The **Bmi-1 Oncogene Induces Telomerase Activity and Immortalizes Human Mammary Epithelial Cells**

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**ABSTRACT**

The vast majority of breast cancers are carcinomas that arise from mammary epithelial cells (MECs). One of the key early events in tumorigenic transformation is the ability of cells to overcome replicative senescence. However, the precise genetic changes that are responsible for this event in MECs is largely unknown. Here, we report that Bmi-1, originally identified as a c-Myc cooperating oncoprotein, can bypass senescence, extend the replicative life span, and immortalize MECs. Furthermore, Bmi-1 was overexpressed in immortal MECs and several breast cancer cell lines. Overexpression of Bmi-1 in MECs led to activation of human telomerase reverse transcriptase (hTERT) transcription and induction of telomerase activity. Telomerase induction by Bmi-1 was an early event in the extension of the replicative life span and immortalization. Bmi-1 was not overexpressed in hTERT-immortalized MECs, suggesting that Bmi-1 functions upstream of hTERT. Although, c-Myc has been reported to induce telomerase in MECs, Bmi-1 appeared to act independently of c-Myc binding sequences in the hTERT promoter. Deletion analysis of the Bmi-1 protein suggested that the RING finger, as well as a conserved helix-turn-helix-turn domain, were required for its ability to induce telomerase and immortalize MECs. These data suggest that Bmi-1 regulates telomerase expression in MECs and plays a role in the development of human breast cancer.

**INTRODUCTION**

Normal human cells, with few exceptions, undergo replicative senescence whereby they irreversibly cease proliferation after a finite number of divisions (1). Senescence constitutes a strong natural tumor suppressor mechanism and a barrier to spontaneous immortalization (1–3). Escape from senescence is a critical step in the progression of a normal cell into a tumor cell (4–6). The primary cause of replicative senescence in human cells is thought to be the progressive erosion of telomeres (7, 8), the short repetitive sequences that cap the ends of chromosomes and prevent chromosome end-to-end fusion (reviewed in Ref. 9). In most cells, telomeres shorten with each cell cycle as a consequence of DNA replication. In germ cells and some stem cells, however, telomere length is stabilized by the enzyme telomerase. Activation of telomerase is a very common occurrence in tumor cells (5, 9–12).

Telomerase is repressed in most human somatic cells. This enzyme is a ribonucleoprotein complex containing an RNA component and an essential protein catalytic subunit (hTERT) that has reverse transcriptase motifs (9). Although the RNA component is expressed in most cell types, hTERT is generally expressed in only a few cell types, including the germ line (5, 9–12). hTERT is frequently induced during immortalization (13), and ectopic expression of hTERT can restore telomerase activity and prevent telomere erosion in many cell types (14–16). These findings suggest that hTERT is the only limiting factor for telomerase activity in normal cells, and its expression is the most common means by which cells acquire replicative immortality.

The cellular proto-oncogene c-Myc was found recently to induce telomerase activity and immortalize certain human MECs and fibroblasts (17). Direct support for c-Myc as an inducer of telomerase came from studies of the hTERT promoter. The hTERT promoter contains several c-Myc-binding sites (CAGCTG) through which c-Myc can activate hTERT transcription (18, 19). Despite being the only cellular gene identified thus far that can activate telomerase, c-Myc is amplified or overexpressed in only ~15% of breast cancers (reviewed in Ref. 20). However, the majority of breast cancers express telomerase. These findings suggest that additional pathways of telomerase induction must exist.

Bmi-1 was cloned as a c-myc cooperating oncopogene in murine lymphomas (21, 22). It was shown subsequently to be a transcriptional repressor belonging to the PcG of proteins (Ref. 23; reviewed in Ref. 24). Consistent with its oncogenic potential, Bmi-1 also regulates cell proliferation (25). Bmi-1-deficient MEFs overexpressed the Ink4a encoded genes p16 and p19ARF (murine homologue of human p14ARF) and underwent premature senescence in culture. Conversely, overexpression of Bmi-1 reduced expression of p16, and to a lesser extent p19ARF, and immortalized MEFs. Bmi-1 overexpression in human fibroblasts also extended the replicative life span but did not result in immortalization. The mechanism by which Bmi-1 represses p16 and p19ARF in MEFs appears to be transcriptional but is not well understood (25). Recently, it was shown that by repressing p19ARF, Bmi-1 inhibits the proapoptotic function of c-Myc and thus collaborates with c-Myc in tumorigenesis (26).

Bmi-1 contains a conserved RF domain at the NH2 terminus, which is required for its ability to cooperate with c-Myc in tumorigenesis and regulate cell proliferation (27). Bmi-1 also contains a conserved centrally located helix-turn-helix-turn (H-T-H-T) motif (22), which is required for transcriptional repression but not transformation, in rat embryo fibroblasts (28). Bmi-1 was recently reported amplified in certain mantle cell lymphomas (29), providing evidence for a role in human tumorigenesis. In addition, Bmi-1 is overexpressed in other human cancers, such as non-small cell lung cancer and B-cell non-Hodgkin lymphoma (30, 31).

In human fibroblasts, where senescence mechanisms have been studied extensively, intact pRb/p16 and p53/p21 checkpoints are required to establish and maintain the senescent phenotype (32–34).
Inactivation of either checkpoint results in an extension of replicative life span but not immortalization (32, 33). These checkpoints appear to contribute to the limited replicative life span of MECs as well (35). Overexpression of \( kTERT \) alone can immortalize certain human fibroblasts and retinal epithelial cells without inactivating the pRb/p16 or p53/p21 pathways (14). It has therefore been argued that inactivation of these checkpoints is not necessary for immortalization by telomerase (3).

When mammary tissue is explanted into tissue culture medium (DFCI-1 or an equivalent), a heterogeneous population of MECs, termed preselection cells, emerges (reviewed in Ref. 36). This population proliferates for 10–15 doublings before the majority of cells undergo senescence. However, regular feeding of preselection cells eventually gives rise to a homogeneous population, termed postselection MECs, which appear to arise from relatively rare cells in the preselection population (36–38). The emergence of postselection MECs is associated with, and believed attributable to, progressive methylation of the p16 gene (39, 40). Thus, p16 is silenced and not expressed in postselection MECs. Nonetheless, postselection MECs eventually undergo replicative senescence, after an additional 30–40 doublings, and do not spontaneously immortalize (36–38). Recently, it was reported that postselection MECs display signs of genetic instability as they approach senescence (41). However, consistent with earlier reports, neither the preselection nor the postselection MECs underwent spontaneous immortalization (36, 41).

Ectopic expression of \( hTERT \) failed to immortalize preselection MECs or keratinocytes that contained an intact p16/pRb pathway but readily immortalized postselection MECs and keratinocytes in which the p16/Rb pathway was inactivated because of p16 methylation or expression of the HPV E7 oncogene (36, 42, 43). We have shown that the HPV E6 oncogene, which abrogates the p53/p21 checkpoint, efficiently immortalizes postselection MECs (44, 45). E6 also targets other tumor suppressors, which may contribute to its immortalizing activity (reviewed in Ref. 36). For example, E6-induced degradation of a Rap GAP protein (E6TP1) strongly correlates with its immortalizing activity of MECs (46). Moreover, E6 can induce telomerase activity, which also correlates well with its ability to immortalize cells (47).

The mechanism by which E6 induces telomerase activity is unknown. Here, we report that \( Bmi-1 \) is overexpressed in several human breast cancer cell lines, suggesting a possible role for this gene in breast cancer and MEC immortalization. Consistent with this notion, we show that overexpression of an exogenous \( Bmi-1 \) gene extends the replicative life span of postselection MECs, leading to immortalization. \( Bmi-1 \) overexpression induced telomerase activity at an early stage in MEC immortalization and appeared to do so by transcriptional activation of \( hTERT \) that was independent of c-Myc binding sites present in the \( hTERT \) promoter.

### MATERIALS AND METHODS

**Vectors, Viruses, and Cell Culture.** MEC strains 76N (36, 37) and 184 (48), obtained from Dr. M. Stimpfer (Lawrence Berkeley Laboratory, Berkeley, CA), were grown in DFCI-1 medium (37, 44). For immortalization assays, cells were subcultured in a modified DFCI-1 medium termed D2, which lacks serum and bovine pituitary extract, contains 0.05% BSA, and supports the growth of immortal or life span extended, but not normal, cells (44). MEC senescence was determined as described for fibroblasts (49). Cells were considered senescent when labeling indices, determined by \( [\text{H}] \) thymidine incorporation, were <10% and cells acquired a flat morphology and stained positive for the SA-β-gal (49). WI-38 and BJ normal human fibroblasts were grown as described (49).

Human \( Bmi-1 \) was amplified by the PCR using cDNA from normal human fibroblasts (WI-38). \( Bmi-1 \) deletion MTs, generated by PCR, were: ARF, deletion of the NH$_2$-terminal RF domain; ΔHTE, deletion of the H-T-H-T domain; ΔHTNLS2, deletion of the H-T-H-T domain and NLS2. WT and MT \( Bmi-1 \) DNAs were cloned into the pBabe-puro retroviral vector (pb0), obtained from Dr. H. Land (Imperial Cancer Research Fund, London, United Kingdom). Retroviruses were produced by transient transfection, as described (50). Virus-containing supernatants were centrifuged to remove cell debris and stored at -70°C. pB0 control or pb-Bmi-1 retroviral supernatants were quick thawed at 37°C and used to infect MECs and fibroblasts for three sequential infections, each for 4 h, in the presence of Polybrene (4 μg/ml). Virally transduced cells were selected in 0.5 μg/ml puromycin for 3–5 days. This protocol typically yielded 60–80% infection efficiency. Cells were passaged every 5–7 days at 2 × 10$^5$–2 × 10$^5$ cells/cm$^2$ flask and plated at 5 × 10$^5$–10$^5$ cells/mm dish to determine the percentage of cells that incorporated \( [\text{H}] \) thymidine over a 48-h interval (%LN) and/or stained for SA-β-gal, as described (49).

**Western Blotting, Antibodies, and Immunostaining.** Denatured protein lysates (40 μg) in Laemmli loading buffer were analyzed by gradient (4–15%) SDS-PAGE and Western blotting, as described (50). Western blots were probed with F6, a mouse mAb against Bmi-1 (51), mAb anti-α-tubulin (Calbiochem, Cambridge, MA), polyclonal anti-p14ARF (Ab-1), or mAb anti-p16 (Ab-1; NeoMarkers, Union City, CA), mAb anti-p53 (DO-1), polyclonal anti-Mad1 (C-19), or mAb (9E10) or polyclonal (N-262) anti-c-Myc (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunostaining was carried out using anti-Bmi-1 F6 mAb, as described (51).

**Northern and RT-PCR Analyses.** Total cellular RNA was prepared using the RNAeasy midi kit (Quagen, Inc., Valencia, CA) and quantified; 10 μg of RNA were separated on 1% agarose-formaldehyde gels, transferred to Hyb-on-N membranes (Ammersham), and hybridized to \( [\text{32P}] \)-labeled cDNA probes. \( Bmi-1 \) and 36B4 probes were made by digesting, and gel purifying fragments, from pB-Bmi-1 and pEGMs/36B4, respectively. To determine \( hTERT \) expression, total RNA was isolated from control and Bmi-1 overexpressing cells at passages 1 and 50. RT-PCR was performed using \( hTERT \) primers, sense, 5'-GACTCGACACCGTGTCACCTACGT-3'; antisense, 5'-TGACAGGCCTCGTGTTGTCTGCTCTC-3', which amplify a 193-bp cDNA fragment and 733-bp genomic fragment, and β-Actin primers, as described (50). The \( hTERT \) signal was detected by Southern blot analysis of the PCR products using a labeled \( hTERT \) cDNA probe.

**DNA Damage Checkpoint Analysis.** To determine the DNA damage checkpoint response, cells were treated with 0.5 μg/ml ADM or solvent (DMSO) for 24 h. Total cell lysates were prepared and analyzed for p53 and p21 by Western blotting (50). To determine the %LN, cells were pulsed with \( [\text{3H}] \) thymidine for 6 h, fixed, and processed for autoradiography as described (49).

**Measurement of Telomerase Activity.** Telomerase activity was determined using a TRAP (10) assay kit (Intergen Co., Purchase, NY).

**Reporter Assays.** Reporter assays were performed as described (52). Transient transfections were carried out using the FuGENE transfection reagent, as described by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). The \( hTERT \) promoter-luciferase vector and a derivative with mutations in the c-Myc binding sites of the promoter (19) were obtained from Dr. R. Dalla-Favera (Columbia University, New York, NY). Reporter vectors were cotransfected with a bacterial β-galactosidase (pH 7.5) reporter vector for normalization. Cell extracts were prepared 48 h after transfection, analyzed using commercial kits for luciferase (Promega Corp., Wisconsin, WI) and β-galactosidase (Clontech Laboratories, Palo Alto, CA) activities and normalized as described (52).

**Telomere Length Assay.** Telomere lengths were analyzed as described (7, 14). Briefly, genomic DNA was isolated using a kit (Qagen), 2–5 μg of DNA digested with \( Hinf1 \) and \( Rsa1 \), and analyzed by Southern blotting using a labeled telomeric (TTAGGG)$_n$ probe. The size ranges and intensities of the telomeric signals were analyzed using a PhosphoImager (Molecular Dynamics).

### RESULTS

**Bmi-1 Is Overexpressed in Breast Cancer Cell Lines.** As a first step to assess a potential role for \( Bmi-1 \) in MEC transformation, we asked whether \( Bmi-1 \) is overexpressed in breast cancer cell lines. For this purpose, we analyzed total RNA from several human breast cancer cell lines by Northern blotting using a human \( Bmi-1 \) cDNA probe. Compared with normal MECs (76N), most of the breast cancer
cell lines overexpressed the Bmi-1 mRNA (Fig. 1). The MCF7, MDA-MB-231, SKBR-3, and ZR-75-1 cell lines showed the highest level of expression, whereas MDA-MB-453-, MDA-468-, T47D-, and E6-immortalized 76N cells showed modestly increased expression. HBL100, a nontumorigenic immortal breast cell line, showed only a slight overexpression, compared with normal cells. Bmi-1 expression in another normal MEC strain (184) was also much lower than the immortal and breast cancer cells (not shown). Thus, Bmi-1 is overexpressed in several human breast cancer cell lines, including E6-immortalized MECs.

**Bmi-1 Extends the Replicative Life Span of MECs.** The overexpression of Bmi-1 in breast cancer cells and E6-immortalized MECs suggested a possible association with immortalization. To test this hypothesis, we used two independently isolated strains of post-selection MECs, 76N and 184. These cells were infected at passage 12 with pB0 control or pB-Bmi-1 retroviruses. After puromycin selection, the cells were cultured in D2 medium, and overexpression of Bmi-1 was confirmed by Western analysis (Fig. 2A). As expected, 76N cells infected with the control retrovirus proliferated for a limited number of passages (8–10 PDs after selection) and then underwent senescence. At PD 8, control cells showed a decline in both the rate of cell growth (not shown) and the %LN (Fig. 2B); no significant labeling was observed at PD 18. In contrast, Bmi-1 overexpressing cells continued to proliferate well beyond PD 18 (Fig. 2B). Extended proliferation of Bmi-1-overexpressing cells was seen in three independent experiments, indicating that Bmi-1 overexpression increases the replicative life span of MECs.
Bmi-1 and MEC Immortalization

Consistent with their extended replicative life span, the percentage of Bmi-1-overexpressing cells that stained positive for SA-β-gal at PDs 12–18 was much lower compared with control cells (Fig. 2C). At PDs 12–18, control cells had ceased growth. The remaining cells did not attach well to plastic dishes and thus were not available for staining. However, at these PDs, Bmi-1-overexpressing cells showed a progressive loss of SA-β-gal staining, indicating bypass of senescence.

We regularly subcultured 76N cells overexpressing Bmi-1 in D2 medium at 1:4 (initial passages) to 1:10 (later passages) cell-splitting ratios. The cells were capable of continuous proliferation over >100 PDs without any features of senescence and thus appear to be immortal (Fig. 2D). At present, they have undergone well over 200 PDs (not shown). Similarly, Bmi-1 overexpression in normal 184 cells caused a significant extension of replicative life span (Fig. 2E). Bmi-1 overexpressing 184 cells also continue to proliferate (presently at PD 100), whereas control cells proliferated for only 8–10 PDs after drug selection.

The emergence of continuously proliferating Bmi-1-overexpressing MECs occurred without a significant lengthy crisis period. However, a mixture of small, tightly clustered, mitotically active colonies among large senescing cells were noticeable during early passages (Fig. 2F, upper panel). As cells expressing higher levels of Bmi-1 may have a growth advantage and retroviruses integrate randomly into genomic sites with varying transcriptional activities, the rapidly growing, mitotically active colonies may derive from those cells in which the retrovirus integration site produced optimal levels of Bmi-1. At later passages, Bmi-1-overexpressing cells had a uniform distinct morphology. Notably, they were small in size and grew in tighter colonies (Fig. 2F, lower panel), similar to the morphology of epithelial cells immortalized by MT p53 (53).

Bmi-1 Overexpression Does Not Down-Regulate p14ARF in MECs. Bmi-1 down-regulates the expression of two important tumor suppressors, p16 and p19ARF (25), which are encoded by a single locus, Ink4a (reviewed in Ref. 54). Mutation or inactivation of Ink4a is associated with extension of replicative life span and cell immortalization (55). In postselection MECs, the p16 promoter is methylated and thus inactive. We therefore assessed expression of p14ARF (human homologue of mouse p19ARF) in control and Bmi-1-overexpressing cells by Western analysis. The results of three independent experiments indicated that p14ARF is expressed at equally low levels in control and Bmi-1-overexpressing cells (Fig. 3). Because endogenous levels of p14ARF are very low in MECs, we used extracts from p14ARF-overexpressing cells as a positive control for the analysis. Thus, Bmi-1 does not appear to act by down-regulating p14ARF expression in postselection MECs.

Bmi-1 Induces Telomerase Activity in MECs. A number of studies have linked telomerase to immortalization. We therefore asked whether Bmi-1 overexpression leads to derepression of telomerase in MECs. We assessed telomerase activity in control and Bmi-1-overexpressing 76N cells immediately after puromycin selection (passage 1). There was significant telomerase activity in Bmi-1-overexpressing cells, compared with control cells, which were devoid of detectable telomerase activity under identical conditions (Fig. 4A, left panel). Because E6 is known to induce telomerase activity in MECs, its overexpression using a pLSXN retrovirus served as a positive control. Overexpression of Bmi-1 also induced telomerase activity in the other MEC strain, 184 (Fig. 4A, right panel). Thus, the ability of Bmi-1 to induce telomerase activity was not limited to a single MEC strain.

Telomerase induction by Bmi-1 was confirmed using hTERT reporter assays. We cotransfected a hTERT promoter-luciferase vector with a pCMVβ-gal normalization vector into control or Bmi-1-overexpressing 76N cells at passage 1. Cell lysates were prepared 48 h later, and luciferase activity was assayed and normalized, as described in “Materials and Methods.” Consistent with the assays for telomerase activity, control cells exhibited very little luciferase activity, whereas the Bmi-1-overexpressing cells displayed substantial luciferase activity (Fig. 4B). Male results suggest that Bmi-1 induces telomerase activity by activating hTERT transcription.

To determine the effect of Bmi-1 overexpression on the endogenous hTERT gene, RNA was isolated from control and Bmi-1-overexpressing cells at passages 1 and 50 and analyzed by RT-PCR. These results confirmed that Bmi-1 overexpression leads to activation of transcription of the endogenous hTERT gene (Fig. 4C).

The above results indicate that telomerase is derepressed in Bmi-1-overexpressing cells and suggest that Bmi-1 acts upstream of telomerase in the immortalization process. We therefore measured Bmi-1 expression in 76N cells that had been immortalized by hTERT, E6 or E7, and an γ-irradiation transformed 76N cell line, 76-R30 (56). Northern analysis indicated that, although Bmi-1 was overexpressed to varying degrees in all immortalized cells compared with 76N cells, it was not overexpressed in the hTERT-immortalized 76N cells. Thus, there was no selection for Bmi-1 overexpression when hTERT was ectopically expressed (Fig. 4D). Bmi-1 expression was particularly prominent in E7-immortalized cells, which may be attributable to further up-regulation of Bmi-1 through inactivation of the pRb pathway by E7. Taken together, the lack of Bmi-1 overexpression in hTERT-immortalized cells, its overexpression in other immortalized and tumor cells (Figs. 1 and 4D), and the increased hTERT reporter activity in Bmi-1-overexpressing cells indicate that Bmi-1 acts upstream of telomerase.

Telomerase Activity in Bmi-1-overexpressing Cells Increases with Passage. Our RT-PCR analysis suggested that hTERT mRNA levels increase as Bmi-1-overexpressing cells are passaged. To determine whether there was a similar increase in telomerase activity, we performed TRAP assays at different passages. Indeed, telomerase activity increased with increasing passages (Fig. 4E). Quantification of the TRAP products by densitometry (normalized to the internal control band) indicated that, compared with cells at passage 1, telomerase activity was roughly 12-fold higher in cells at passage 20, 14-fold higher at passage 30, and 19-fold higher at passage 50 (Fig. 4E). The activity remained relatively constant thereafter (not shown).

Bmi-1 Induction of Telomerase Is Cell Type Specific. Bmi-1 was reported to extend the replicative life span of human fibroblasts, but contrary to its activity in MECs, it did not immortalize these cells (25). We therefore asked whether the differential ability of Bmi-1 to...
Fig. 4. Bmi-1 overexpression induces telomerase in MECs but not human fibroblasts. A, control (BO), Bmi-1-overexpressing 76N (Bmi-1, left panel) and 184 (Bmi-1, right panel) cell lysates were prepared at passage 1 after puromycin selection. Extracts made from equal numbers of cells (2 × 10⁶) were analyzed for telomerase activity using a TRAP assay kit. IC, internal control for the PCR reaction. + Heat, heat-inactivated (70°C, 30 min) extract. +/− Control, lysis buffer in place of cell extracts; +/− Control, cell extract provided with the kit. B, 76N control and Bmi-1-overexpressing 76N cells (both at passage 1) were transfected with pGL2 (luciferase gene lacking a promoter) or hTERT-luc (hTERT promoter driving luciferase) and the pCMVβ-gal normalization vector as described in “Materials and Methods.” Extracts were made 48 h after transfection, and luciferase and bacterial β-galactosidase activities were determined using commercial kits. Luciferase activity was normalized for β-galactosidase activity and expressed as fold over the normalized luciferase produced by pGL2. C, Bmi-1 overexpression leads to activation of transcription of endogenous hTERT. RT-PCR analysis of control (BO) and Bmi-1-overexpressing cells at passages 1 and 50 was performed using primer specific for hTERT and β-actin as described in “Materials and Methods.” D, endogenous Bmi-1 is not overexpressed in hTERT immortalized cells. The Bmi-1 mRNA levels in 76N and 76N-hTERT, HPV16 E6 or E7 immortalized MEC strain 76N, and radiation-transformed 76N (76-R30) cells (as indicated) were determined by Northern blot analysis, as described in “Materials and Methods.” E, telomerase activity of Bmi-1-overexpressing cells increases with passage number. TRAP assays using extracts from different passages (as indicated) were done as described for A. To determine relative telomerase activity, we exposed the autoradiograms for different intervals to obtain signals in the linear range. We then performed densitometric analysis of TRAP products, which was normalized to the value of the internal control (IC) in each reaction. The signal from 76N-Bmi-1-overexpressing cells at passage 1 was arbitrarily set at a value of 1.0. F, Bmi-1 overexpression does not lead to telomerase induction in human fibroblasts. Telomerase assays using extracts from control and Bmi-1-overexpressing and WI-38 and BJ fibroblasts were performed as described in A.

Immortalize cells was attributable to a cell type-specific ability to induce telomerase. Two different strains of commonly used human fibroblasts, WI-38 (from fetal lung) and BJ (from neonatal foreskin), were infected with control (pB0) or Bmi-1-expressing (pB-Bmi-1) retroviruses. After puromycin selection, total cells extracts were prepared, overexpression of Bmi-1 was confirmed by Western blot analysis (not shown), and telomerase activity was measured. The results indicated that Bmi-1 did not induce telomerase in human fibroblasts (Fig. 4F).

Bmi-1 Induction of Telomerase in MECs Is Not Mediated by c-Myc Binding Sites Present in the hTERT Promoter. c-Myc is known to activate hTERT transcription, and Bmi-1 cooperates with c-Myc in cancer development. It was therefore possible that induction of hTERT transcription by Bmi-1 was mediated by c-Myc. To address this possibility, we compared luciferase activity driven by the WT hTERT promoter (WT) with an hTERT promoter in which the c-Myc binding sites were mutated (MT). The MT hTERT promoter was even more active than the WT hTERT promoter in Bmi-1-overexpressing cells (Fig. 5A). To confirm that, in 76N MECs, the WT hTERT promoter is c-Myc responsive whereas a MT hTERT promoter is not, we transiently transfected the hTERT reporter vector into pB0 (control) and c-Myc-overexpressing (pBabe-c-Myc) MECs. After 48 h, luciferase activity was measured and normalized to the transfection control vector (pCMVβ-gal). The results indicated that indeed the WT hTERT promoter is c-Myc responsive, whereas the MT hTERT promoter is truly insensitive to c-Myc in the MECs used in our study (Fig. 5B). Similar results were obtained in Bmi-1-immortalized MECs (not shown).

We also assessed the expression of c-Myc and the c-Myc antagonist Mad1 (reviewed in Ref. 57) in control and Bmi-1-overexpressing cells. c-Myc protein levels were slightly higher in Bmi-1-overexpressing cells (Fig. 5C). However, Mad1, which competes with c-Myc for dimerization with Max (57), was also higher in Bmi-1-overexpressing cells (Fig. 5C), indicating that the c-Myc/Mad1 ratio is similar in control and Bmi-1-
overexpressing cells. Thus, the induction of telomerase activity by Bmi-1 is independent of c-Myc binding sites in the hTERT promoter, and Bmi-1 overexpression does not lead to significant induction of endogenous c-Myc protein.

**Bmi-1 Immortalized Cells Maintain DNA Damage Checkpoint.**
Immortalization of cells with agents such as E6, E7, or p53 MTs causes abrogation of the DNA damage checkpoint (45, 58, 59). To assess the impact of Bmi-1-induced immortalization on this checkpoint, control and Bmi-1-overexpressing 76N cells were treated with 0.5 μg/ml ADM for 24 h and assessed for their ability to incorporate [3H]thymidine (an indication of DNA synthesis). As expected, control cells failed to incorporate [3H]thymidine after ADM treatment, indicating cell cycle arrest. Notably, ADM treatment also inhibited DNA synthesis in Bmi-1-overexpressing cells, and this was true at both early and late passage (passages 3 and 40; Fig. 7A). We also assessed the levels of p53 and its transcriptional target p21 after ADM treatment. ADM induced comparable levels of p53 and p21 in both control and Bmi-1-overexpressing cells (Fig. 7B). Taken together, the data indicate that Bmi-1-overexpressing cells have an intact DNA damage checkpoint.

**Deletion Analysis of Bmi-1.** Bmi-1 contains an NH2-terminal RF domain, COOH-terminal H-T-H-T domain and two NLSs, NLS1 and NLS2 (Fig. 8A). To determine the specificity and Bmi-1 domains that are required for telomerase induction and immortalization, we generated Bmi-1 deletion MTs lacking the RF domain (ΔRF), H-T-H-T domain (ΔH-T), or both the H-T-H-T domain and NLS2 (ΔH-T-NLS2; Fig. 8A). The WT and ΔH-T proteins retain both NLS1 and NLS2; the ΔRF MT lacks NLS1, and ΔH-T-NLS2 retains NLS1 but lacks NLS2. These MTs were introduced into 76N cells using retroviruses. Western analysis showed that the MT proteins were overexpressed and had

overexpressing cells. Thus, the induction of telomerase activity by Bmi-1 is independent of c-Myc binding sites in the hTERT promoter, and Bmi-1 overexpression does not lead to significant induction of endogenous c-Myc protein.
overexpress WT or MT Bmi-1 proteins. After puromycin selection, we stained the infected cells using an anti-Bmi-1 antibody. WT Bmi-1, and all of the MTs except HT-NLS2, showed prominent nuclear staining. The ΔHT-NLS2 MT showed mostly cytoplasmic staining with some nuclear staining, which may be attributable to endogenous Bmi-1 (Fig. 8C). These results indicate that NLS2 is sufficient for proper nuclear localization of human Bmi-1, as reported previously (53, 59). To verify the expected sizes (Fig. 8B), we determined their subcellular localization, we stained the infected cells using an anti-Bmi-1 antibody. WT Bmi-1, and all of the MTs except ΔHT-NLS2, showed prominent nuclear staining. The ΔHT-NLS2 MT showed mostly cytoplasmic staining with some nuclear staining, which may be attributable to endogenous Bmi-1 (Fig. 8C). These results indicate that NLS2 is sufficient for proper nuclear localization of human Bmi-1, as reported previously (28).

We next determined the replicative life span of 76N cells that overexpress WT or MT Bmi-1 proteins. After puromycin selection, the cells were cultured in D2 medium, and the number of PDs was determined over 60–120 days. Only WT Bmi-1 was capable of inducing significant extension of replicative life span and immortalization (Fig. 8D). Control (B0) cells, as well as cells expressing the ΔHT and ΔRF proteins, underwent senescence and completely ceased growth within 10 PDs after selection. Thus, both the RF and H-T-H-T domains of Bmi-1 appear to be required for the immortalization of MECs.

We next examined the ability of WT and MT Bmi-1 proteins to induce telomerase activity. Telomerase activity was determined by TRAP assay immediately after puromycin selection (passage 1). Only WT Bmi-1 was capable of inducing significant activity; cells expressing the ΔRF and ΔHT proteins, similar to control cells, were devoid of telomerase activity (not shown). Taken together, these data suggest that both the RF and H-T-H-T domains of Bmi-1 are required for telomerase induction, as well as MEC immortalization, supporting a critical role for these conserved domains in mediating the oncogenic capacity of Bmi-1 (21, 25, 27).

**DISCUSSION**

Replicative senescence is widely acknowledged as a tumor suppressor mechanism, and genetic alterations that extend replicative life span are very likely important events during oncogenesis. The nature of such events in epithelial cells is poorly understood. We show here that overexpression of the Polycomb transcriptional repressor Bmi-1 extends the replicative life span and induces immortalization in two different postselection MEC strains. We also show that Bmi-1 is overexpressed in most breast cancer cell lines and in MECs immortalized by other oncogenes. Together, our data suggest a potential role for Bmi-1 in MEC immortalization and breast cancer.

Bmi-1 is known to immortalize and cooperate with c-Myc in transforming MEFs (25, 26). In contrast, Bmi-1 overexpression only extends the replicative life span of human fibroblasts and does not immortalize them (25). Our data also indicate that Bmi-1 cannot induce telomerase in human fibroblasts (Fig. 5F). In this respect, Bmi-1 differs from c-Myc, which has been reported to activate telomerase in human MECs as well as fibroblasts (17) but functions similarly to the HPV E6 oncoprotein, which also cannot activate telomerase in human fibroblasts (47). Telomerase induction by Bmi-1, thus, is cell type specific. Differential regulation of telomerase by Bmi-1 is very likely responsible for its failure to immortalize human fibroblasts. The reason for this cell type specificity is not yet known. One possibility is that Bmi-1 down-regulates an epithelial-specific repressor of telomerase, because Bmi-1 is a member of the PcG family of transcription repressors. This observation may have important implications for cancer development, because it would suggest that Bmi-1 may be able to induce cancers of epithelial origin but not mesenchymal origin (fibroblasts). This may also shed light on the prevalence of carcinomas, as opposed to sarcomas, in humans, because there may be other genes that can function similarly to Bmi-1 by inducing telomerase selectively in epithelial cells.

Bmi-1 effects on the replicative life span of mouse and human fibroblasts are apparently the result of its ability to repress the *INK4a* locus, which encodes the p16 and p19ARF genes (26). The postselection MECs used in our study do not express p16. They do, however, express low levels of p14ARF, but Bmi-1 did not down-regulate p14ARF in these cells. Thus, there appear to be cell type- and species-specific differences in the pathways that prevent immortalization. We are currently determining whether down-regulation of p16 in postselection MECs confers susceptibility to immortalization by Bmi-1. Our preliminary data using preselection MECs indicate that Bmi-1 can repress p16, induce weak telomerase activity, and extend the replicative life span of these cells.

In preselection MECs, senescence (termed M0; Ref. 40) is presumed because of the expression of p16. Recent findings suggest that M0 senescence may also reflect inadequate culture conditions (60). Thus, M0 senescence is independent of telomere length. In postselection MECs, senescence is associated with telomere attrition. Although it is possible that genes other than p16 are methylated in postselection cells, hTERT expression is sufficient to immortalize them (17, 36, 42). Thus far, the only other cellular gene reported to immortalize postselection MECs is c-myc, apparently acting through its ability to induce telomerase (17). The E6 viral oncoprotein is highly efficient at immortalization and also induces telomerase in MECs (36, 47). In this respect, it is of interest that Bmi-1 was overexpressed in E6-immortalized MECs (Figs. 1 and 4C). In contrast, dominant-negative MTs of p53 immortalize postselection MECs inefficiently (53, 59), as does overexpression of ZNF-217, which encodes a zinc finger protein and is amplified in breast cancer (61). Apparently, induction of telomerase is a late event in these cases, which may explain the infrequent immortalization. Here, we show that Bmi-1 overexpression...
efficiently immortalizes MECs, apparently via early activation of \( hTERT \) transcription and induction of telomerase activity.

Unlike E6, Bmi-1 overexpression did not abrogate the DNA damage checkpoint, and p53 and p21 remained inducible by DNA damage. Bmi-1 overexpression also did not confer anchorage-independent growth (not shown), suggesting that Bmi-1 can immortalize but not transform MECs. In this respect, immortalization by Bmi-1 is similar to that induced by \( hTERT \), which also does not cause transformation (19, 43). Nonetheless, Bmi-1 was overexpressed in all breast cancer cell lines tested and also in MECs immortalized by HPV E6 and E7. However, Bmi-1 was not overexpressed in MECs immortalized by exogenous \( hTERT \). Because, in cells expressing exogenous \( hTERT \), telomerase expression is independent of its native promoter/regulation, up-regulation of Bmi-1 would not confer a growth advantage. Although E6 has numerous biological activities, it is possible that E6 induces telomerase by up-regulating Bmi-1. Together, these data suggest that Bmi-1 functions upstream of \( hTERT \) in mediating immortalization.

Telomerase induction by Bmi-1 appears to be attributable to transcriptional activation of the gene encoding the catalytic subunit, \( hTERT \), which is rate-limiting for telomerase activity in most somatic cells. This conclusion is based on results of \( hTERT \) promoter-reporter assays. Compared with control cells, \( hTERT \) promoter activity was substantially higher in Bmi-1-overexpressing cells. Among several cellular genes that were tested, only c-myc was able to induce telomerase in postselection MECs (17). Although telomerase regulation by c-Myc may depend on the cell type (62), c-Myc is a transcription activator and has been shown to bind and activate the \( hTERT \) promoter (18, 19). Moreover, telomerase appears to be at least partially regulated by c-Myc in the MECs used in our study. However, we found that Bmi-1 induces telomerase independent of the c-Myc binding sites in \( hTERT \) promoter. Our studies do not rule out a possible indirect involvement of c-Myc in Bmi-1-mediated induction of telomerase. Interestingly, the \( hTERT \) promoter that contained MT c-Myc binding sites was more active than the WT promoter in both Bmi-1-immortalized cells (Fig. 5A) and normal cells (Fig. 5B). It is possible that these c-Myc binding sites function as repressor elements in MECs. This possibility is consistent with the recent finding that Max-Mad complexes compete with Max-Myc complexes for c-Myc binding sites, and Max-Mad complexes inhibit \( hTERT \) transcription (63–65).

Although Bmi-1 induced \( hTERT \) transcription independently of c-Myc binding sites, it is possible that c-Myc and Bmi-1 cooperate to induce high levels of telomerase activity in late-passage cells and immortalization. Indeed, our preliminary data suggest that late-passage cells have 3–5-fold higher c-Myc levels compared with early passage Bmi-1-overexpressing cells. It is also possible that Bmi-1-overexpressing cells accumulate additional epigenetic changes or mutations that cooperate with Bmi-1 in inducing full immortalization. Such changes could be responsible for the increased c-Myc expression and altered composition of Max-Myc and Max-Mad complexes and contribute to the increased telomerase activity seen at late passage.

Bmi-1 is a member of the family of Polycomb group (PcG) proteins, which form repressor complexes. These complexes are thought to bind PcG response elements and silence the expression of neighboring genes (24, 66). Thus, it is unlikely that Bmi-1 binds the \( hTERT \) promoter directly and activates it. Preliminary chromatin-immunoprecipitation linked PCR assays failed to demonstrate Bmi-1 at the \( hTERT \) promoter (not...
shown). We speculate that Bmi-1 represses the expression of another gene, the product of which negatively regulates hTERT expression.

Although other functions of Bmi-1 may contribute to MEC immortalization, it is likely that induction of telomerase activity plays an important role. Indeed, telomeres initially shortened in Bmi-1-overexpressing cells but then stabilized at 3–4 kb and did not undergo the further shortening characteristic of telomerase-negative MECs undergoing senescence. E6 and c-Myc immortalized MECs and breast cancer cells show similar telomere dynamics (17, 42). The reason for the initial telomere shortening, despite telomerase activity, is not clear. Recent data suggest that a limiting level of telomerase activity produced by exogenous hTERT preferentially maintains the shortest telomeres, allowing cells with very short telomeres to proliferate (67). Similarly, it is possible that the limited telomere induced by Bmi-1 in early-passage MECs is just sufficient to maintain the shortest telomeres, whereas other telomeres shorten. At later passages, other mechanisms, such as c-Myc up-regulation, might increase telomerase activity and stabilize all of the telomeres. On the other hand, telomere end-binding proteins and negative regulators of telomerase might counteract the telomerase induced by Bmi-1 in early-passage cultures (68–71). Regardless of the mechanism, induction of telomerase by Bmi-1 in MECs, similar to the situation in many tumor cells, led to maintenance of a short but stable telomere length.

Mutational analysis of Bmi-1 indicated that the H-T-H-T and RF domains of Bmi-1 are essential for telomerase activation as well as immortalization of MECs. The RF domain is thought to mediate interactions with other PcG proteins, such as human Polycomb 2 (HPC2), RING1, and human polyhomeotic, to assemble a repressor complex (Ref. 51; reviewed in Ref. 66). We envision that targets of the Bmi-1 repressor complex may include a telomerase repressor, and down-regulation of this repressor results in induction of the hTERT gene. Future work will be needed to test the validity of this model.

In conclusion, we demonstrated that overexpression of the PcG transcriptional repressor Bmi-1 immortalizes human MECs and induces telomerase, apparently via transcriptional up-regulation of the hTERT gene. Similar to hTERT-immortalized MECs, Bmi-1 immortalized MECs have an intact DNA damage checkpoint. Our data also suggest that Bmi-1 regulates hTERT independent of the c-Myc binding sites in its promoter. We speculate that this may add to the powerful collaboration between c-Myc and Bmi-1 in tumorigenesis. Finally, the availability of Bmi-1-immortalized MECs should help elucidate the sequence of events that occur during the neoplastic transformation of human mammary epithelial cells.

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REFERENCES


The *Bmi-1* Oncogene Induces Telomerase Activity and Immortalizes Human Mammary Epithelial Cells

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