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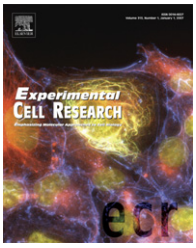

Research Article

# Bmi-1 cooperates with human papillomavirus type 16 E6 to immortalize normal human oral keratinocytes

*Reuben H. Kim*[a,1], *Mo K. Kang*[a,b,1], *Ki-Hyuk Shin*[a,b], *Zin M. Oo*[a], *Thomas Han*[a], *Marcel A. Baluda*[c], *No-Hee Park*[a,b,c,*]

[a]*School of Dentistry at UCLA, Los Angeles, CA 90095, USA*
[b]*Jonsson Comprehensive Cancer Center at UCLA, Los Angeles, CA 90095, USA*
[c]*David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA*

ARTICLE INFORMATION

*Article Chronology:*
Received 26 July 2006
Revised version received
21 October 2006
Accepted 23 October 2006
Available online 28 October 2006

*Keywords:*
Bmi-1
HPV-16 E6
Telomerase activity
Immortalization
Keratinocytes

ABSTRACT

Bmi-1 is a member of the polycomb group (PcG) transcription repressors and is implicated in human carcinogenesis. In normal human oral keratinocytes (NHOK), we found that exogenous Bmi-1 expression significantly extended the replicative life span without causing cellular immortalization. Immortalization of NHOK occurs only in combination with human papillomavirus type 16 E6 (HPV-16 E6) but not with E7. During immortalization of NHOK by sequential expression of exogenous Bmi-1 and E6, telomerase activation was observed only after the cells had overcome crisis. Genetic analysis with E6 deletion mutants revealed that the intact second zinc finger domain (amino acids 118–122) was necessary for its cooperative effects with Bmi-1 in the immortalization process. Using these mutants, we found that the increased telomerase activity was closely associated with cell immortalization by Bmi-1 and E6, whereas p53 degradation was not. Using microarray analysis, we identified genes that are immortalization-specific and may participate in the process of NHOK immortalization by Bmi-1 and HPV-16 E6. Our results provide new information on the roles of Bmi-1 and HPV-16 E6 in the multi-step process of oral epithelial carcinogenesis.



## Introduction

Carcinogenesis is a multi-step process that occurs via an accumulation of a series of discrete, irreversible, complementary events. These events include the disruption of the tumor suppressor genes, e.g., p53 and pRb, which are frequently found to be inactivated during the development of human cancers. 'High-risk' human papillomaviruses (HPVs), such as HPV-16 and HPV-18, are closely associated with human cancers [1,2]. The expression of the 'high-risk' HPV E6 and E7 oncogenes can efficiently immortalize several different cell types [3–8], but individually E6 or E7 immortalizes only certain cell types, e.g., human mammary epithelial cells (HMEC), human embryonic fibroblasts, and human foreskin keratinocytes (HFK) [9–11]. This suggests that HPV E6 and E7 participate in immortalization in a cell-type-specific manner.

'High-risk' HPV E6 and E7 participate in the multi-step process of carcinogenesis by interfering with the protective mechanisms of p53 and pRb. The 'high-risk' HPV E7 oncoprotein binds and inactivates pRb, causing E2F release from pRb

* *Corresponding author*. UCLA School of Dentistry, CHS 53-038, 10833 Le Conte Ave. Los Angeles, CA 90095-1668, USA. Fax: +1 310 825 3290.
E-mail address: npark@dent.ucla.edu (N.-H. Park).
[1] Reuben Kim and Mo Kang contributed equally.

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doi:10.1016/j.yexcr.2006.10.025

and the eventual cell cycle progression. The E6 oncoprotein binds and inactivates p53, facilitating the latter's degradation via the ubiquitin pathway [12,13]. E6 also induces telomerase activity which is greatly elevated in the vast majority of immortal cells and cancers if compared to their normal counterparts [14,15]. Telomerase is activated immediately after the cells escape from the replicative crisis, and its activity prevents further shortening of telomere DNA, suggesting that telomerase activation is an important early event during the process of immortalization and carcinogenesis [16–18].

The cellular *Bmi-1* gene also plays a role in carcinogenesis. Initially identified as a c-myc-cooperating cellular gene in murine lymphomas and as a polycomb group (PcG) transcription repressor gene, *Bmi-1* is expressed during normal replication of primary human cells but its expression level decreases as the cells enter senescence, during which both the $p16^{INK4A}$ and $p14^{ARF}$ genes become overexpressed [19]. $p16^{INK4A}$ and $p14^{ARF}$ inhibit cyclin D-Cdk4/6 kinase complexes and MDM2-mediated p53 degradation, respectively. Bmi-1 plays at the upstream of $p16^{INK4A}$ and $p14^{ARF}$, abolishing both the Rb and the p53 pathway by inhibiting transcription of the *INK4a* gene [20]. As a result, it leads to the inhibition of senescence and the extension of the life span. Bmi-1 has also been implicated in telomerase activation in epithelial cells [21], suggesting that it may play another fundamental role in carcinogenesis. However, Bmi-1 does not induce telomerase activity in fibroblasts, indicating that it is a cell-type-specific event possibly restricted to cells from epithelial origin [21]. At the clinical level, overexpressed Bmi-1 is frequently detected in many human cancers [22–24], suggesting that Bmi-1 plays an important role in tumor formation. Whether Bmi-1 has these roles in different cell types requires further examination.

As part of our studies to elucidate the mechanism(s) of immortalization in normal human oral keratinocytes (NHOK), we sought to characterize the roles of Bmi-1 and HPV-16 oncoproteins E6 and E7 in this process. We overexpressed Bmi-1 in NHOK and demonstrated that Bmi-1 alone extended their life span significantly but failed to immortalize them. In NHOK overexpressing Bmi-1, HPV-16 E6, but not E7, enabled the cells to overcome crisis and become immortalized. Using in-frame deletion single mutants of E6, we found that the second zinc finger domain (amino acids 118–122) of E6 was responsible for immortalization. Also, we found that telomerase activity, but not the p53 degradation, was closely associated with immortalization, implying that telomerase activation was the critical event necessary to overcome the replication crisis and to induce immortalization. Using microarray analysis, we found several genes that were specifically upregulated in Bmi-1/E6 cells but neither in Bmi-1/LXSN nor Bmi-1/E6Δ118–122 cells, indicating that these genes may contribute to the immortalization of Bmi-1/E6 cells.

## Materials and methods

### *Cells and cell culture*

Primary NHOK were prepared from keratinized oral epithelial tissues according to methods described elsewhere [25]. Detached cells were seeded onto collagen-treated flasks and cultured in Keratinocyte Growth Medium (KGM) containing a low level of calcium (0.15 mM) and a supplementary bullet kit (Cambrex, East Rutherford, NJ). The cumulative population doublings (PDs) and replication kinetics were determined based on the number of NHOK harvested at every passage. HOK-16B, an immortalized counterpart of NHOK harboring the HPV-16 genome [26], and HOK-16B B(a)P-T, a tumorigenic counterpart of NHOK [27], were included in the study.

### *Retroviral vector construction and transduction of cells*

The following retroviruses were used in this study: RV-B0, RV-Bmi-1, RV-LXSN, RV-16E6, RV-16E7, RV-16E6Δ8S9A10T, RV-16E6Δ118–122, and RV-16E6Δ146–151. Retroviruses expressing Bmi-1 were constructed from pBabe-puro containing Bmi-1 cDNA, which was kindly provided by Dr. G. Dimri (Evanston Northwestern Healthcare Research Institute, Evanston, IL). Retroviruses expressing HPV-16 E6 and E7 were constructed from pLXSN-16E6 and pLXSN-16E7, which were kindly provided by Dr. D. Galloway (Fred Hutchinson Cancer Center, University of Washington, Seattle, WA). RV-16E6Δ8S9A10T, RV-16E6Δ118–122, and RV-16E6Δ146–151 were also constructed as described previously [28]. The retroviral expression plasmids were transfected into GP2-293 universal packaging cells (Clonetech, Mountain View, CA) with pVSV-G envelope plasmid using a calcium-phosphate transfection kit (Invitrogen, Carlsbad, CA). Two days after transfection, the virus supernatant was collected and concentrated by ultracentrifugation. The virus pellet was resuspended in KGM and was used for infection or stored in −80°C for later use.

Secondary NHOK cultures were infected with RV-B0, RV-Bmi-1, RV-LXSN, RV-16E6, and RV-16E7 in the presence of 6 μg/ml polybrene for 3 h. Selection of cells began at 48 h after infection with 1 μg/ml puromycin (for RV-B0 and RV-Bmi-1) or 200 μg/ml G418 (for RV-LXSN, RV-16E6, and RV-16E7). The drug-resistant cells were maintained in subcultures as described above. NHOK infected with RV-Bmi-1 was superinfected with RV-LXSN, RV-16E6, RV-16E7, RV-16E6Δ8S9A10T, RV-16E6Δ118–122, and RV-16E6Δ146–151, and selected with 200 μg/ml G418.

### *Reverse transcription (RT)-PCR*

Total RNA was isolated from the cultured cells using Trizol™ reagent (Invitrogen) and was subjected to RNases-free DNase I digestion at 37°C for 2 h to eliminate any contaminating genomic DNA. DNA-free total RNA (5 μg) was dissolved in 15 μl DEPC-$H_2O$, and the RT reaction was performed in first strand buffer (Invitrogen) containing 300U Superscript II (Invitrogen), 10 mM dithiotrietol, 0.5 μg random hexamer (Promega Corporation, Madison, WI) 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 $H_2O$.

The following primers were used for PCR amplification: E6 primers, 5′-ATGTTTCAGGACCCACAG-3′ (forward), 5′-CAGGACACAGTGGCTTTT-3′ (reverse); E7 primers, 5′-GATCGGATCCATGCATGGAGATACA-3′ (forward), 5′-CTAGGTCGACTTATGGTTCTGAGA-3′ (reverse); Bmi-1 primers, 5′-AGCAGAAATGCATCGAACAA-3′

(forward), 5′-CCTAACCAGATGAAGTTGCTGA-3′ (reverse); GAPDH primers, 5′-GACCCC TTCATTGACCTCAAC-3′ (forward), 5′-CTTCTCCATGGTGGTGAAGA-3′ (reverse); and hTERT primers, 5′-GCCTGAGCTGTACTTTGTCAA-3′ (forward), 5′-CGCAAACAGCT TGTTCTC CATGTC-3′ (reverse). To verify microarray data, we used the primers shown in Table 1.

### *Western blotting*

Whole cell extracts (WCE) from NHOK cultures expressing Bmi-1 and/or E6 were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon protein membrane (Millipore, Billerica, MA). Immobilized membrane was incubated with primary antibodies, i.e., Bmi-1 (F6; Upstate, Charlottesville, VA), p53 (DO-1; Santa Cruz, CA), p21 (Ab-1, EMD Biosciences, Inc., San Diego, CA), Rb (IF8, Santa Cruz) and β-actin (I-19, Santa Cruz), and probed with the respective secondary antibodies.

### *Analysis of hTERT reporter activity*

A pGL3B-TRTP containing the 1670-bp fragment (−1665 to +5) of full-length hTERT promoter upstream of the firefly luciferase gene in the pGL3-basic (Promega Corporation), kindly provided by Dr. J. Carl Barrett (National Institute of Environmental Health Science), was used for the *hTERT* reporter assay. Prior to transfection, a six-well plate with approximately $5\times10^4$ cells per well was inoculated and cultured for 24 h. The pGL3B-TRTP vector (1 μg/well) was transfected alone or together with pBABE-Bmi-1 or pLXSN-16E6 vector (1 μg/well) using Lipofectin Reagent (Invitrogen). For better comparison among cells with different transfection efficiencies, pRL-SV40 plasmid (0.001 μg/well), which has the Renilla Luciferase gene under the transcriptional control of SV40 enhancer/promoter, was also transfected into each cell and used for normalization of the activities shown by the pGL3B-TRTP construct. Cells were collected 48 h after transfection, and the cell lysates were prepared using Dual-Luciferase Reporter Assay System (Promega Corporation). Luciferase activity was measured using a luminometer.

### *Telomeric repeat amplification protocol (TRAP) assay*

Telomerase activity was determined by using the TRAP-eze Telomerase Detection Kit (Chemicon, Temecula, CA, USA) according to manufacturer's guidelines. Cell lysate was extracted using 1× CHAPS buffer, and aliquoted and frozen in liquid nitrogen for subsequent TRAP assay. The cellular extracts of HOK-16B B(a)P-T containing telomerase activity served as positive control.

The telomerase reaction mixture was prepared by adding the cell lysate containing 1 μg of cellular protein to 24 μl solution comprising 1× TRAP reaction buffer. The telomerase reaction product was allowed for 30 min at 30°C and amplified by PCR using a DNA Thermal Cycler (Perkin-Elmer, Foster City, CA). The following conditions were used for PCR: 32 cycles at 94°C for 30 s and 59°C for 30 s, followed by one delayed extension cycle at 72°C for 10 min. The PCR products were electrophoresed in 12.5% non-denaturing polyacrylamide gel in 1× Tris–borate EDTA for 90 min at 60 W. After drying the gel, the radioactive signal was detected by Phosphor Imager (Molecular Dynamix Inc., Sunnyvale, CA, USA).

### *Telomere length determination by Southern blot hybridization*

Genomic DNA isolated from NHOK cultures at different PDs was extracted by the conventional method as previously described [26]. 10 μg of DNA digested with the restriction enzyme HinfI was electrophoresed in a 0.8% agarose gel and transferred to Hybond nylon membrane (Amersham, Arlington Heights, IL). After rinsing with 2× SSPE, the filter was baked at 80°C for 2 h in a vacuum oven. The filter was soaked in 2× SSPE for 5 min, and prehybridized in 20 ml of prehybridization buffer (0.5 M $NaH_2PO_4$, pH 7.2, 7% SDS, 1% BSA, 0.5 mM EDTA) at 65°C for 4 h. Hybridization was performed with a $^{32}$P-labeled telomere probe in the above buffer at 65°C for 16 to 24 h. Subsequently, the filter was washed with 2× SSPE–0.1% SDS at room temperature for 15 min, and with 0.1× SSPE–0.1% SDS twice at 65°C for 15 min each. The radioactive signals were detected by Phosphor Imager (Molecular Dynamix Inc.), and densitometric analysis of each lane was performed using NIH Image v1.60 (National Institutes of Health, Bethesda, MD).

### *Microarray analysis*

The microarray probe synthesis, hybridization, and scanning were done at the UCLA Microarray Core Facility as previously described [29]. Briefly, HOK/Bmi-1 cells were infected with RV-LXSN or RV-16E6 and selected with 100 μg/ml G418. Total cellular RNA was isolated from the actively proliferating Bmi-1/LXSN and Bmi-1/E6 cells using Qiagen RNeasy total RNA isolation kit (Qiagen Inc.). cDNA was synthesized from 10 μg total RNA and the biotin-labeled antisense cRNA was

**Table 1 – Primers used in RT-PCR for microarray data validation**

| Genes | Forward primer | Reverse primer |
|---|---|---|
| *DNAJC12* | 5′-CAGACAAGCATCCTGAAAACCC-3′ | 5′-TCGCCAGTGGTCATAGCGGGC-3′ |
| *TNFSF11* | 5-TGGATCACAGCACATCAGAGCAG-3 | 5-TGGGGCTCAATCTATATCTCGAAC-3 |
| *UGT1A6* | 5′-GGAAAATACCTAGGAGCCCTGTGA-3′ | 5′-AGGAGCCAAATGAGTGAGGGAG-3′ |
| *CCL5* | 5′-CTCATTGCTACTGCCCTCTGCGCTCCTGC-3′ | 5′-GCTCATCTCCAAAGAGTTGATGTACTC-3′ |
| *MEST* | 5′-TCAAAGATGGAGGTGTGCTG-3′ | 5′-CTCCTGCTGGCTTCTTCCTA-3′ |
| *PODXL* | 5′-CCGTGGTCGTCAAAGAAATC-3′ | 5′-GTCGTCCTTGGTCAGGTTGT-3′ |
| *ATF3* | 5′-CTCCTGGGTCACTGGTGTTT-3′ | 5′-GTCGCCTCTTTTTCCTTTCA-3′ |
| *c-fos* | 5′-GAATAAGATGGCTGCAGCCAAATGCCGCAA-3′ | 5′-CAGTCAGATCAAGGGAAGCCACAGACATCT-3′ |
| *Notch 1* | 5′-GGCCACCTGGGCCGGAGCTTC-3′ | 5′-GCGATCTGGGACTGCATGCTG-3′ |
| *DAPK1* | 5′-GACACCGGCGAGGAACTTGGC-3′ | 5′-AAAGTCAATGATCTTGATCCGA-3′ |

produced by in vitro transcription using the ENZO BioArray HighYield kit. cRNA (15 μg) was fragmented and hybridized to Affymetrix HG-U133 Plus 2.0. The chip was stained with streptavidin–phycoerythrin and analyzed using GCOS 1.2 software for pairwise comparison of gene expression. The statistical significance for each gene was evaluated by ANOVA single-factor analysis using Microsoft Excel 2000 and the fold difference >4.0 as well as $p<0.05$ were considered significant. The microarray data is available in Gene Expression Omnibus (GEO) with an access number GSE6075.

## Results

### *Bmi-1 extended the replicative life span of NHOK but was not sufficient for immortalization*

To determine the effects of Bmi-1 on the replication capacity of NHOK, exponentially replicating NHOK at PD 10 were infected either with the pBABE-based empty retroviral vector (RV-B0) or with that expressing Bmi-1 (RV-Bmi-1). The infected cells were selected with puromycin (1 μg/ml), serially subcultured, and denoted HOK/B0 and HOK/Bmi-1, respectively. Both parental NHOK and HOK/B0 cells spontaneously arrested replication after PD 16 and reached replicative senescence, while HOK/Bmi-1 cells continued to divide until PD 43 and reached "delayed senescence," representing a 2.7-fold increase in the replicative life span (Fig. 1A). The HOK/Bmi-1 cells were maintained in culture for a period of 1 month after replication arrest, but we did not observe any replicating cells. The same experiment was performed with two other independent primary cultures of NHOK with similar results. Thus, ectopic expression of Bmi-1 extended the life span of NHOK but failed to cause immortalization.

### *HPV-16 E6, but not E7, immortalized Bmi-1-expressing NHOK*

To further investigate the mechanism by which NHOK could be immortalized, we investigated the effect of either HPV-16 E6 or E7 on the replicative capacity of HOK/Bmi-1 cells. HOK/Bmi-1 cells were superinfected with a retroviral vector expressing either E6 or E7 of HPV-16 (RV-16E6 or RV-16E7). After selection, the infected cells were serially subcultured and denoted Bmi-1/E6 and Bmi-1/E7, respectively. Both Bmi-1/E6 and Bmi-1/E7 cells further extended their proliferation beyond the replicative senescence, eventually reaching to delayed senescence after approximately 60 PDs for Bmi-1/E6 and 35 PDs for Bmi-1/E7 (Fig. 1A). However, unlike Bmi-1/E7, exponentially

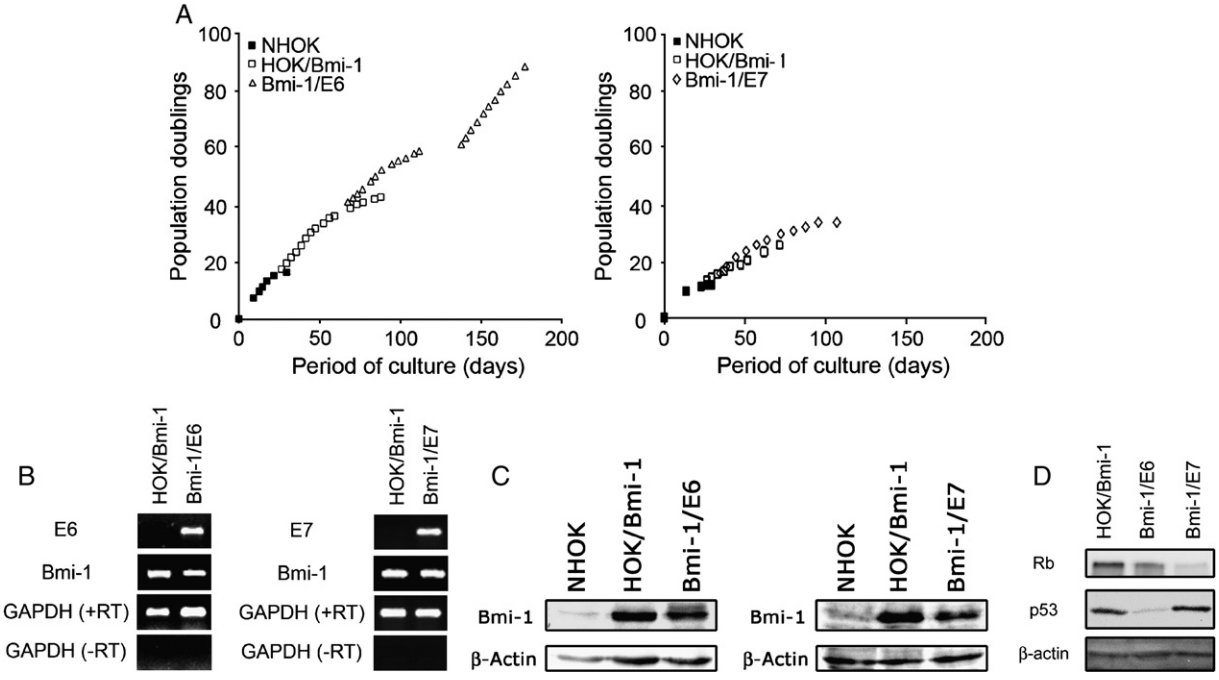


**Fig. 1 – Bmi-1 extended the replicative life span of NHOK, but HPV-16 E6, not E7, is required for cellular immortalization. (A) Secondary NHOK cultures (strain 04–10, left panel; strain 05–10, right panel) were infected with RV-B0 (not shown) or RV-Bmi-1, selected with puromycin (1 μg/ml), and maintained in serial subcultures to obtain the population doublings curve. Immediately after selection with puromycin, the NHOK-expressing Bmi-1 (HOK/Bmi-1) were superinfected with RV-16E6 (left panel) or RV-16E7 (right panel) along with the control, RV-LXSN (not shown), and selected with G418 (200 μg/ml). The HOK/Bmi-1 cells expressing E6, but not E7, underwent additional PDs and eventually became immortalized. (B) Total RNAs were prepared from HOK/Bmi-1, Bmi-1/E6, and Bmi-1/E7 cells after selection. Reverse-transcriptase PCR was performed. GAPDH was used as a control with or without reverse transcriptase (RT). (C) Protein extracts were prepared from the same samples and analyzed for Bmi-1 expression by Western blotting. β-Actin was used as a loading control. (D) Protein extracts were prepared from HOK/Bmi-1, Bmi-1/E6, and Bmi-1/E7 cells and analyzed by Western blotting to confirm that E6 and E7 are fully functional. β-Actin was used as a loading control.**

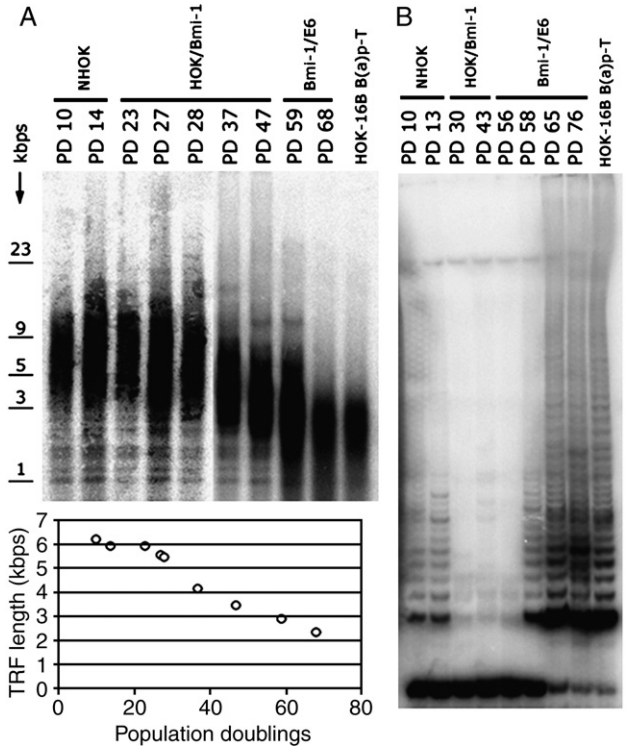


**Fig. 2 – Expression of exogenous Bmi-1 in NHOK did not induce telomerase activity, which was activated only upon immortalization by E6. (A) Using Southern blot hybridization, telomere length was measured in NHOK at various PD levels during immortalization with Bmi-1 and E6 expression. TRF length was quantitated and plotted against PDs. (B) Telomerase activity was measured by the TRAP assay at different PDs during immortalization in cells. HOK-16B B(a)P-T cells were used as a positive control.**

proliferating clones arose from the Bmi-1/E6. These Bmi-1/E6 cells bypassed the crisis and continued to replicate beyond PD 100 and became immortalized, suggesting that E6 had a cooperative role with Bmi-1 in immortalizing NHOK. The expression of E6 or E7 was confirmed by RT-PCR and Western blotting (Figs. 1B and C). The functionality of E6 and E7 in Bmi-1/E6 and Bmi-1/E7 cells was confirmed by Western blotting (Fig. 1D). HPV E6 or E7 alone cannot immortalize primary human keratinocytes [30], and we confirmed this finding in NHOK (data not shown).

### *Expression of exogenous Bmi-1 in NHOK did not induce telomerase activity, which was activated only upon immortalization by Bmi-1 and E6*

Of all the functions of E6 and E7 related to their immortalizing potential, induction of telomerase activity is specific to 'high-risk' HPV E6 and closely associated with cell immortalization [31–33]. This prompted us to examine the status of telomerase activity and telomere length at different culture passages of NHOK during immortalization with Bmi-1 and E6.

Cultured NHOK maintain a telomere length of approximately 6.0 kb until they reach replicative senescence [34]. During the early period of extended life span of NHOK by Bmi-1 (PDs 23 to 28), this telomere length remained constant but progressively shortened to approximately 3 kb at PD 47 (Fig. 2A). Shortening of telomere length continued even in the presence of E6, eventually reaching approximately 2.3 kb when the Bmi-1/E6 cells bypassed the crisis and became immortalized (Fig. 2A).

Telomere length was correlated with telomerase activity in Bmi-1/E6 cells by TRAP assay. Exponentially replicating NHOK at PD 10 and PD 13 demonstrated a moderate but significant level of telomerase activity, which was diminished in the Bmi-

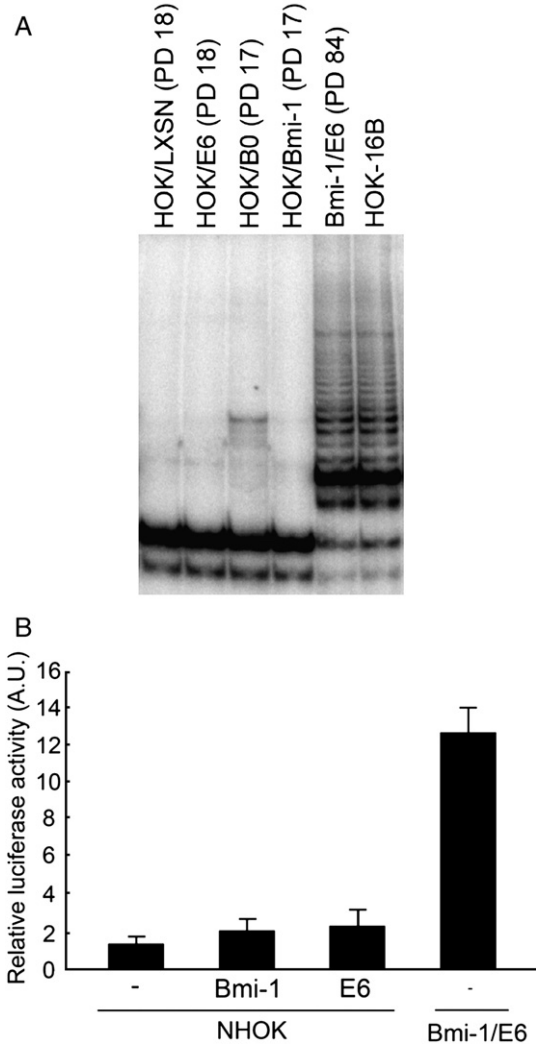


**Fig. 3 – Bmi-1 or HPV-16 E6 alone was not sufficient to cause telomerase activation in NHOK. (A) A secondary NHOK culture (strain 06-3) was infected with RV-B0 or RV-Bmi-1, as well as RV-LXSN or RV-16E6. Immediately after selection with respective selection reagents, cells were passed once and harvested for the TRAP assay. Bmi-1/E6 (post-crisis) and HOK-16B cells were used as positive controls. (B) A secondary NHOK culture (strain 06-1) was transfected with *hTERT*-luc reporter plasmid alone, or co-transfected with pBABE-Bmi-1 or pLXSN-16E6 (all at the passage 1). pRL-SV40 vector was also used to normalize the transfection efficiency. 48 h after transfection, cells were harvested, and luciferase activity was measured. The experiment was performed in triplicates.**

1-expressing cells between PDs 30 to 56 (Fig. 2B). Thus, the expression of exogenous Bmi-1 did not induce telomerase activity, indicating that the extension of life span by Bmi-1 expression was independent of this activity which decreased during senescence [25,34]. When E6 was ectopically expressed in HOK/Bmi-1 cells, telomerase activity was not induced immediately, but it reappeared at a moderate level in the Bmi-1/E6 cells during crisis at PD 58. After PD 65, or after overcoming the crisis, Bmi-1/E6 cells exhibited the highest level of telomerase activity similar to that of HOK-16B B(a)P-T cells, an immortalized and tumorigenic derivative of NHOK (Fig. 2B). This observation was consistent with our previous finding that HPV-16 genome did not induce telomerase activity in NHOK until the crisis stage [18]. These data

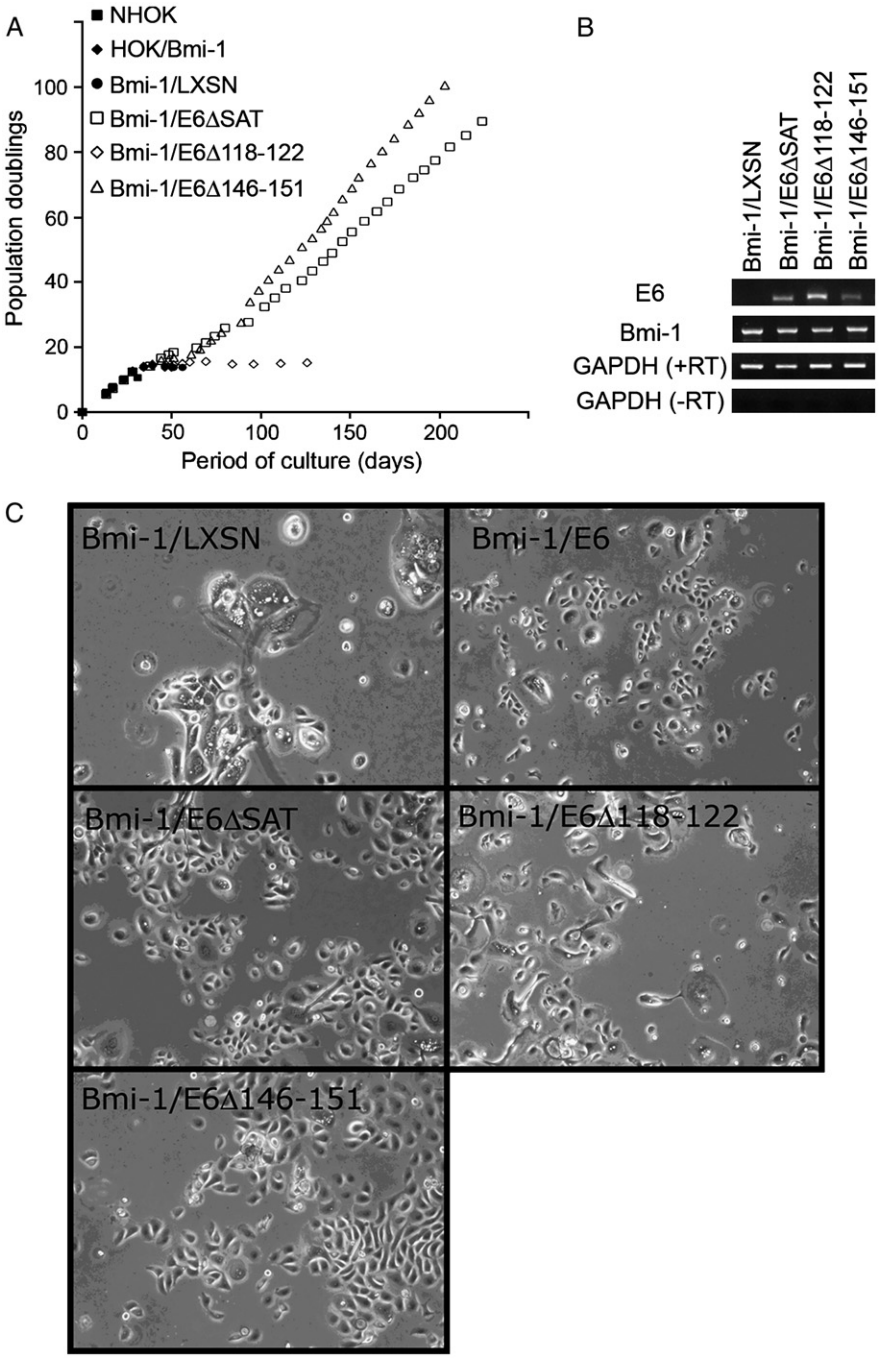


**Fig. 4 – The second zinc finger domain of HPV-16 E6 (amino acids 118–122) was necessary to immortalize HOK/Bmi-1 cells. (A) HOK/Bmi-1 (strain 05-04) cells are superinfected with retroviruses capable of expressing E6 mutants—E6Δ8S9A10T, E6Δ118–122, and E6Δ146–151. After selecting with G418 (200 μg/ml), cells were maintained in serial subcultures. Only cells expressing Bmi-1/E6Δ8S9A10T and Bmi-1/E6Δ146–151 were immortalized. (B) The expression of Bmi-1 and E6 mutants was verified using RT-PCR. GAPDH was used as a positive control. (C) Morphology of HOK/Bmi-1 cells expressing different E6 mutants. Photographs were taken under phase-contrast microscopy (magnification, ×100).**

indicated that telomerase activity was induced during the crisis stage in the presence of both Bmi-1 and E6, allowing the cells to become immortalized.

In Fig. 2B, NHOK showed no telomerase activation when retrovirally infected with Bmi-1 or E6. To further confirm our finding, we accessed telomerase activity by performing the TRAP assay in the HOK/B0 and HOK/Bmi-1 as well as in the HOK/LXSN and HOK/E6 immediately one passage after retroviral infection and selection. No significant telomerase activity was observed in Bmi-1- or E6-expressing NHOK (Fig. 3A). We also examined the transcriptional activity of an exogenous *hTERT* promoter in NHOK by co-transfecting *hTERT* promoter reporter plasmid together with pBABE-Bmi-1 or pLXSN-16E6. In the presence of Bmi-1 or E6, NHOK showed a slight increase in the luciferase activity, whereas the post-crisis Bmi-1/E6 cells caused approximately 13-fold induction in luciferase activity (Fig. 3B), further confirming that the effect of Bmi-1 or E6 on telomerase activation is minimal in the mortal NHOK population.

### *The second zinc finger domain of HPV-16 E6 (118–122) was necessary to bypass the delayed senescence and to immortalize the HOK/Bmi-1 cells*

Many functions of HPV-16 E6 contribute to its immortalization potential including the activation of telomerase and degradation of cellular proteins such as p53 and PDZ-containing proteins [35]. To confirm that induction of telomerase activity by E6 is necessary in the E6-mediated immortalization of HOK/Bmi-1 cells, we used in-frame HPV-16 E6 deletion mutant E6Δ118–122 along with two other E6 mutants, E6Δ8S9A10T and E6Δ146–151. E6Δ118–122 mutant binds and degrades p53 but is defective in telomerase activation; E6Δ8S9A10T mutant cannot target p53 for degradation but activates telomerase activity; and E6Δ146–151 mutant cannot bind to PDZ-containing cellular proteins which are involved in the organization of epithelial architecture [36–38].

HOK/Bmi-1 cells were infected with the appropriate retroviruses, selected and serially subcultured (Fig. 4A). As expected, HOK/Bmi-1 cells overcame replicative senescence but underwent delayed senescence. Expression of E6Δ8S9A10T permitted HOK/Bmi-1 cells to bypass the delayed senescence and crisis, and allowed the cells to proliferate beyond 100 PDs to become immortalized. HOK/Bmi-1 cells expressing E6Δ146–151 also became immortalized and exhibited phenotype similar to those of Bmi-1-expressing cells superinfected with wild-type E6 (Fig. 4C). In contrast, expression of E6Δ118–122 failed to immortalize HOK/Bmi-1 cells, which ceased proliferation at PD 17 (Fig. 4A), indicating that the second zinc finger domain of E6 corresponding to amino acids 118–122 is responsible for telomerase activation and immortalization. The presence of each E6 mutant type as well as Bmi-1 was confirmed by RT-PCR (Fig. 4B).

### *The reduced level of p53 was not necessary to cause immortalization of Bmi-1/E6-expressing cells*

Degradation of p53 by E6 is known to be associated with cellular immortalization [37–39]. To address whether down-regulation of p53 was required for immortalization by Bmi-1 and E6, p53 expression level was analyzed in all experimental cells including both wild-type E6 and different E6 mutant types (Fig. 5A). The wild-type E6, as well as E6Δ146–151, caused decreased p53 level in HOK/Bmi-1 cells and eventually led to immortalization. The wild-type E6 also led to efficient down-regulation of p53 in NHOK without ectopic Bmi-1 expression

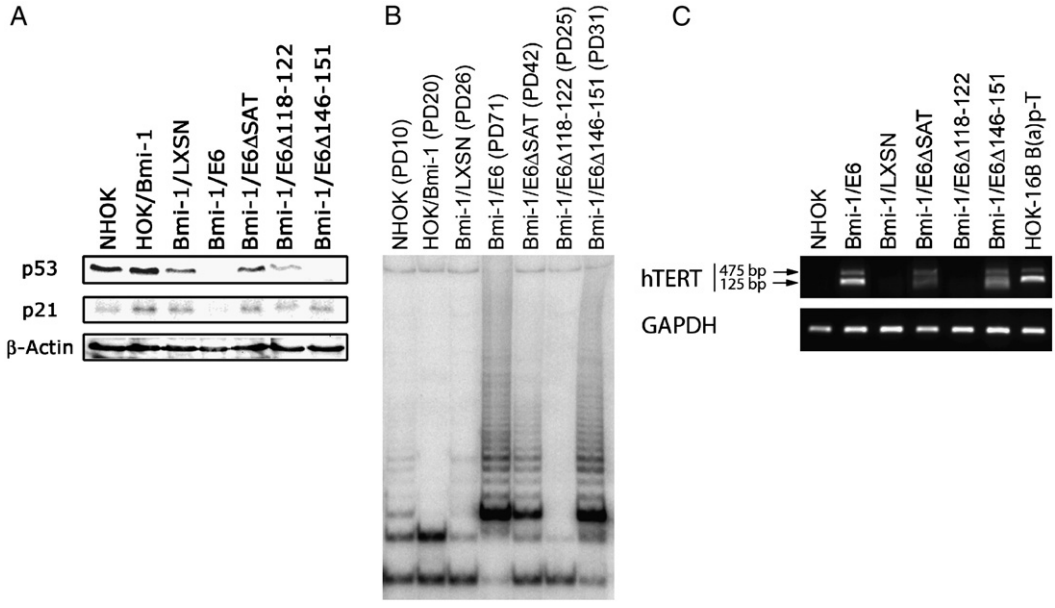


**Fig. 5 – Telomerase activity, but not the expression of p53, was associated with the immortalization induced by Bmi-1 and E6 in NHOK. (A) Protein extracts were obtained from each E6 mutant-expressing cells after the selection with G418 (200 μg/ml). The levels of p53 and p21 were analyzed by Western blotting. β-Actin was used as a loading control. (B) Telomerase activity was measured using the TRAP assay on all cells at or after the crisis. (C) The same samples were subject to RT-PCR analysis for *hTERT* expression. HOK-16B B(a)P-T cells were used as a positive control, and GAPDH as a loading control.**

(data not shown). However, E6Δ8S9A10T caused immortalization of HOK/Bmi-1 cells without lowering the p53 level. Conversely, E6Δ118–122 failed to cause immortalization although it lowered the level of p53. Therefore, the reduced expression level of p53 was not necessary for immortalization. We also examined the status of telomerase activity in these cells. The TRAP assay showed that only the immortalized cells had an increased telomerase activity whereas the cells which did not become immortalized had a decreased or unchanged telomerase activity (Fig. 5B). The status of telomerase activity was correlated with the presence of *hTERT* transcripts, a catalytic domain of telomerase responsible for its activity (Fig. 5C). These data indicated that telomerase activation was associated with immortalization.

### *Identification of gene differentially expressed by E6 in HOK/Bmi-1*

To identify the genes that might be involved in the immortalization process that are relevant to overcoming crisis, we compared the global gene expression profiles between actively proliferating Bmi-1/LXSN and Bmi-1/E6 cells by utilizing microarray-based gene expression analysis. Using Affymetrix, we screened more than 47,000 transcripts and found 32 genes that were upregulated and 5 genes that were downregulated by greater than 4-fold (Table 2). To validate the microarray data, we selected 10 genes (six genes from Table 1 and the other four genes that are associated with carcinogenesis and differentially expressed less than 4-fold but greater than 2-fold) and performed semi-quantitative RT-PCR (Fig. 6). Consistent with the microarray data, E6-expressing HOK/Bmi-1 cells showed a marked induction of DnaJ (Hsp40) homolog (*DNAJC12*), tumor necrosis factor (ligand) superfamily (*TNFSF11*), UDP glucuronosyltranferase 1 family (*UGT1A6*), chemokine ligand 5 (*CCL5*), mesoderm-specific transcript homolog (*MEST*), and podocalyxin-like (*PODXL*) as well as a marked reduction of activating transcription factor 3 (*ATF3*), *c-fos*, *Notch1*, and death-associated protein kinase 1 (*DAPK1*). Interestingly, several genes (*CCL5*, *MEST*, *PODXL*, and *Notch1*) were differentially regulated in Bmi-1/E6 and Bmi-1/E6Δ118–122 cells, suggesting that the E6 domain corresponding to amino acids 118–122 may be

**Table 2 – Genes differentially expressed by HPV-16 E6 in NHOK expressing Bmi-1**

| Gene description | Gene ID | Fold change | Category |
|---|---|---|---|
| DnaJ (Hsp40) homog, subfamily C, member 12 (*DNAJC12*) | 56521 | 39.4 | Protein folding |
| Tumor necrosis factor (ligand) superfamily, member 11 (*TNFSF11*) | 8600 | 24.3 | Immune response |
| CD302 antigen (*CD302*) | 9936 | 21.1 | Cell receptor |
| Glutathione S-transferase kappa 1 (*GSTK1*) | 76263 | 14.9 | Metabolism |
| Potassium voltage-gated channel, subfamily H, member 7 (*KCNH7*) | 90134 | 11.3 | Membrane protein |
| Solute carrier family 7, member 2 (*SLC7A2*) | 6542 | 11.3 | Metabolism |
| Lymphocyte antigen 75 (*LY75*) | 4065 | 9.2 | Cell receptor |
| Zyg-11 homolog A (*ZYG11A*) | 440590 | 9.2 | Cell division |
| UDP glucuronsyltransferase 1family, polypeptide A6 (*UGT1A6*) | 54578 | 8.6 | Metabolism |
| Hypothetical gene (*LOC388494*) | 388494 | 8.6 | N/D |
| Chemokine (C-C motif) ligand 5 (*CCL5*) | 6352 | 8.0 | Chemokine |
| Hypothetical protein (*LOC92659*) | 92659 | 7.5 | N/D |
| Hypothetical protein (*FLJ20449*) | 54937 | 7.5 | N/D |
| Hypothetical protein (*LOC441168*) | 441168 | 7.5 | N/D |
| Dysferlin interacting protein 1 (*DYSFIP1*) | 116729 | 7.0 | N/D |
| Ubiquitin carboxyl-terminal esterase L1 (*UCHL1*) | 7345 | 7.0 | Protein catabolism |
| Zinc finger protein 24 (*ZNF24*) | 7572 | 7.0 | N/D |
| Mesoderm specific transcript homolog (*MEST*) | 4232 | 6.5 | Imprinting gene |
| Collagen, type VIII, alpha 2 (*COL8A2*) | 1296 | 6.1 | Structural protein |
| Internexin neuronal intermediate filament protein, alpha (*INA*) | 9118 | 6.1 | Structural protein |
| Tudor domain containing 9 (*TDRD9*) | 122402 | 6.1 | Metabolism |
| CCR4-Not transcription complex, subunit 4 (*CNOT4*) | 4850 | 5.7 | Transcription regulator |
| H19, imprinted maternally expressed untranslated mRNA (*H19*) | 283120 | 5.3 | Imprinted gene |
| Synaptogyrin 3 (*SYNGR3*) | 9143 | 5.3 | Membrane protein |
| Carboxypeptidase, vitellogenic-like (*CPVL*) | 54504 | 4.9 | metabolism |
| Intercellular adhesion molecule 1 (*CD54*), human rhinovirus receptor (*ICAM1*) | 3383 | 4.9 | Cell surface glyco-proein |
| MAX gene associated (*MGA*) | 23269 | 4.9 | Transcription regulator |
| Hemoglobin, alpha 2 (*HBA2*) | 3040 | 4.3 | Metabolism |
| Matrix metallopeptidase 9 (*MMP9*) | 4318 | 4.3 | Enzyme |
| Ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (*ARIH1*) | 25820 | 4.0 | Metabolism |
| Hypothetical protein (*LOC441168*) | 441168 | 4.0 | N/D |
| Interferon, alpha-inducible protein (*G1P3*) | 2537 | −4.0 | Immune response |
| Carbonic anhydrase II (*CA2*) | 760 | −4.3 | Metabolism |
| Tumor necrosis factor (ligand) superfamily, member 4 (*TNFSF4*) | 7292 | −4.9 | Signaling |
| Activating transcription factor 3 (*ATF3*) | 467 | −5.3 | Transcription factor |
| Tripartite motif-containing 22 (*TRIM22*) | 10346 | −6.5 | Transcription factor |

*Note.* Differential gene expression profiles were compared between BM-1/pLXSN and Bmi-1/E6 cells by using Affymetrix microarray ($P<0.05$). Genes that were differentially expressed by a factor of 4 were identified. Abbreviation; N/D; not determined.

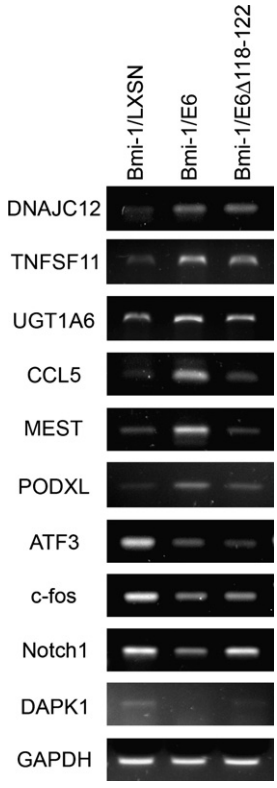


**Fig. 6 – Identification of genes differentially expressed by E6 in HOK/Bmi-1 cells. To validate the microarray data, 10 genes, either upregulated or downregulated, were analyzed using RT-PCR. GAPDH was used as a control.**

important in regulation of these genes. These results indicate that these genes may be associated with immortalization of Bmi-1/E6 cells.

## Discussion

In this paper, we characterized the role of Bmi-1 and HPV-16 oncoproteins E6 and E7 in NHOK. We provide evidence that (1) Bmi-1 alone caused the bypass of replicative senescence but was not sufficient for immortalization; (2) Bmi-1, along with E6 but not with E7, further extended the replication capacity beyond the "delayed senescence" and crisis, resulting in cellular immortalization; (3) the second zinc finger domain (amino acids 118–122) of E6 was necessary for immortalization with Bmi-1; and (4) immortalization was associated with telomerase activation but not with the aberrant diminution of p53 expression level.

In serially cultured normal MECs and nasopharyngeal cells, Bmi-1 overexpression alone is sufficient for immortalization [21,40]. This was not the case in NHOK possibly because the Bmi-1-induced immortalization mechanism is tissue-dependent or because the cultured MECs and nasopharyngeal cells had already undergone critical steps towards immortalization. Apparently, NHOK requires additional steps besides Bmi-1 overexpression for immortalization. This notion is supported by our observations that there is another senescing stage, delayed senescence, controlled by Bmi-1 expression in NHOK. Also, telomerase activity was not induced after Bmi-1 overexpression in NHOK while it was induced in MECs and nasopharyngeal cells by Bmi-1 overexpression [21,40].

Interestingly, HPV-16 E6 did not cause immediate telomerase activation, as confirmed by *hTERT* reporter assay, the TRAP assay, and microarray analysis. Rather, it induced telomerase activity at a moderate level in the pre-crisis Bmi-1/E6 cells, eventually leading to immortalization. This is consistent with our previous finding with NHOK in which the transfection of HPV-16 genome caused extended life span without significant increase in telomerase activity [18]. This observation, together with delayed senescence in the immortalization process of NHOK, may provide an important clinical implication. For instance, HPV-associated oral cancers, despite many challenges by chemical and physical stresses, are shown to have a lower incidence and prevalence if compared to HPV-associated anal or cervical cancers [1,2,41,42]. In this regard, NHOK culture system, along with their distinct replicative stages with Bmi-1 and E6, may provide a useful tool to investigate senescence, replicative crisis, and immortalization processes.

Bmi-1 and E6 appear to act independently, targeting different cellular pathways that separately contribute to the multiple steps of the immortalization process. Recently, PcG proteins, including Bmi-1, are shown to target and repress differentiation-specific genes, thereby maintaining stem cell characteristics and preventing cells from differentiation [43]. In NHOK, the global effects of Bmi-1 may program the cellular machinery to overcome terminal differentiation without successfully inducing telomerase activation and maintaining continual proliferation and immortalization. HPV E6 oncoprotein cooperated with Bmi-1 to cause immortalization of NHOK most likely by activating telomerase pathways, as confirmed by our study that (1) E6 mutant Δ118–122, which is defective in telomerase activation, failed to immortalize NHOK-expressing Bmi-1, and (2) all the cells immortalized with the E6 mutants retain their ability to activate telomerase. Effects of the reduced p53 level by E6 seem to be negligible since cells harboring Bmi-1 and E6Δ8S9A10T caused immortalization in the presence of high level of p53.

The E6 mutant Δ118–122 demonstrated equivocal results on immortalization of MECs as one group successfully immortalized MECs with E6Δ118–122 alone while others failed [38,44]. In NHOK, multiple experiments confirmed the lack of immortalizing capacity of E6Δ118–122 in the Bmi-1-expressing NHOK, which failed to show any increase in telomerase activity. Thus, this domain appears to be necessary for the cooperative effects of E6 with Bmi-1 on telomerase activation and immortalization in NHOK. It is possible that there are unknown cellular target genes regulated by E6 through the second zinc finger domain (amino acids 118–122) that are critical in the keratinocyte immortalization process.

Microarray data analysis using HPV E6 has been previously reported by several research groups [45–48]. Here, we identified genes that may contribute to immortalization process in the context of Bmi-1. To do so, we compared the global gene expression profiles between HOK/Bmi-1 cells infected with either RV-LXSN or RV-16E6 and found 32 upregulated genes and 5 downregulated genes by greater than 4-fold. Among genes that were differentially expressed, *PODXL* (3.7-fold) and

*CCL5* are known to be regulated through p53 [49–53]. Several genes showed up- or downregulation that were specific to the wild-type E6, e.g.,*CCL5*, *MEST*, *PODXL*, and *Notch1*, suggesting a notion that these genes are specifically regulated via E6 domain Δ118–122 and may be involved in the telomerase activation pathway. It is also worthwhile to note that among upregulated genes are imprinting genes, *MEST* and *H19*, which are associated with severe genetic aberrations and controlled by epigenetic regulation. Whether E6 targets genes associated with telomerase activation, or whether it directly regulates epigenetic alteration, remains to be determined.

Finally, HPV infection is suggested to be specific to the cells in the basal layers of the epithelia [54,55]; it is believed to occur when basal cells are exposed to HPV after micro-wounds of the epithelium [56]. Basal cells possess indefinite capacity to divide into either daughter cells that migrate into the differentiating layers or to basal cells themselves, indicating that they possess stem cell-like characteristics. Immediately after HPV enters basal stem cells, early transcripts, including E6 and E7 oncogenes, are expressed at significant levels, altering cell cycle control and contributing to HPV-induced oncogenesis. Hence, the cooperative effects of Bmi-1 and HPV-16 E6 may also provide possible suggestion as to why HPV infection exhibits its tropisms to the basal stem cells.

## Acknowledgments

We thank Dr. Dimri for the Bmi-1 construct and Dr. Galloway for the HPV constructs. This work was supported in part by grants DE14147 (N.-H. P.), DE15316 (M.K.K.), and DE07296 from the National Institute of Dental and Craniofacial Research (NIDCR) and by the Oral and Maxillofacial Surgery Foundation Grant (M.K.K.).

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