Figures 3A and 5). Comparison of the replication kinetics of the cells infected with the LV-GFP or LV-Bmi-1i also demonstrates clear difference in the cellular response between the normal and OSCC cells.

**DISCUSSION**

Figure 4  Inhibition of endogenous Bmi-1 causes premature senescence in NHOK. (A) Rapidly proliferating NHOK (05-10) was infected with LV-GFP or LV-Bmi-1i. Phase contrast photographs, SA β-Gal staining, and GFP fluorescence were obtained 10 days after virus infection. Original magnification, ×100. (B) Proliferation kinetics of NHOK infected with LV-GFP or LV-Bmi-1i was determined and plotted against time in culture. (C) NHOK cultures were harvested at 10 days after infection with LV-GFP or LV-Bmi-1i, and the expression levels of GPR1, Cyc2A, MMP-1, and MMP-3 were determined by semi-quantitative RT–PCR. The band intensities were quantitated and plotted by Scion Image software against those of GAPDH amplification. (D) NHOK (05-10) and SCC4 cells infected with LV-GFP or LV-Bmi-1i were harvested at 10 days after infection, and Western blotting was performed with 100 μg WCE for p16INK4A, CDK4 was detected as a loading control.
NHOK and the BaP-T cells (Figures 4B and 6). Our result is consistent with a recent report demonstrating cancer-specific cytotoxic effect of Bmi-1 knockdown in neuroblastoma cells (Liu et al, 2006). The differential effect of Bmi-1 knockdown in the normal and cancer cells may be beneficial if Bmi-1 were to be targeted for anticancer therapy.

The LV-Bmi-1i vector was effective against the BaP-T cells, which express HPV type 16 E7 (Park et al, 1995). Thus, Bmi-1-mediated growth inhibition may not require intact p16\(^{\text{INK4A}}\)/pRb checkpoint pathway, as it is also reflected in the absence of p16\(^{\text{INK4A}}\) induction in the NHOK and SCC4 cells after Bmi-1 knockdown. The inverse correlation between Bmi-1 and p16\(^{\text{INK4A}}\) protein expression levels was not evident in the OSCC cell lines in our study (Figure 1B). Furthermore, we recently reported that expression of exogenous Bmi-1 in NHOK led to significant extension (2.7 fold) of replicative life span but did not efficiently downregulate the expression of p16\(^{\text{INK4A}}\) (Kim et al, 2006). It is possible that Bmi-1 targets other important regulators of cell division in NHOK, such as p14\(^{\text{ARF}}\) or p15\(^{\text{INK4B}}\), as previously suggested (Jacobs et al, 1999). Several recent studies also showed that the aberrant Bmi-1 expression is not necessarily associated with downregulation of p16\(^{\text{INK4A}}\) expression (Dukers et al, 2004; Breuer et al, 2005). Also, Bracken et al (2006) recently identified numerous cellular genes targeted by Bmi-1 other than p16\(^{\text{INK4A}}\) using microarray-based expression screening and chromatin immunoprecipitation (ChIP)-on-chip analysis. These findings support the possibility that Bmi-1 elicits its oncogenic property during oral carcinogenesis in p16\(^{\text{INK4A}}\)-independent manner.

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