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TITLE: Role of p53 in Mammary Epithelial Cell Senescence

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14. ABSTRACT

The tumor suppressor p53 plays an important role in a variety of cancers including breast cancer. It inhibits the growth of malignant cells either by inducing G1 arrest, apoptosis or senescence. We are determining the role of p53 in human mammary epithelial cell (HMEC) senescence and the requirement of p53 inactivation in transformation of HMECs. In this report, we have found that p53 downregulation is required to overcome H-Ras induced senescence during transformation of HMECs. Downregulation of p53 resulted due to co-overexpression of Bmi-1 with H-Ras in transforming MCF10A strain of HMECs. We also continued to perform chromatin immunoprecipitaion linked PCR (ChIP) assay to identify targets of p53 involved in replicative senescence of HMECs. ChIP assays were performed using senescent 76N cells. The DNA obtained after chromatin IP of senescent cells using p53 antibody was amplified using linkers and cloned in a pGEM plasmid vector. Several clones were selected and sequenced to identify p53 regulated genes. Many clones contained p53 binding sites suggesting that genes represented in these particular clones are regulated by p53 during HMEC senescence. These genes are likely to have a role in p53-medaited tumor-suppression.

15. SUBJECT TERMS
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INTRODUCTION

In most cases, breast cancer is a carcinoma arising from the transformation of mammary epithelial cells. Transformation is a complex multistep process involving several molecular genetic changes [1, 2]. It is believed that the first molecular genetic change entails bypass of cellular senescence followed by the immortalization of cells [1, 2] . After completing a certain number of divisions, normal cells enter a state of irreversible growth arrest and altered function, known as cellular senescence [2]. In somatic cells, telomerase remains repressed and telomere length keeps shortening at each round of DNA replication. Short telomeres signal cells to stop further proliferation and invoke a permanent growth arrest phenotype known as replicative senescence [2]. Two important tumor suppressor pRb and p53 are required for the maintenance and genesis of senescent phenotype. Apart from telomere length -dependent senescence, senescence- like phenotype is also induced by non-telomeric signals [2]. For example, it has been shown that many potent oncogenes such as H-Ras induces senescence in primary cells [2]. This type of senescence is known as Oncogene-Induced Senescence, which is also a potent tumor-suppressor mechanism [2]. Recently, OIS has been shown to occur in vivo [3, 4].

The p53-p21 pathway is an important mediator of cellular senescence as well as senescence induced by non-telomeric signals such as OIS [2]. The p53 protein is a typical transcription factor and contains an N-terminus transactivation, a centrally located DNA binding and a C-terminus oligomerization domains [5, 6] . Transcriptionally active p53 binds to a consensus site 5' -RRRCA/TA/TYYY-3', often present in pairs in p53 regulated genes [7, 8] . Tumor derived mutants of p53 are always defective in sequence-specific transactivation, thus attesting the importance of transcription activation function of p53 [9]. Activation of these transcriptional targets of p53 results in apoptosis, G1 and G2 cell cycle arrest or senescence [10, 11]. Mutations in p53 and genes of p53 pathway are also of common occurrence in breast cancer [12]. However, the cooperation between mutant p53 or downregulation of p53 and its targets with other oncogenic mutations during breast cancer progression is not very well understood. In this report, we studied the cooperation between H-Ras and Bmi-1 oncoproteins in HMECs transformation, and the effect of H-Ras and Bmi-1 overexpression on p53 pathway.

When mammary tissue is explanted in an appropriate tissue culture medium, a heterogeneous cell population emerges. This heterogeneous population proliferates for 3-5 population doublings before a majority of cells undergoes senescence. Regular feeding of these cells (sometimes) give rise to a homogeneous population which is referred to post-selection HMECs, while the original heterogeneous mixture is referred to as pre-selection cells [1]. The post-selection cells are p16 negative but still undergo senescence and never spontaneously immortalize [1]. We have shown in our previous report, that p53 and p21 is significantly upregulated during senescence in post-selection HMECs but not in pre-selection HMECs. These data has been confirmed by a recent report [13].

Several targets of p53 has been identified using various techniques such as microarray analysis, SAGE analysis, bioinformatics and ChIP (chromatin-IP) on chip analysis [9]. Many of these targets are common to all p53 functions such as G1 arrest, p53 and senescence, while some of the targets may be specific for apoptosis, G1 arrest or senescence. Because cells need to overcome senescence in order to become transformed and p53 and p53 targets regulate senescence, it is important to identify p53 targets genes that are specific to senescence induction. Using ChIP approach in senescent cells, we can identify in vivo targets of p53 that are relevant to senescence. Here we used ChIP approach to study the targets of p53 in senescent HMECs.

BODY:

Methods

76N and MCF10A cells were cultured as described [14]. Senescence was determined using senescence associated beta-galactosidase (SA-β-gal) assay and using ^{3}H-thymdine incorporation assay (% labeled nuclei or %LN) as described [14]. Cells were considered early passage when >70% cells incorporated ^{3}H-thymidine and less than 5% cells were SA-β-gal positive. Conversely cells were considered senescent when SA-β-gal index was >70% and %LN were 10-15%. MCF10A cells co-overexpressing H-Ras and/or Bmi-1 were generated by infecting cells with retroviral vector expressing H-Ras and/or Bmi-1, and selecting cells in puromycin and/or G418. The retroviruses were generated by transfecting a particular retroviral vector with a packaging plasmid pIK into tsa54 as described [14]. The culture supernatant containing retrovirus was collected, filtered and either used to infect recipient cells right away or stored at -80^{0}C. Western blot analyses to determine the expression of various genes was done as described [14]. Soft-agar and Matrigel assays to determine the transformed features of HMECs were done as described [15].

To perform ChIP analysis 76N cells were serially passaged in culture until senescence. To perform ChIP assay a kit from Upstate Biotechnology was used. Briefly, chromatin DNA was prepared from HCHO treated senescent 76N cells. The p53-bound chromatin was immunoprecipitated using DO-1 p53 antibody (Santa Cruz Biotech). The immunoprecipitated chromatin DNA was then purified using phenol-chloroform and treated with T4 DNA polymerase for 15 min at 16 °C to generate blunt ends. The DNA was purified and ligated to *Eco*RI adaptor (NEB). The ligated DNA was PCR amplified using primer designed from the *Eco*RI adaptor and size fractionated on an agarose gel. The DNA fragments (>300 bp) were gel purified, cloned in pGEM-T Easy vector (Promega) and sequenced. The sequences were analyzed for putative p53 binding site

Results

1. Bmi-1 expression bypasses OIS caused H-Ras overexpression in HMECs-

To understand the role of p53 in H-Ras induced premature senescence in HMECs, we overexpressed H-Ras in MCF10A cells. We also overexpressed Bmi-1 in MCF10A cells and MCF10A-H-Ras cells. These MCF10A-derived and control cells were studied for OIS, p53 induction and other growth regulatory pathways (Fig. 1A and B). Results showed that H-Ras induces p53 in MCF10A cells similar to other primary cells and although, MCF10A cells do not express p16^{INK4a}, H-Ras overexpression in these cells still induced senescence (OIS) and inhibited cell proliferation (Fig. 2A and B). Bmi-1 co-overexpression with H-Ras resulted in bypass of H-Ras-mediated OIS (Fig. 2A and B) and p53 downregulation suggesting that Bmi-1 cooperates with H-Ras to transform HMECs, by inhibiting H-Ras-mediated OIS and downregulating p53. Consistent with these data, we found that only Bmi-1+H-Ras expressing cells exhibited features of transformation such as growth in soft-agar and disorganized morphology of acini in Matrigel (Fig. 3). We are further analyzing expression of p53 targets in H-Ras, Bmi-1 and H-Ras+Bmi-1 overexpressing cells to determine the role of p53 pathway in transformation of HMECs and cooperation between H-Ras and Bmi-1 in transforming HMECs via downregulation of p53 and inhibition of OIS.

2. Identification of potential p53 targets in senescent HMECs using ChIP cloning approach-

Before proceeding with cloning of p53-bound chromatin, we verified the presence of p53 targets in p53-immunoprecipitated (IPed) chromatin using primers for p21, DDB2 (DNA damage-inducible protein 2), and RPS27L, which we have previously found upregulated in senescent HMECs. Results confirmed that p53-IPed chromatin contained these target genes (Fig. 4). Next, the p53-IPed chromatin was cloned in pGEM-T easy vector and several clones were sequenced. Sequence of several clones confirmed the presence of p53 binding sites in insert present in these clones. The various genes and the p53 binding site that we identified are summarized in table 1.

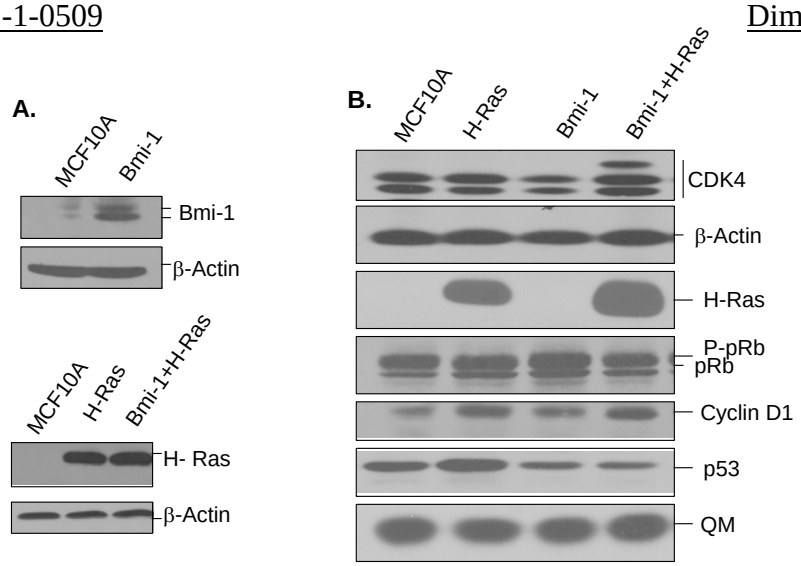

Figure 1: Generation of MCF10A cells overexpressing Bmi-1 (A, upper panel) and overexpressing H-Ras and Bmi-1+H-Ras (A, lower panel). MCF10A control and MCF10A-derived cells were studied for p53, pRb, CDK4, And Cyclin D1. Results show upregulation of p53 in H-Ras expressing cells, while Bmi-1 and Bmi-1+H-Ras cells showed p53 downregulation suggesting that Bmi-1 downregulates p53. QM is a loading control.

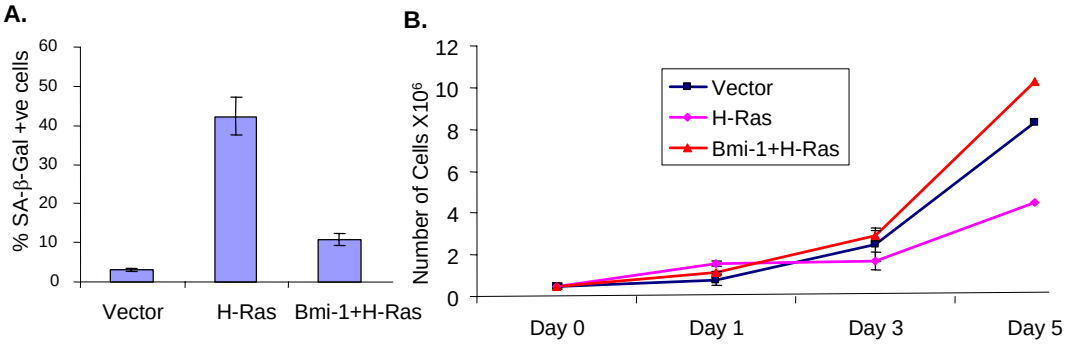

Figure 2: H-Ras overexpression in MCF10A leads to partial OIS (Oncogene Induced Senescence), which is compromised by Bmi-1 overexpression. **(A)** vector control or MCF10A cells expressing H-Ras alone or co-overexpressing Bmi-1 and H-Ras at passage 2 after selection were plated in multiwell plates, grown for 24-48 hrs, fixed, and stained for SA-β-Gal marker as described in Methods. **(B).** MCF10A cells with vector, and MCF10A-H-Ras, and MCF10A-Bmi-1+H-Ras cells were plated (5X105 cells/P100) at day 0 in 3 sets, harvested using trypsin-EDTA at day 1, day 3 and day 5, and counted using a Coulter-counter. The cell number at different days was plotted to measure the short-term growth potential of MCF10A-derived cells.

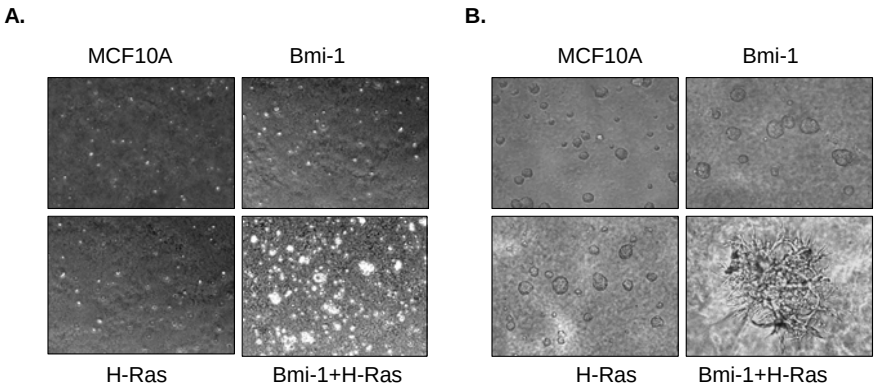

Figure 3: Transformed phenotype of MCF10A cells expressing Bmi-1+H-Ras. (A) MCF10A and MCF10A cells expressing H-Ras alone, Bmi-1 alone or Bmi-1 together with H-Ras (as indicated), at passage two (after Ras selection) were analyzed under light microscope for anchorage-independent growth using soft-agar assays, and photographed (4X). (B). MCF10A and MCF10A-derived cells (as indicated) at passage 2 were analyzed for acini formation using Matrigel assays and photographed (6X)

Table 1: Putative p53 targets obtained ChIP cloning

S. No	Chrom. No.	Location	Description	Putative P53 binding Site Sequence
1	22q11.21	5′ UTR	armadillo repeat gene deletes in velocardiofacial syndrome (ARVCF)	AGGCAGGTGA-1-GGAGTGCCC
2	15q25-q26	Internal	membrane alanine aminopeptidase precursor	AGCCATGGGC-5-GGCACCCCC
3	1p36.33	5′UTR	similar to myosin XV	GGCCATGGCT-38-GGCAGGAGT
4	8	Internal	Homo sapiens chromosome 8, clone RP11-301G7	AGACACTCCT-8-AGACAGGGTC
5	6	Internal	Human DNA sequence from clone RP3-322A24. fibronectin type III domain containing 1	TTTCATGGCT-74-TGGTTTGCCT
6	12	Internal	Homo sapiens 12 BAC RP11-513P18	TAACTTGTGT-x-TGAAATGCTT
7	5	Internal	Homo sapiens chromosome 5 clone CTD-2210P15	AGGCAGGTTG-28-AGGCATCCTA
8	12	Internal	Homo sapiens 12q BAC RP11-798P24	AGACATAACA-26-AGCCATGTGT TGCCAGGCTT-12-ATGCTGGCCT
9	5	5″UTR	Homo sapiens chromosome 5 clone CTC-454D3, spermatogenesis associated 9 isoform a	AGGATTGTTC-3-AGCCTTTTCC AGGCCTCTCT-5-AGTTGTGCCT
10	5	Internal	Homo sapiens chromosome 5 clone CTB-43D14, collagen, type XXIII, alpha 1	TTACATGTGC-7-CGGCTCGTCA TGACTTCTCC-6-TGTGATGTCT ATGTATGTCT-16-AAACACGATT
11	3	Internal	Homo sapiens 3 BAC RP11-190F16	TGATTTGTTT-2-TGACCTGGCT
12	15	Internal	Homo sapiens chromosome 15, clone RP11-114H24	AGGCCAGGCA-10-AAACACGGCA ATGCGTGACC-2-GGGCAAGTGA
13	1	Internal	Human DNA sequence from clone RP11-349E20	AGGCATGTGC-9-TGGCTTGACA
14	3	Internal	Homo sapiens 3 BAC RP11-139K4	GAGCAAGACC
15	3	Internal	Homo sapiens 3 BAC RP11-139K4	GGTCTTGCTC-11-GGACCTTTCT
16	Xq23	Internal	Homo sapiens PAC clone RP1-170D19 from	TAACTTGCCA-x-GTTCATGTCA
17	7	Internal	Homo sapiens BAC clone RP11-302C22 from 7	AAACTGGTCT
18	Xp11	Internal	Human DNA sequence from clone RP1-169I5, the 3' end of the DDX3 gene for DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, the NYX gene for nyctalopin, a gem (nuclear organelle) associated protein 7 (GEMIN7) pseudogene and two CpG islands	GGGCAGGCCC-2-GGACCTGACA
19	19	Internal	glucose phosphate isomerase (GPI)	GGGTCTGCCT-x-GGGCATGGCT AGGGATGGCC-0-CGTCTAGCCC

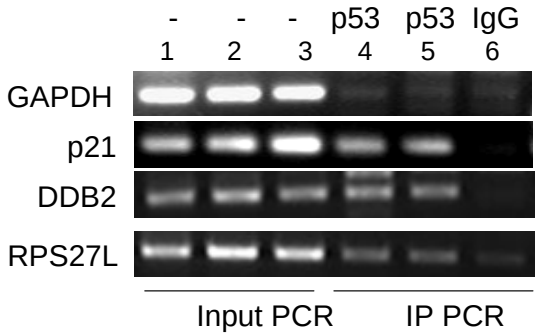

Figure 4: ChIP analysis of p53-IPed Chromatin using primers specific for p21, DDB2 and RPS27L. GAPDH primers set was used as a negative control. Lane 1 and 4 -76N senescent cells, lane 2, 3, 5 and 6- adriamycin treated 76N hTERT cells. Lane 4 and 5 are IPed with p53 antibody and lane 6 is an IgG control

We are continuing to sequence more clones and analyze sequences for p53 binding sites. We area also refining the ChIP cloning procedure by introducing binding of DNA sequences obtained after ChIP to GST-p53 to reduce the background and then clone DNA fragments that binds to GST-p53.

KEY RESEARCH ACCOMPLISHMENTS:

The key research accomplishments during this progress period are as following-

- p53 mediates H-Ras induced premature senescence (OIS) in HMECs.
- Bmi-1 co-overexpression with H-Ras overcomes p53-medaited OIS.
- Bmi-1 cooperates with H-Ras to transform HMECs by inhibiting H-Ras induced OIS and downregulating p53.
- We have identified several new putative genes that contain putative p53 binding sites in 5' untranslated region and in internal non-coding regions.

REPORTABLE OUTCOMES:

The Career Development Award "DAMD17-02-1-0509" from USAMRMC has been instrumental in advancing my academic career (CV is enclosed in Appendices). Following publications resulted during this progress period (06-07), which were partially supported by DAMD17-02-1-0509 award.

Peer Reviewed Publications:

1. Guo, W-J., Datta, S., Band, V. and **G. P. Dimri** (2007). Mel-18, a polycomb group protein regulates cell proliferation and senescence via transcriptional repression of Bmi-1 and c-Myc oncoproteins. **Mol. Biol. Cell** 18: 536-546.
2. Guo, W-J., Zeng, M. -S., Yadav, A., Song, L-B., Guo, B.-H, Band, V and **G. P. Dimri** (2007) Mel-18 acts as a tumor suppressor by repressing Bmi-1 expression and downregulating Akt activity in breast cancer cells. **Cancer Res.** 67(11):5083-5089.
3. Datta, S., Hoenerhoff, M. J., Bommi, P., Sainger, R., Guo, W.-J., Dimri, M., Band, H. Band, V., Green, J. E. and **G. P. Dimri**. (2007) Bmi-1 cooperates with H-Ras to transform human mammary epithelial cells via dysregulation of multiple growth regulatory pathways. **Cancer Res.** In Press.

Book Chapters:

1. Itahana, K., Campisi, J. and **G. P**. **Dimri.** (2007) Methods to detect biomarkers of cellular senescence: the senescence-associated β-galactosidase. **Methods in Molecular Biology volume on "Biological Aging: Methods and Protocol",** The Humana Press Inc., Totowa, NJ, pp 21-31.
2. Itahana, K. and **G. P**. **Dimri.** Senescence and Cancer. In **Encyclopedia of Public Health.** Elsevier Ltd, Oxford, UK. In Press.

3. Dellambra, E. and **G.P. Dimri.** Cellular Senescence and Skin Aging. In **Skin Aging Handbook: Market Perspectives, Pharmacology, Formulation, and Evaluation Techniques**, ed., N. Dayan, William Andrew Publishers NY. In Press
4. Dimri, M and **G. P. Dimri.** Cellular Senescence, Apoptosis and Cancer. In **"The Molecular Basis of Human Cancer, Second Edition"**. Humana Press Inc., Totowa, NJ. In Press.

CONCLUSIONS:

p53 an important mediator of cellular senescence, which plays a role in telomere length dependent senescence. p53 is also induced during oncogene induced senescence (OIS) as an anti-onoogenic response. In order to become transformed, cells need to overcome p53-mediated OIS. Thus p53 plays a role in telomere-dependent senescence as well as telomere-independent senescence such as OIS. In both cases, it acts as potent tumor-suppressor.

In the first year of the grant, we proposed to study the DNA binding activity, its expression level and posttranslational modifications during senescence in HMECs. We have completed the proposed studies. However, we have not found any significant differences in posttranslational modifications using limited number of antibodies that we used.

In the second year, we started using p53 RNAi approach to study the role of p53 in senescence. We generated post-selection HMECs cells with p53 and p21 knockdown using RNAi approach. The study of replicative life span of these cells suggest that p53 plays an important role in senescence of post-selection HMECs and other target genes of p53 are possibly involved in senescence.

In year 3, we performed ChIP analysis and identified several known targets of p53 that were upregulated in senescent HMECs. Expression of these targets was confirmed using RT-PCR analysis.

In year 4, we showed that H-Ras overexpression results in p53 upregulation and OIS in HMECs, and Bmi-1 overcome H-Ras induced OIS in HMECs by downregulating p53. Thus *Bmi-1* is a p53 counteracting oncogene. Furthermore, we identified several p53 targets in HMECs that are possibly involved in p53-mediated senescence in HMECs. These different targets of p53 may mediate its tumor-suppressor function.

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14. Dimri, G.P., et al., *The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells.* Cancer Res, 2002. **62**(16): p. 4736-45.
15. Dimri, M., et al., *Modeling breast cancer-associated c-Src and EGFR overexpression in human MECs: c-Src and EGFR cooperatively promote aberrant three-dimensional acinar structure and invasive behavior.* Cancer Res, 2007. **67**(9): p. 4164-72.

CURRICULUM VITAE

PART I. GENERAL INFORMATION

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Place of Birth:	Koladungri, UP, India
Citizenship:	United States
Marital status:	Married (two daughters, ages 2 and 10)

Part II. EDUCATION AND TRAINING

Year	Degree (Field)	Institution
1981	B.S. (Chemistry, Botany, Zoology)	University of Garhwal, Srinagar, India
1984	M.S. (Life Sciences)	J.N. University, New Delhi, India
1985	M.Phil. (Environmental Sciences)	J.N. University, New Delhi, India (Mentor: Dr. H. K. Das)
1990	Ph.D. (Environmental Sciences)	J.N. University, New Delhi, India (Mentor: Dr. H. K. Das)

POSTDOCTORAL TRAINING:

Year	Title	Specialty/Discipline	Place of Training
1989-90	Research Fellow	Biochemistry	University of California, Berkeley, CA (Mentor: Giovanna Ames, Ph.D).
1991-1994	Research Fellow	Cell and Molecular Biology	Lawrence Berkeley National Laboratory, Berkeley, CA (Mentor, Judith Campisi, Ph.D.)
1995-1999	Scientist	Cancer Biology	Lawrence Berkeley National Laboratory, Berkeley, CA (Mentor, Judith Campisi, Ph.D.)

PART III. PROFESSIONAL APPOINTMENTS, SERVICES AND ACHEIVMENTS

ACADEMIC APPOINTMENTS:

Year	Academic Title	Institution
1999-2003	Assistant Professor of Radiation Oncology	Tufts University-New England Medical Center (NEMC) Boston, MA
1999-2003	Special and Scientific Staff, Radiation Oncology	Tufts-New England Medical Center, Boston, MA
2003-	Assistant Professor of Medicine	Feinberg School of Medicine, Northwestern University, Evanston, IL
2003-	Assistant Professor of Medicine	Division of Cancer Biology, Department of Medicine, Evanston, IL
2006-	Member	IBiS Graduate Program, Northwestern University, Evanston, IL

HOSPITAL AND AFFILIATED INSTITUTION SERVICE RESPONSIBILITIES:

Year	**Role**	**Institution**
2003-	Senior Scientist	Division of Cancer Biology, ENH Research Institute, Evanston, IL
2003-	Invention Disclosure and Patent Review Committee	ENH Research Institute Evanston, IL

PEER REVIEW SERVICES:

2005-	Ad-hoc Member	NIH Fellowship study Section FO5
2005	Member	DOD BCRP (Breast Cancer Research Program) Cell Biology 3 (CBY3) Review Panel
2006	Member	DOD BCRP Molecular Biology and Genetics 1 (MBG1) Review Panel
2005:	Mail Reviewer	Austrian Science Fund (FWF), Vienna, Austria
2005:	Mail Reviewer	Doctoral Scholarship Program of the Austrian Academy of Sciences, Vienna, Austria
2005:	Mail Reviewer	Pilot Research Projects for "Center for Genetics and Molecular Medicine', Univ. of Louisville, KY
2006:	Mail Reviewer	The Italian Association for Cancer Research" (AIRC), Milan, Italy
2006:	Mail Reviewer	Netherlands Organization for Scientific Research (NOW, the Dutch Research Council)
2006:	External Evaluator	Faculty Appointments (Tenure-Track Assistant and Associate Professors), UCLA School of Dentistry, Los Angeles, CA

2007:	Mail Reviewer	DOD Ovarian Cancer Research Program, Concept Grants
2007:	Member	DOD BCRP Training #4 Review Panel
2007:	Ad-hoc Teleconference Reviewer	DOD BCRP Epidemiology Review Panel
2007:	Mail Reviewer	Association for International Cancer Research (AICR), St. Andrews, UK
2007:	Mail Reviewer	Medical Research Council (MRC), UK

EDITORIAL BOARDS AND JOURNAL PEER REVIEW SERVICES:

1994-	Ad-hoc Reviewer	Exp. Cell Res. & J. Geron.
2000-	Ad-hoc Reviewer	Cancer Res. & Biogerontology
2003-	Ad-hoc Reviewer	Cancer Letters & J. Biol. Chem.
2004-	Ad-hoc Reviewer	J. Mol. Cell Life Sciences, Mech. Aging and Development, J. Clin. Investigation, J. Lab Investigation, Mol. Cell Biol., Life Sci., Analytical Biochem., Biotech., Apoptosis, FASEB J., British J. Cancer, Mol. Cancer Therapeutics, Breast Cancer Res., Cancer Biol. and Therapy, Oncogene.
2001-	Editorial Board Member	Biogerontology
2006-	Editorial Board Member	Research & Reviews in BioSciences
2007-	Editor-in-Chief	Breast Cancer: Basic and Clinical Research

PROFESSIONAL SOCIETIES:

Year	Role	Society
1994-	Member	American Association of Advancement of Science (AAAS)
1995-	Member	American society of Cell Biology (ASCB)
1996-	Member	American Society for Microbiology (ASM)
2005-	Member	American Association for Cancer Research (AACR)

AWARDS AND HONORS:

Year	Award	Institution
1082-84	Merit Scholarship	J. N. University, New Delhi, India
1984-1986	Junior Research Fellowship	University Grants Commission, New Delhi, Commission India
1987-1989	Senior Research Fellowship	University Grants Commission, New Delhi, India
1994	Travel Award	National Institute on Aging and Gordon Conferences

RESEARCH PATENTS:

U.S. Patent # 5,491,069 and 5,795,728; "Biomarkers of Cell Senescence"

RESEARCH, TEACHING AND CLINICAL CONTRIBUTIONS:

Research:

Postdoctoral training period

The most important contribution that I made during my postdoctoral training period at Lawrence Berkeley National laboratory was the discovery of a senescence marker known as the Senescence Associated- Beta- Galactosidase (SA- -gal) marker. Prior to the discovery of this biomarker, there was no reliable method to detect senescent cells in vitro and in vivo. The marker is widely used in cancer biology and aging research. The original paper describing the method (Dimri, et al. (1995),

Proc. Natl. Acad. Sci. USA, 92: 9363-9367) has received 1356 citations until today. According to PNAS web site (http://www.pnas.org/reports/mfc1.dtl), this publication is ranked #25 among top 50 articles ever published in Proc. Natl. Acad. Sci. USA. Apart from this major contribution, I also discovered the role of E2F family of transcription factors in regulating cellular senescence and proliferation (Dimri et. al. (1994), J. Biol. Chem. 269: 16180-16186, number of citations- 44; Dimri et. al. (1996), Mol. Cell Biol. 16: 2987-2997, number of citations- 68; Dimri et. al. (2000), Mol. Cell Biol. 20: 273-285; number of citations- 193).

Independent faculty period

Following two major research contributions were made during my independent faculty tenure-

1. We showed that polycomb group protein Bmi-1 plays an important role in breast cancer and that it can impart limitless proliferation capacity to human mammary epithelial cells by regulating telomerase (Dimri, G. P. et al. (2002), Cancer Res. 62: 4736-4745; number of current citations- 79). This was the first indication in the literature that Bmi-1 may be involved in maintaining and generating stem cell phenotype.

2. We demonstrated that Bmi-1 regulate cellular senescence by repressing p16 in human fibroblasts and based on our finding proposed that Bmi-1 regulates extrinsic senescence (senescence induced by external factors that lead to p16 upregulation) and not the intrinsic senescence (senescence induced by telomere dysfunction). We also proposed that senescence in fibroblasts occurs by two distinct mechanisms- 1. Via p53-p21 pathway and 2. Via p53-p21 and p16 pathways (Itahana et al. Mol. Cell Biol. 23: 389-401; number of current citations- 132).

Ongoing research

Current research projects in my laboratory are as followings-

1. Identification of novel targets of p53-p21 pathway of senescence in human mammary epithelial cells- Using chromatin-IP approach (ChIP) and ChIP on CHIP, we are interested in identifying novel targets of p53, which are involved in senescence.

2. Regulation of Bmi-1 expression- We are exploring the mechanism of Bmi-1 upregulation in tumors and cancer cell lines, and the molecular basis of telomerase induction of Bmi-1. Among the various regulators of Bmi-1, we are focusing on Myc and E2F family of transcription factors.

3. Identification of Bmi-1 cooperating oncogenic lesions- Using a defined cell culture model of breast epithelial transformation, we have found that Bmi-1 cooperates with other oncogenes such as H-Ras and PcG proteins during oncogenesis. We are studying the mechanism of oncogenic cooperation between Bmi-1 and other oncogenes. We are also interested in identifying such lesions using an unbiased genetic screen such as an RNAi approach.

4. Functional role of Bmi-1 related polycomb proteins during oncogenesis- We have also cloned and characterized other PcG family members, which are closely related to Bmi-1. These proteins have over-lapping and non-overlapping functions in regulating senescence and oncogenesis; some members regulate p53-p21 pathway of senescence, while others regulate pRb-p16 pathway of senescence. These proteins also modulate activity of each other, and can cooperate to cause cellular transformation. We are further studying the molecular basis of these diverse functions of PcG proteins.

5. Developing molecular and biochemical reagents to inhibit polycomb expression/function- Finally, we are interested in developing reagents that can inhibit the function and/or expression of pro-oncogenic PcG proteins. In particular, we have identified a class of genotoxic drugs that can inhibit Bmi-1 function and expression. We are characterizing the mechanism of Bmi-1 downregulation by these drugs. Such drugs/reagents may help in developing novel strategies to treat breast and other cancers.

Teaching:

1999-2003 Participated in Teaching Residents and Postdoctoral Fellows, Department of Radiation Oncology, NEMC, Boston, MA

2003- Participated in Teaching Postdoctoral Fellows, ENH Research Institute Evanston, IL

Advisory and Supervisory Responsibilities (Research Assistants, Graduate and Undergraduate Students, Residents and Postdoctoral Fellows):

During Postdoctoral Fellow position:

1991-1994: Trishia Chandra, Research associate, Lawrence Berkeley National Laboratory, Berkeley, CA

1991-1993: Ying Lee, Research associate, Lawrence Berkeley National Laboratory, Berkeley, CA

1994-1999: Meilleen Acosta, Research associate, Lawrence Berkeley National Laboratory, Berkeley, CA

1998-1999: Ying Zou, Research associate, Lawrence Berkeley National Laboratory, Berkeley, CA

During Independent Investigator position:

2000-2002: Dr. Jose-Luis Martinez, Postdoctoral Fellow, New England Medical Center, Boston, MA

2002-2003: Dr. Suresh Kumar, Postdoctoral Fellow, New England Medical Center, Boston, MA

2002-2004: Dr. Libing Song, Postdoctoral Fellow, New England Medical Center, Boston, MA; Postdoctoral Follow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2003-2006: Dr. Sonal Datta, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2004-2007 Dr. Ajay Kumar, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2005-2007 Dr. Wei Jian Guo, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2006- Dr. Rachana Sainger, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2006- Dr. Anag Sahasbudere, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2005- Prashant Bommi Reddi, Research Assistant, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2006- Hema, RamKumar, Pre-Med Undergraduate Student, Northwestern University

CLINICAL: Not applicable

FUNDING INFORMATION:

Active:

1.

Title: Role of p53 in Mammary Epithelial Cell Senescence
Role: PI
Funding Agency: US ARMY Medical Research and Material Command, DOD

Direct cost: $221,549
Duration: 05/01/02-04/30/07

2.

Title: Role of Bmi-1 in Telomerase Regulation and Breast Cancer
Role: PI
Funding Agency and Grant Number: NIH, 1RO1 CA094150
Total Direct Cost: $1,000,000
Duration: 09/17/03-08/30/08

Pending:

1. Title: Transcriptional reprogramming to convert human somatic cells into Stem Cell like Cells
Role: PI
Funding Agency and Grant Number: NIH, 1R21AG031342-01
Total Direct Cost: $275,000

2. Title: The role of Polycomb group (PcG) proteins in breast oncogenesis
Role: PI
Funding Agency and Grant Number: USAMRMC, DOD- BC073732
Total Direct Cost: $300,000

3. Title: A genetic screen for tumor suppressors involved in early steps of breast cancer progression
Role: PI
Funding Agency and Grant Number: USAMRMC, DOD- BC073729
Total Direct Cost: $300,000

Past:

1. Title: Senescence-specific Promoter Vectors
Role: PI
Funding Agency and Grant Number: NIH, AG165851-01
Total Direct Cost: $50,000
Duration: 03/01/99-02/28/01

2. Title: Polycomb Proteins and Breast Epithelial Cell Transformation
Role: PI
Funding Agency and Grant Number: USAMRMC, DOD- BC032256
Direct Cost- $75,000
Duration: 07/01/04-06/30/05

REGIONAL, NATIONAL, OR INTERNATIONAL CONTRIBUTIONS, INVITED SPEAKER

1994: Biology of Aging, Special interest subgroup meeting, American Society for Cell Biology, Thirty Fourth Annual Meeting, San Francisco, CA

1998: Cardinal Bernardin Cancer Center, Loyola University Medical Center, Maywood, IL, " Cellular and Molecular Biology of senescence"

1999: California Pacific Medical Center, San Francisco, CA
"Role of Cellular Senescence in Aging and Cancer"

1999: Center for Aging, University of Alabama at Birmingham, AL
"Role of Senescence in Aging and Cancer "

1999: New England Medical Center, Boston, MA
"Mechanism of Cellular Senescence in Human Cells"

2003: 8th World Congress on Advances in Oncology and
6th International Symposium on Molecular Medicine
16-18th October, 2003, Creta Maris, Hersonissos, Crete, Greece
"Molecular Mechanisms of Cellular Senescence in Human Cells".

2005: "What has senescence got to do with cancer?"
Children's Memorial Research Center, Northwestern University, Chicago, IL

2005: "The role of Bmi-1 and Bmi-1 related genes in Senescence and Proliferation", Gheens Center for Research in Aging, University of Louisville, School of Medicine, Louisville, KY

2006: "The role of Bmi-1 and Bmi-1-related Polycombs in Senescence and Oncogenesis". National Cancer Institute, NIH, Bethesda, MD

2007: "Control of cell proliferation and oncogenesis by Bmi-1 and related Polycomb proteins", University of Vermont, Burlington, VT

2007: "Regulation of cell proliferation and senescence by Bmi-1 and related Polycomb proteins", University of Maryland, Baltimore, MD

2007: "Control of cell proliferation and oncogenesis by Bmi-1 and related Polycomb proteins", University of Omaha, Omaha, NB.

2007: " Cellular Senescence and Skin Aging" HBA Global Exposition and Educational Conference, Sept. 18-20, 2007; Jacob K. Javits Convention Center, New York City, NY

2007: "Regulation of cell proliferation and senescence by Polycomb group (PcG) of proteins".
12th World Congress on Advances in Oncology and 10th International Symposium on Molecular Medicine, 11-13 October, 2007, Creta Maris, Hersonissos, Crete, Greece

PART IV. BIBLIOGRAPHY

Original Reports:

1. Phadnis, S. H., **Dimri, G. P.** and H. K. Das (1988) Segregation characteristics of multiple chromosomes of *Azotobacter vinelandii*. **J. Genet.** 67: 37-42.

2. **Dimri, G. P.,** Roy, K. B. and H. K. Das (1988) Cloning of ferredoxin I gene from *Azotobacter vinelandii* using synthetic oligonuclotide probes. **J. Biosc.** 13: 323-327.

3. **Dimri, G. P.** and H. K. Das (1988) Transcriptional regulation of nitrogen fixing genes by DNA supercoiling. **Mol. Gen. Genet.** 212: 360-363.

4. **Dimri, G. P.** and H. K. Das (1990) Cloning and sequence analysis of *gyrA* gene of *Klebsiella pneumoniae.* **Nucl. Acids Res.** 18: 151-156.

5. **Dimri, G. P.,** d. Ari, L., Ames, G. -F. L. and J. C. Rabinowitz (1991) Physical mapping of *Escherichia coli* gene encoding the bifunctional enzyme 10-Methylentetrahydrofolate hydrogenase/ 5-10 Methenyl tetrahydrofolate cyclohydrolase. **J. Bacteriol.** 173: 5251.

6. **Dimri, G. P.,** Rudd, K. E., Morgan, M., Bayat, H. and G. -F. L. Ames (1992) Physical mapping of REP sequences in *Escherichia coli*, Phylogenetic distribution among *E.coli* strains and other enteric bacteria. **J. Bacteriol.** 174: 4583-4593.

7. **Dimri, G. P.** and J. Campisi (1994) Altered profile of transcription factors binding activity during cellular senescence. **Exp. Cell Res.** 212: 132-140.

8. **Dimri, G. P.,** Hara, E. and J. Campisi (1994) Regulation of two E2F related genes in presenescent and senescent human fibroblasts. **J. Biol. Chem.** 269:16180-6186.

9. **Dimri, G. P.,** Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Rubelj, I., Pereira-Smith, O. M., Peacocke, M. and J. Campisi (1995) A Novel biomarker identifies senescent human cells in culture and in aging skin in vivo.

Proc. Natl. Acad. Sci. USA 92: 9363-9367.

10. Hara, E., Uzman, A., **Dimri, G. P.,** Nehlin, J., Testori, A. and J. Campisi (1996) The HLH protein ID1 complements an Rb binding deficient T antigen for stimulation of DNA synthesis in senescent human fibroblast. **Dev. Genet.** 18: 161-172.

11. **Dimri, G. P.,** Nakanishi, M., Desprez, P., Smith, J. R. and J. Campisi (1996) Inhibition of E2F activity by the cyclin dependent protein kinase inhibitor p21. **Mol. Cell. Biol.** 16: 2987-2997.

12. Good, G., **Dimri, G. P.,** Campisi, J. and K. Y. Chen (1996) Regulation of dihydrofolate reductase and E2F genes in human diploid fibroblasts during senescence in culture. **J. Cell. Physiol.** 168: 580-588.

13. **Dimri,G. P.,** Itahana, K., Acosta, M. and J. Campisi (2000). Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14ARF tumor suppressor. **Mol. Cell. Biol.** 20: 273-285.

14. Itahana, K., **Dimri, G. P.** and J. Campisi (2001) Regulation of cellular senescence by p53. **Eur. J. Biochem.** 268: 2784-2791.

15. Li, B., Goyal, J., Dhar, S., **Dimri, G. P**., Evron, E., Sukulmar, S. and V. Band (2001) CpG methylation in exon 3 as a basis for breast tumor specific loss of NES1 expression. **Cancer Res.** 61: 8014-8021.

16. Itahana, K., **Dimri, G. P.** Itahana, Y., Zou, Y., Hara, E., Desprez, P. Y., and J. Campisi (2002) A role for p53 in maintaining and establishing quiescence growth arrest in human cells. **J. Biol. Chem.** 277: 18206-18214.

17. **Dimri, G. P.,** Martinez, J. L., Jacobs, J. L, Keblusek, P., Itahana, K., van Lohuizen, M., Campisi, J. Wazer, D. E., and V. Band (2002) Bmi-1 oncogene induces telomerase and immortalizes human mammary epithelial cells. **Cancer Res.** 62: 4736-4745.

18. Kumar, A., Zhao, Y., Meng, G., Zeng, M., Srinivasan, S., Gao, Q., **Dimri, G.,** Weber, G., Wazer, D., Band, H., and V. Band (2002) Human papilloma virus oncoprotein E6 inactivates the transcriptional coactivator human ADA3. **Mol. Cell. Biol.** 22: 5801-5812.

19. Zeng, M., Kumar, A., Meng, G., Gao, Q., **Dimri, G.,** Wazer, D., Band, H., and V. Band (2002) HPV16 E6 oncoprotein inhibits RXR-mediated transactivation by targetting human ADA3 coactivator. **J. Biol. Chem.** 277: 45611-45618.

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senescence in human fibroblast by p16 and the polycomb protein Bmi-1. **Mol. Cell. Biol.** 23: 389-401.

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22. Maurelli, R., Bondanza, S., Guerra, L., Abbruzzese, C., **Dimri, G.**, Gellini, M., Zambruno, G. and Dellambra, E. (2006) Inactivation of $p16^{Ink4a}$ immortalizes primary human keratinocytes by maintaining cells in the stem cell compartment. **FASEB J**. 20(9): 1516-8. Epub 2006 Jun 5.

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24. Zhang, Y., Gurumurthy, C. B, Kim, J. H, Bhat, I., Gao, Q., **Dimri, G**, Lee, S. W., Band, H and V. Band (2006). The human ortholog of Drosophila ecdysoneless protein interacts with p53 and regulates its function. **Cancer Res.** 66: 7167-7175.

25. Kang, M. K, Kim, R. H., Kim, S. J., Yip, F. K., Shin, K. H., **Dimri, G. P.**, Christensen, R., Han,T. and N. H. Park. (2006). Elevated Bmi-1 expression is associated with dysplastic cell transformation during oral carcinogenesis and is required for cancer cell replication and survival. **Br. J. Cancer** 96: 126-133.

26. Guo, W-J., Datta, S., Band, V. and **G. P. Dimri** (2007). Mel-18, a polycomb group protein regulates cell proliferation and senescence via transcriptional repression of Bmi-1 and c-Myc oncoproteins. **Mol. Biol. Cell** 18: 536-546.

27. Nag, A., Germaniuk-Kurowaska, A., Dimri, M., Sassack, M., Gurumurthy, C.B., Gao, Q., **Dimri, G.,** Band, H. and V. Band (2007). An essential role of human ADA3 in p53 acetylation. **J. Biol. Chem.** 282(12):8812-8820.

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Modeling breast cancer-associated c-Src and EGF receptor overexpression in human mammary epithelial cells: c-Src and EGFR cooperatively promote aberrant three- dimensional acinar structure and invasive behavior. **Cancer Res.** 67(9):4164-4172

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32. Datta, S., Hoenerhoff, M. J., Bommi, P., Sainger, R., Guo, W.-J., Dimri, M., Band, H. Band, V., Green, J. E. and **G. P. Dimri**. Bmi-1 cooperates with H-Ras to transform human mammary epithelial cells via dysregulation of multiple growth regulatory pathways. **Cancer Res.** In Press.

Peer Reviewed and Invited Reviews:

32. Campisi, J., **Dimri, G. P.,** Nehlin, J. O., Testori, A. and K. Yoshimoto (1996) Coming of age in culture. **Exp. Geron.** 31: 7-12.

33. **Dimri, G. P.,** Testori, A. and J. Campisi (1996) Replicative senescence, aging and growth regulatory transcription factors. **BioSig.** 5:154-162.

34. Itahana, K., Campisi, J. and **G. P**. **Dimri** (2004) Mechanisms of cellular senescence in human and mouse cells. **Biogeron.** 5: 1-10.

35. **Dimri G. P.** (2004) The search for biomarkers of aging: next stop INK4a/ARF locus. **Sci Aging Knowledge Environ. (**On-line sister publication of **Science)** Nov 03;2004(44):pe40.

36. **Dimri, G. P.**, Band, H and V. Band (2005) Mammary epithelial cell transformation: insights from cell culture and mouse models. **Breast Cancer Res.** 7: 171-179.

37. **Dimri, G.P.** (2005) What has senescence got to do with cancer? **Cancer Cell** 7: 505-512.

Proceedings and Book Chapters:

38. Reddy, A. N., Phadns, S. H., **Dimri, G. P.,** Jaferi, S., Medhora, M. M., and H. K. Das (1986) Complexity of the Genome of *Azotobacter vinelandii.* In Biotechnology in Agriculture Eds Natesh, S., Chopra V. L., and S. Ramachandran, pp 15-19; Oxford &IBH Publishing Co. Pvt. LTD., New Delhi.

39. **Dimri, G. P.,** and J. Campisi (1995) Molecular and cell biology of replicative senescence. In Cold Spring Harbor Laboratory Symposium in Quantitative Biology: **Molecular Genetics of Cancer,** 54: 67-73.

40. Campisi, J. **Dimri, G. P.** and E. Hara (1996) Control of replicative senescence. In **Handbook of Biology of Aging,** 4th ed., Eds Schneider, E. and J. W., Rowe, pp121- 149.

41. Itahana, K., Campisi, J. and **G. P**. **Dimri.** (2007) Methods to detect biomarkers of cellular senescence: the senescence-associated -galactosidase. **Methods in Molecular Biology volume on "Biological Aging: Methods and Protocol",** The Humana Press Inc., Totowa, NJ, pp 21-31.

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43. Dellambra, E. and **G.P. Dimri** Cellular Senescence and Skin Aging. In **Skin Aging Handbook: Market Perspectives, Pharmacology, Formulation, and Evaluation Techniques**, ed., N. Dayan, William Andrew Publishers NY. In Press

44. **Dimri, G. P.** Cellular Senescence, Apoptosis and Cancer. In **"The Molecular Basis of Human Cancer, Second Edition"**. Humana Press Inc., Totowa, NJ. In Press.

Abstracts:

1. **Dimri, G. P.** and J. Campisi (1994) Transcriptional control of cellular replicative senescence. Molecular Biology of the Cell. 5, 386 a.

2. Danahy, J. F., Lee, X., Scott, G., **Dimri, G. P.**, Campisi, J. and M. Peacocke (1994) A biomarker of human cellular aging in vivo and in vitro. AFCR, Clinical Research Meeting, April 29-May 2.

3. **Dimri, G. P**., Hara, E., Acosta, M., Desprez, P., Nakanishi, M., Smith, J. R. and J. Campisi (1995) The role of p53 in cellular replicative senescence. In FASEB J., 9, Abs # 1202.

4. **Dimri, G. P**., Acosta, M. and J. Campisi (1996) Regulation of E2F related genes during cellular senescence. Molecular Biology of the Cell. 9, Abs# 3102.

5. **Dimri, G. P.** and J. Campisi (1997) Extension of replicative life span and induction of crisis in E6 expressing fibroblasts by E2F1 overexpression. In Molecular and Genetic Strategies for Treatment of Age-Related Diseases. NMHCC Conference. July 15-16 Seattle, WA.

6. **Dimri, G. P.** and J. Campisi (1998) Extension of replicative life span and induction of crisis in E6 expressing fibroblasts by overexpression of E2F1. In FASEB J., 12: No. 4, Abs. #1846.

7. Krtolica, A., Yip, D., **Dimri, G. P**., Desprez, P. and J. Campisi (2000) The double-edged sword of replicative senescence: Senescent fibroblasts stimulate pre-malignant epithelial cell growth. AACR 91st Annual Meeting April 1-5, San Francisco, CA.

8. Li, B., Goyal., J., Dhar, S., **Dimri, G.,** Evron, E., Sukumar, S., Wazer, D. E., and V. Band (2002) CpG methylation as a basis for breast tumor-specific loss of NES1/Kallikrein 10 expression. Era of Hope Meeting, Abs# P8

9. Datta, S., Band, V. and **Dimri, G. P.** (2005) Polycomb proteins and Breast Epithelial cell transformation. Era of Hope, Dept. of Defense, Breast Cancer Research Program Meeting. Abst # P11-9.

10. Yadav, A., Datta, S., Band, V. and **Dimri, G. P.** (2005) Role of p53 in Mammary Epithelial Cell Senescence. Era of Hope, Dept. of Defense, Breast Cancer Research Program Meeting. Abst # P27-8.

11. **Dimri, G.P.** (2007) Regulation of cell proliferation, senescence and oncogenesis by polycomb group of proteins. Int. J. Mol. Medicine 20: Suppl. Abs #175.

Database entries/articles:

1. **Dimri, G.P. and H.K. Das (1990)** Genebank # X16817**,** Klebsiella pneumoniae gyrA gene for DNA gyrase subunit A (EC 5.99.1.3).

2. **Dimri, G.P.** PCGF4 (BMI-1). In Targeted Protein Database (TPdb), Current BioData Ltd., London, UK. In Press.

3. **Dimri, G.P.** MYC (c-MYC). In Targeted Protein Database (TPdb), Current BioData Ltd., London, UK. In Press.

4. **Dimri, G.P.** MYCN (N-MYC). In Targeted Protein Database (TPdb), Current BioData Ltd., London, UK. In Press.

5. **Dimri, G.P.** CDKN2A (p16INK4a). In Targeted Protein Database (TPdb), Current BioData Ltd., London, UK. In Press.

6. Dimri, M. and **G.P. Dimri.** SRC (c-Src). In Targeted Protein Database (TPdb), Current BioData Ltd., London, UK. In Press.

7. Dimri, M. and **G.P. Dimri.** FAK. In Targeted Protein Database (TPdb), Current BioData Ltd., London, UK. In Press.

Theses:

1985: M.Phil. thesis titled "Transposon Tn10 Mutagenesis of *Azotobacter vinelandii"*

1990: Ph.D. thesis titled "Genetics of Some Aspects of Regulation of Nitrogen Fixing Genes in *Klebsiella pneumoniae* and *Azotobacter vinelandII"*

Mel-18 Acts as a Tumor Suppressor by Repressing Bmi-1 Expression and Down-regulating Akt Activity in Breast Cancer Cells

Wei-Jian Guo,[1] Mu-Sheng Zeng,[4] Ajay Yadav,[1] Li-Bing Song,[4] Bao-Hong Guo,[4] Vimla Band,[1,2,3] and Goberdhan P. Dimri[1,2]

[1]Division of Cancer Biology and Department of Medicine, ENH Research Institute; [2]Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine; [3]Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois; and [4]State Key Laboratory of Oncology in Southern China and Departments of Experimental Research, Sun Yat-sen University Cancer Center, Guangzhou, China

Abstract

The *Bmi-1* oncogene is overexpressed in a number of malignancies including breast cancer. In addition to Bmi-1, mammalian cells also express four other polycomb group (PcG) proteins that are closely related to Bmi-1. Virtually nothing is known about the role of these PcG proteins in oncogenesis. We have recently reported that Mel-18, a Bmi-1–related PcG protein, negatively regulates Bmi-1 expression, and that its expression negatively correlates with Bmi-1 in proliferating and senescing human fibroblasts. Here, we report that the expression of Bmi-1 and Mel-18 inversely correlates in a number of breast cancer cell lines and in a significant number of breast tumor samples. Overexpression of Mel-18 results in repression of Bmi-1 and reduction of the transformed phenotype in malignant breast cancer cells. Furthermore, the repression of Bmi-1 by Mel-18 is accompanied by the reduction of Akt/protein kinase B (PKB) activity in breast cancer cells. Similarly, Bmi-1 knockdown using RNA interference approach results in down-regulation of Akt/PKB activity and reduction in transformed phenotype of MCF7 cells. Importantly, we show that overexpression of constitutively active Akt overrides tumor-suppressive effect of Mel-18 overexpression and the knockdown of Bmi-1 expression. Thus, our studies suggest that Mel-18 and Bmi-1 may regulate the Akt pathway in breast cancer cells, and that Mel-18 functions as a tumor suppressor by repressing the expression of Bmi-1 and consequently down-regulating Akt activity. [Cancer Res 2007;67(11):5083–9]

Introduction

Polycomb group (PcG) proteins are chromatin-modifying proteins that play an important role in the development and cancer (1). Overexpression of certain PcG proteins, such as Bmi-1 and EZH2, has been linked to invasive breast and prostate cancer (2–4). Bmi-1 is also overexpressed in several other malignancies such as non–small-cell lung cancer (5), colorectal cancer (6), nasopharyngeal carcinoma (7), and oral cancer (8). Bmi-1 is known to be a key regulator of self-renewal of stem cells (1). In addition, recently, it was shown that Hedgehog signaling via Bmi-1 regulates self-renewal of normal and malignant human mammary stem cells (9).

After a finite number of cell divisions, most normal human cells undergo cellular senescence, whereby cells irreversibly cease to divide (10). Senescence constitutes a powerful barrier to oncogenesis (10). Bmi-1 has been shown to regulate cellular senescence and proliferation in rodent and human fibroblasts (11, 12). In addition, Bmi-1 can also bypass senescence and immortalize human mammary epithelial cells (HMEC; ref. 13). We have recently reported that Bmi-1 is negatively regulated by Mel-18 via repression of c-Myc, and that Mel-18 is overexpressed in senescent fibroblasts (14).

Here, we show that similar to human fibroblasts, expression of Mel-18 negatively correlates with Bmi-1 in a number of breast cancer cell lines and in a significant number of breast tumors. We also report that overexpression of Mel-18 in a commonly used breast cancer cell line MCF7 results in down-regulation of Bmi-1 and reduction of transformed phenotype. Furthermore, down-regulation of Bmi-1 by Mel-18 overexpression and knockdown of Bmi-1 expression by RNA interference (RNAi) approach is accompanied by down-regulation of Akt/protein kinase B (PKB) activity. We also show that overexpression of constitutively active Akt restores malignancy in MCF7 cells, in which Bmi-1 expression is reduced due to Mel-18 overexpression or Bmi-1 knockdown.

Materials and Methods

Cellular reagents, retroviral and short hairpin RNA vectors, virus production, and infection. MCF10A, MCF7, and other breast cancer cells were cultured as described (13). Retroviral vectors overexpressing Bmi-1 and Mel-18 and Bmi-1 short hairpin RNA (shRNA) are described earlier (14). A retroviral vector, pSRα-mAkt expressing constitutively active (myristylated) Akt (mAkt), was obtained from Dr. N. Hay (University of Illinois, Chicago, IL). Stable cell lines expressing *Mel-18* or other gene of interest were generated by infection of the retroviral vectors expressing the particular gene as described (13, 14). The retroviruses were produced by transient transfection of the retroviral vector together with pIK packaging plasmid into tsa 54 packaging cell line as described (14). Soft-agar growth assay to determine the anchorage independence of cells was done as described (4).

Immunologic reagents and methods. Bmi-1 was detected using either F6 mouse monoclonal antibody (mAb) from Upstate Cell Signaling Solutions or 1H6B10G7 mAb from Zymed. Mel-18 was detected by a rabbit polyclonal H-115 (Santa Cruz Biotechnology). For the analysis of the Akt pathway, phosphorylated Akt 1/2/3 (pAkt 1/2/3; Ser^{473}; sc-7985-R), pAkt 1/2/3 (Thr^{308}; sc-16646-R), Akt-1 (B-1; sc-5298), Akt-2 (F-7; sc-5270), glycogen synthase kinase-3β (GSK3β; sc-53931), and cyclin D1 (A-12; sc-8396) antibodies were obtained from Santa Cruz Biotechnology. Rabbit polyclonal against total Akt (#9272) and pGSK3β (#9336) were obtained from Cell Signaling Technology.

To determine Akt activity in synchronized cells, MCF7 cells were serum starved for 48 h and stimulated for 30 min by addition of 10% FCS. MCF10A cells were growth factor deprived using D3 medium (15) for 48 h and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-06-4368

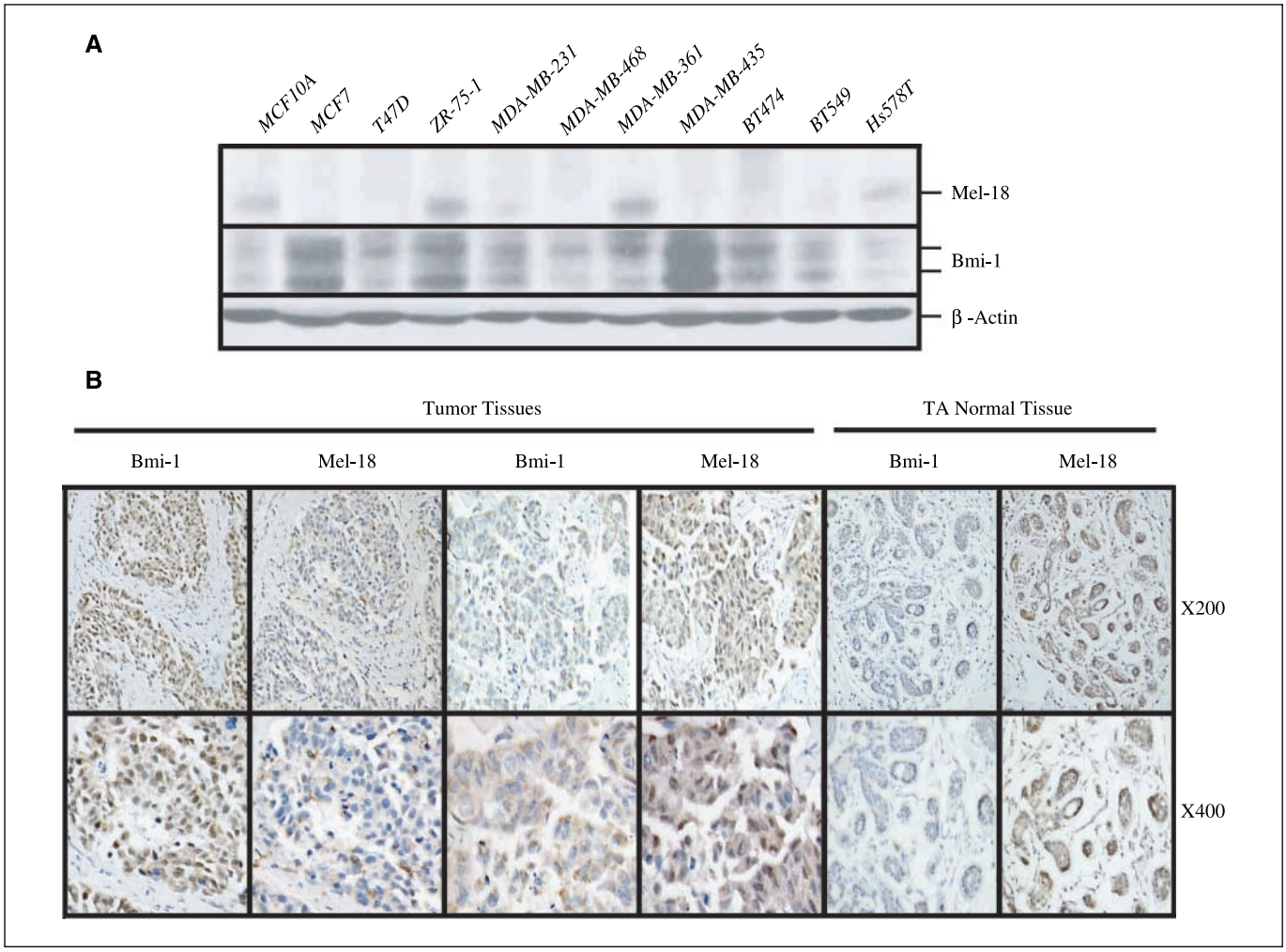

Figure 1. Mel-18 and Bmi-1 expression inversely correlates in breast cancer cell lines and breast tumors. *A,* Bmi-1 and Mel-18 expression in various breast cancer cell lines as detected by Western blot analysis. *B,* representative of two tumor samples: sample 1 expresses high Bmi-1 and low Mel-18, whereas sample 2 expresses high Mel-18 and low Bmi-1 expression. Tumor adjacent (*TA*) normal tissue of a biopsy sample with high Mel-18 and low Bmi-1. Tissues were stained with Bmi-1– or Mel-18– specific antibodies and counterstained with hematoxylin as described in Materials and Methods.

stimulated for 30 min by addition of D medium, which contains 12.5 ng/mL epidermal growth factor (15). For the inhibition of the phosphoinositide 3-kinase (PI3K) pathway, cells were pretreated with LY294002 (20 μmol/L) or Wortmannin (100 nmol/L; Calbiochem) for 1 h before the addition of complete medium. Western blot analyses of total cell extracts were done using antibodies that detect total Akt, pAkt, and various other proteins as described (13, 14).

Clinical samples and immunohistochemical and statistical analyses. A total of 61 invasive breast cancer tissue samples were collected from the archives of the Department of Pathology, Cancer Center, Sun Yat-sen University (Guangzhou, China). For the use of these clinical materials for research purposes, prior patients' consent and approval from the Institute Research Ethics Committee were obtained. Bmi-1 and Mel-18 were detected in paraffin sections of breast cancer tissue as described (7). All slides were interpreted by two independent observers in a blinded fashion. For each sample, one score was given according to the percentage of positive cells as <5% of the cells (1 point), 6% to 35% of the cells (2 points), 36% to 70% of the cells (3 points), >71% of the cells (4 points). Another score was given according to the intensity of staining as negative staining (1 point), weak staining (2 points), moderate staining (3 points), and strong staining (4 points). A final score was then calculated by multiplying the above two scores. If the final score was ≥4, the tumor was considered positive; otherwise, the tumor was considered negative. All statistical analyses were done by using the SPSS 10.0 software package. The Spearman's rank correlation was used to estimate the correlation between Bmi-1 and Mel-18 expression.

Results

Bmi-1 and Mel-18 expression inversely correlates in breast cancer cell lines and breast tumors. Our previous data in cultured human fibroblasts suggest an inverse correlation between Bmi-1 and Mel-18 expression; senescent cells show high expression of Mel-18, whereas proliferating cells show high expression of Bmi-1. These results suggested that breast cancer cell lines might express high Bmi-1 and low Mel-18. To probe this hypothesis, we analyzed expression of Bmi-1 and Mel-18 in several breast cancer cell lines (Fig. 1*A*). Our results suggested that compared with MCF10A, a normal immortal HMEC cell line, the majority of breast cancer cell lines (7 of 10) express high Bmi-1 and low Mel-18 (Fig. 1*A*).

Because Bmi-1 is overexpressed in a large number of breast tumors (2, 3), and because its expression inversely correlates with Mel-18 expression in breast cancer cell lines, we hypothesized that Mel-18 down-regulation may lead to Bmi-1 up-regulation in breast

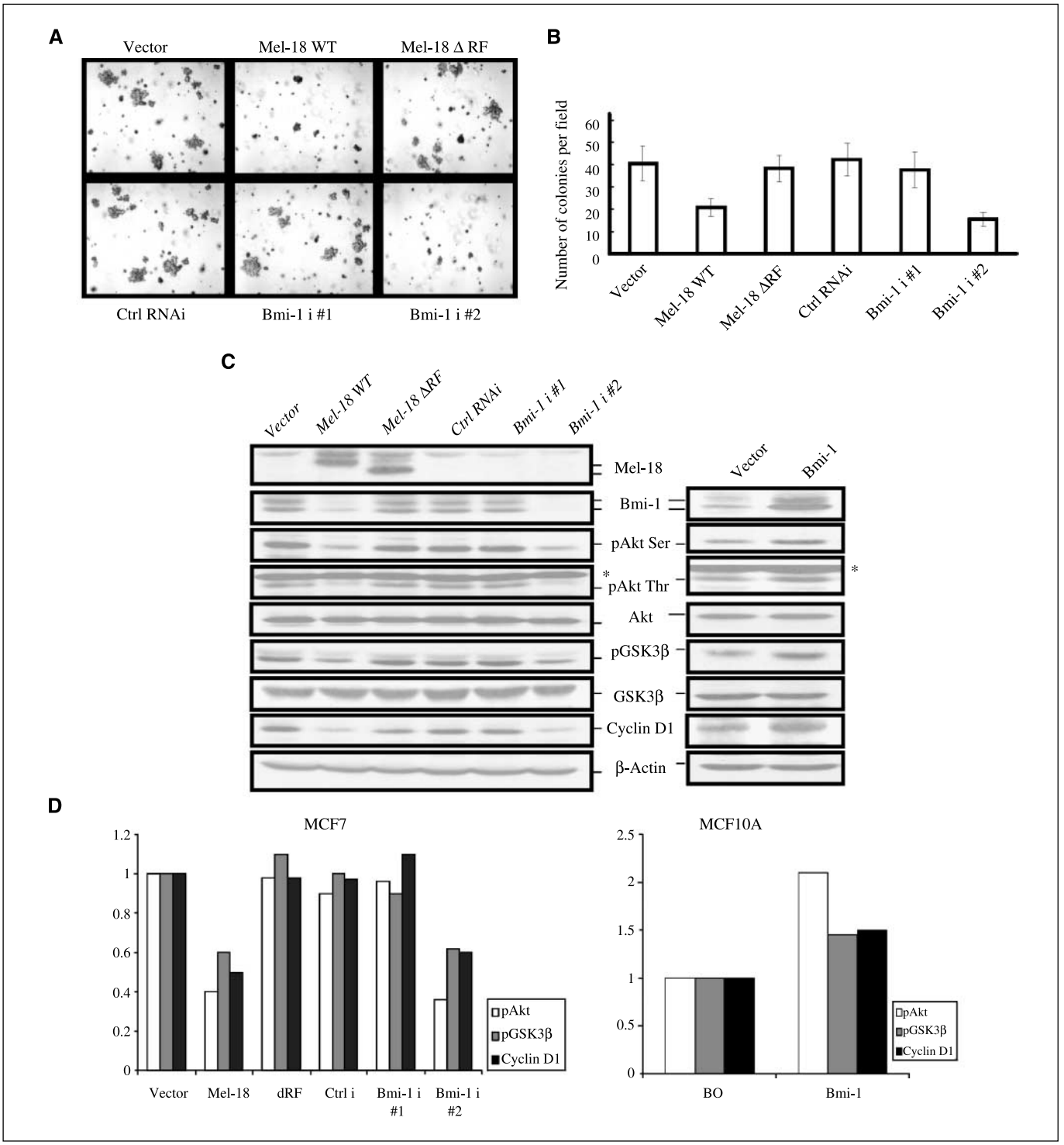

Figure 2. Reduction of transformed phenotype of MCF7 cells by Mel-18 overexpression and knockdown of Bmi-1 expression. *A,* overexpression of Mel-18 and knockdown of Bmi-1 expression in MCF7 decreases colony formation in soft agar. Control or Mel-18–overexpressing MCF7 cells (*top*), and control (*Ctrl RNAi*) or Bmi-1 shRNAs (*Bmi-1 i #1* and *Bmi-1 i #2*) cells (*bottom*) were plated in soft agar to determine the anchorage-independent growth as described in Materials and Methods. *B,* colonies from three different experiments were counted and plotted. *C, left,* Mel-18 and Bmi-1 regulate Akt activity. Bmi-1 knockdown by RNAi approach or its down-regulation by Mel-18 overexpression leads to reduction in pAkt as determined by Western blot (*WB*) analysis using both anti–phosphorylated Ser^{473} and anti–phosphorylated Thr^{308} Akt antibodies. Reduction in Akt activity results in corresponding decrease in pGSK3β and cyclin D1 protein levels. Mel-18, Bmi-1, total Akt, pAkt, pGSK3β, total GSK3β, cyclin D1, and β-Actin (loading control) were detected by Western blot analysis as described in Materials and Methods. *, nonspecific band reacting to pAkt (Thr^{308}) antibody. *Right,* Bmi-1 overexpression up-regulates Akt activity in MCF10A cells. Bmi-1 was overexpressed in MCF10A cells using pBabe-Bmi-1 retrovirus, and vector control and Bmi-1–overexpressing cells were analyzed for the activation of the Akt/GSK3β/cyclin D1 pathway by Western blot analysis as described in Materials and Methods. *D,* quantification of Akt and GSK3β activity. The pAkt and pGSK3β signal in each lane was quantified by densitometric analysis using ImageJ 1.37 software (NIH, Bethesda, MD) and normalized to total Akt and total GSK3β signal of each lane, respectively, and plotted. Similarly, levels of cyclin D1 were quantified using densitometric analysis of signal present in each lane, normalized to β-actin signal of each lane, and plotted.

tumors. To examine this possibility, we studied the expression of Mel-18 and Bmi-1 in 61 breast tumors by immunohistochemistry (Fig. 1*B*; Supplementary Fig. S1). By immunohistochemical analysis, 51 of 61 (83.6%) paraffin-embedded archival breast tumor biopsies showed a positive staining (score of $\geq$4) for Bmi-1, whereas 15 of 61 (24.5%) of the biopsies showed a positive staining (score of $\geq$4) of Mel 18. Of 15 Mel-18–positive and 51 Bmi-1–positive biopsies, only six were positive for both Bmi-1 and Mel-18 (Supplementary Table S1). The correlation between Bmi-1 and Mel 18 expression was further analyzed by Spearman correlation analysis, which showed a strong negative correlation ($r = -0.673$, $P < 0.0001$).

Overexpression of Mel-18 and knockdown of Bmi-1 expression reduce malignancy of breast cancer cells. To examine the possibility that Mel-18 overexpression may reduce or revert the transformed phenotype of malignant cells, we determined the transformation potential of control and Mel-18–overexpressing MCF7 cells using anchorage independence growth assay. The results indicated that Mel-18 overexpression in MCF7 cells led to a decrease in colony formation in soft agar (Fig. 2*A* and *B*). The colonies in Mel-18–overexpressing MCF7 cells were less in frequency and also smaller in size (Fig. 2*A, top*). A RING finger mutant of Mel-18, which does not down-regulate Bmi-1 (14), did not inhibit soft agar colony formation when overexpressed in MCF7 cells (Fig. 2*A, top*).

We also determined the anchorage-independent growth potential of MCF7 cells, which stably express Bmi-1 shRNAs. We used two Bmi-1 shRNAs (Bmi-1 i#1 and Bmi-1 i#2). Western blot analysis of Bmi-1 indicted that Bmi-1 i#2 efficiently knocked down Bmi-1 expression (Fig. 2*C*). Accordingly, we found that stable expression of Bmi-1 i#2 in MCF7 cells led to significant decrease in number of colonies in soft agar, indicating a decrease in transformed phenotype of these cells (Fig. 2*A, bottom* and Fig. 2*B*).

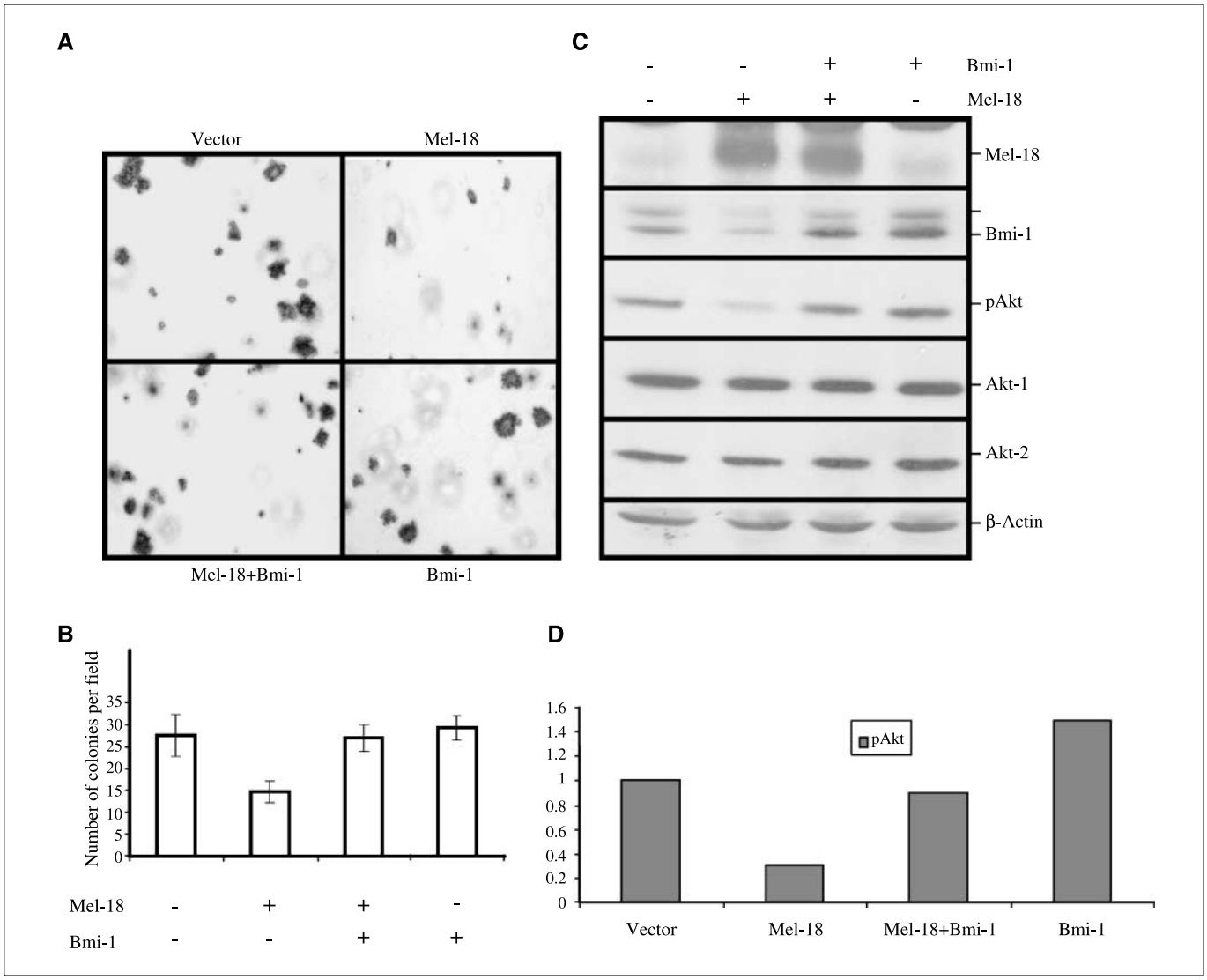

Figure 3. Exogenous Bmi-1 restores Akt activity and anchorage-independent growth potential of Mel-18–overexpressing cells. *A,* MCF7 cells were infected with a control retrovirus or Bmi-1–overexpressing retrovirus. Cells were selected in hygromycin and super-infected with Mel-18–expressing retrovirus. After selection, vector, Mel-18, Bmi-1, and Mel-18 and Bmi-1 coexpressing cells were analyzed for colony formation in soft agar. *B,* numbers of colonies growing in soft agar were quantified per field, and data were plotted. *C,* Western blot analysis of cells expressing Mel-18, Bmi-1, or Bmi-1 and Mel-18 and control cells was done to confirm overexpression as well as restoration of Akt activity as described in Materials and Methods. *D,* quantification of Akt activity in control vector and Mel-18, Mel-18 + Bmi-1, and Bmi-1–overexpressing cells (as indicated). Akt activity was quantified as described in Fig. 2*D*.

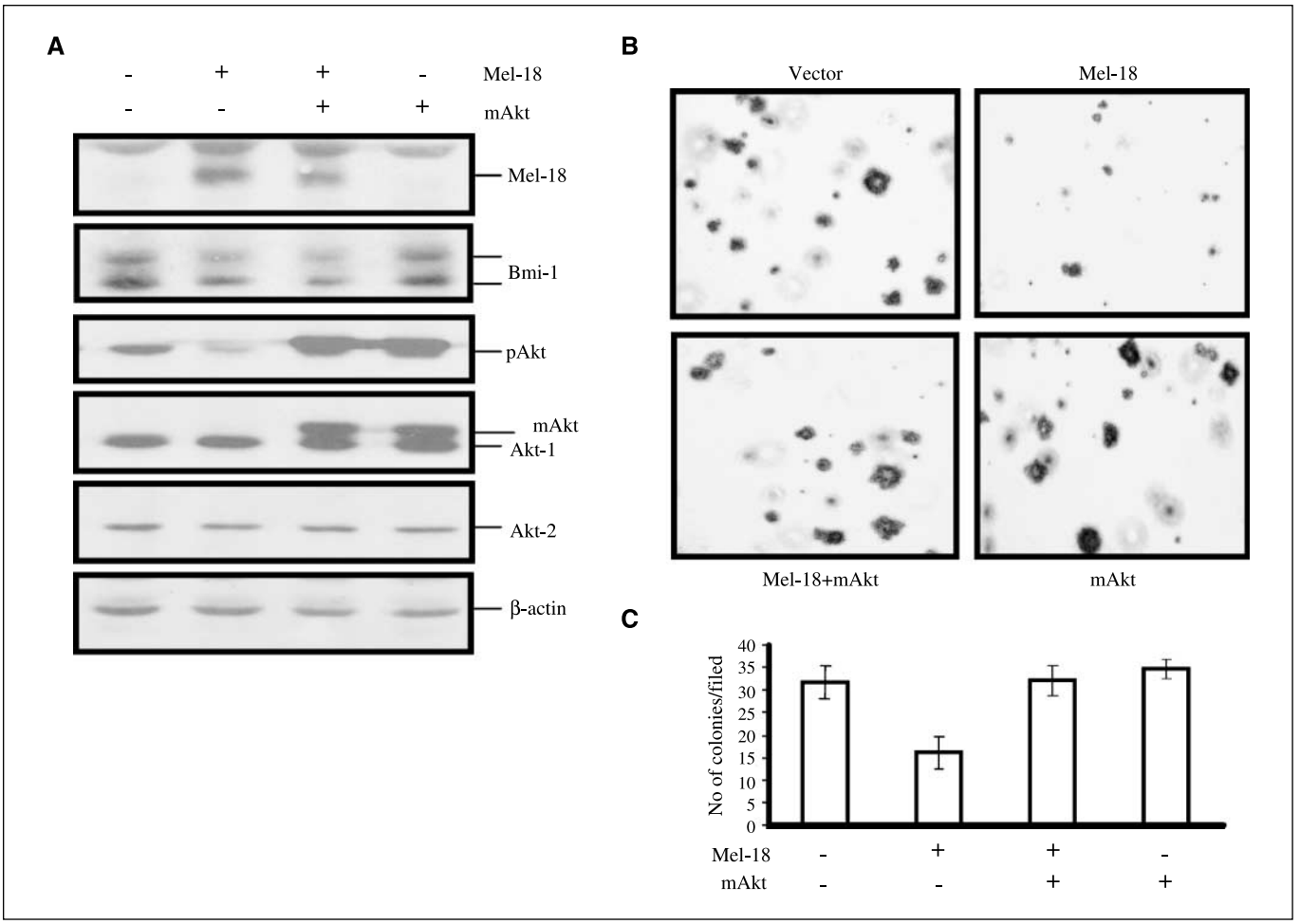

Figure 4. Exogenous overexpression of activated Akt (mAkt) restores anchorage-independent growth potential of Mel-18–overexpressing MCF7 cells. *A,* mAkt was stably expressed in Mel-18–overexpressing cells using a retroviral expression vector as described in Materials and Methods. Cell expressing Mel-18, mAkt, and Mel-18 together with mAkt were analyzed for expression of activated (phosphorylated) Akt by Western blot analysis. *B,* soft agar assay was done to determine anchorage-independent growth potential of MCF7-derived cells done as described in Materials and Methods. Representative photograph of colonies of control MCF7 (vector) and MCF7 derivatives (as indicated) growing in soft agar. *C,* colonies of control MCF7 and MCF7 expressing Mel-18, mAkt, or Mel-18 and mAkt (as indicated) growing in soft agar were counted and plotted from three different experiments.

Mel-18 and Bmi-1 regulate Akt activity in breast cancer cells. To determine the mechanism of inhibition of colony formation in soft agar and growth inhibition by Mel-18 overexpression or knockdown of Bmi-1 expression, we examined various growth regulators in these cells. Our results showed that Mel-18 overexpression did not affect p53 or its target p21 and pRb (Supplementary Fig. S2). Because Akt activity is constitutively high in many cancer cells, including breast cancer cells, we hypothesized that Mel-18 overexpression or Bmi-1 knockdown may reduce transforming phenotype via down-regulation of Akt pathway. To examine this possibility, we determined total Akt and pAkt by Western blot analysis. Our results showed that Bmi-1 down-regulation by Mel-18 overexpression or RNAi approach leads to substantial reduction in pAkt (Ser^{473} and Thr^{308}) in MCF7 cells, suggesting that Bmi-1 regulates Akt activity (Fig. 2*C*; Supplementary Fig. S2). Our results also showed that total Akt levels remained unaffected by inhibition of Bmi-1 expression.

To further confirm the down-regulation of Akt activity by Bmi-1 knockdown or Mel-18 overexpression, we determined the expression of downstream targets of Akt pathway. GSK3β is known to be phosphorylated at Ser^{9} and inactivated by activated Akt (16). Inactivation of GSK3β by Akt mediated phosphorylation at Ser^{9} also results in cyclin D1 up-regulation (16). Hence, we determined GSK3β and cyclin D1 expression in control, Mel-18–overexpressing cells, and Bmi-1 knockdown cells. Consistent with reduction of Akt activity, Western blot analysis of cells with reduced expression of Bmi-1 due to Mel-18 overexpression or Bmi-1 knockdown showed decreased levels of pGSK3β and down-regulation of cyclin D1 (Fig. 2*C, left* and Fig. 2*D*). In MCF7 cells, activation of Akt depends on the presence of estradiol (E2) in the serum, which can be removed by charcoal stripping. Using regular serum (contains E2) and charcoal-stripped serum (no E2), we confirmed that Mel-18 overexpression or Bmi-1 knockdown inhibits activation of Akt (Supplementary Fig. S3), which depends on the presence of E2 in serum.

We also confirmed regulation of Akt activity by Bmi-1 using overexpression studies (Fig. 2*C, right* and Fig. 2*D*). Consistent with Bmi-1 knockdown studies, Bmi-1 overexpression led to up-regulation of Akt activity as determined by Western blot analysis using pAkt and pGSK3β antibodies (Fig. 2*C, right* and Fig. 2*D*). To determine the mechanism of Akt regulation by Bmi-1, we used

PI3K inhibitors LY294002 and Wortmannin. Pretreatment of cells with these inhibitors strongly attenuated Akt activity in both control and Bmi-1–overexpressing cells (Supplementary Fig. S4), indicating that Bmi-1 regulates Akt activity via the PI3K pathway.

Exogenous Bmi-1 expression restores Akt activity and anchorage-independent growth in Mel-18–overexpressing MCF7 cells. Next, we examined whether exogenous expression of Bmi-1 using a retroviral promoter, which is not repressed by Mel-18, can restore Akt activity and full anchorage-independent growth in Mel-18–overexpressing MCF7 cells. The anchorage-independent growth of vector-infected control, Mel-18–overexpressing and Bmi-1–overexpressing MCF7 cells, and MCF7 cells expressing both Bmi-1 and Mel-18 was determined using soft-agar assays. The results (Fig. 3*A* and *B*) indicated that exogenous Bmi-1 could indeed restore anchorage-independent growth in Mel-18–overexpressing MCF7 cells. Western blot analysis of cells expressing both Mel-18 and Bmi-1 suggested that Bmi-1 could restore Akt activity in MCF7 cells (Fig. 3*C* and *D*).

Exogenously expressed mAkt restores full transformed phenotype in Mel-18 overexpressing MCF7 cells. To test the hypothesis that Mel-18 overexpression or Bmi-1 knockdown reduces the transformed phenotype of MCF7 cells by down-regulating Akt activity, we co-overexpressed activated Akt (mAkt) in MCF7 cells with Mel-18 or Bmi-1 shRNA. MCF7 cells were selected for co-overexpression using different antibiotic resistance markers and analyzed for the overexpression of mAkt. Western blot analysis indicated that overexpression of mAkt resulted in high pAkt proteins indicative of activated Akt (Fig. 4*A*; Supplementary Fig. S5*A*). Consistent with Akt acting downstream of Bmi-1, mAkt overexpression did not result in Bmi-1 up-regulation. Next, using soft agar assay, anchorage-independent growth potential of control cells and cells expressing Mel-18, mAkt, or both was examined. Results indicated that mAkt fully restores anchorage-independent growth of MCF7 cells expressing Mel-18 (Fig. 4*B* and *C*) or Bmi-1 shRNA (Supplementary Fig. S5*A*–*C*), without perturbing Bmi-1 expression Collectively, these data indicate that Mel-18 and Bmi-1 shRNA inhibit colony formation in MCF7 cells via down-regulation of Akt activity.

Discussion

Our cell culture data showing an inverse correlation between Bmi-1 and Mel-18 expression prompted us to examine if indeed this inverse correlation exists *in vivo* in breast tumors. Bmi-1 is overexpressed in invasive breast cancer; hence, we reasoned that in such breast tumors where Bmi-1 is highly expressed, Mel-18 expression might be low. Indeed, we found a strong negative correlation between Mel-18 and Bmi-1 expression in invasive breast cancer, which favored high Bmi-1 and low Mel-18 expression. A recent report did not find a negative correlation between Bmi-1 and Mel-18 expression in primary breast cancer samples (17). These authors also did not find negative correlation between Bmi-1 and p16/ARF expression, which has been shown in other cancers such as non–small-cell lung cancer (5) and colorectal cancer (6), and several *in vivo* and culture studies. At present, the reasons of discrepancy between the work published by Silva et al (17) and other studies (5, 6) and our data presented here is unclear. It may reflect tumor heterogeneity in the samples, different stages of tumor progression, and methods of detection and data analysis. All breast cancer samples used in our study were from late-stage invasive breast tumors, most of which had relatively undetectable to low Mel-18 expression compared with Bmi-1 expression as determined by immunohistochemistry. Based on these results, we suspect that this inverse correlation may persist with other cancer types. Analysis of Mel-18 and Bmi-1 coexpression in a large cohort of breast tumors and other cancers remains to be explored. Nonetheless, our studies suggest that Mel-18 is a physiologic regulator of Bmi-1 expression in breast epithelial cells.

It is interesting to note that Akt activity is up-regulated in a number of cancers including breast cancer (18, 19). Bmi-1 is thought to promote oncogenesis primarily by down-regulating the expression of the *p16Ink4a/ARF* locus (20). However, most breast cancer cells, including MCF7 cells that were used in this study, express very little, if any, p16, owing to p16 promoter methylation and/or deletion of the *Ink4a/ARF* locus. Our previous studies (13) and data presented here suggest that Bmi-1 can also promote oncogenesis via p16-independent mechanisms. In particular, Bmi-1 seems to regulate Akt activity in breast cancer cells and breast epithelial cells. Although the detailed mechanism of regulation of Akt activity by Bmi-1 remained to be elucidated, our PI3K inhibitor data and Akt phosphorylation studies suggest that Bmi-1 regulates Akt activity by up-regulating PI3K/3-phosphoinoisitide–dependent kinase-1 pathway. In conclusion, our studies suggest that polycomb proteins, in particular Bmi-1 and Mel-18, can regulate Akt activity in normal breast epithelial and breast cancer cells.

Acknowledgments

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Supplementary Table and Figures

Supplementary Table 1. Correlation between Bmi-1 and Mel-18 expression in invasive breast tumors. For relative comparison, + indicates that number of tumors that had a score of 4 or more, while – indicates the number of tumors that had a score of less than 4 as described in the main text (Materials and Methods). In absolute terms, - does not indicate that the tumor samples did not immunostain for the indicated protein.

		Bmi-1 expressing tumors		P value
		-	+	
Mel-18 expressing tumors	-	1	45	<0.0001
	+	9	6	

Supplementary Figure Legends

Fig. S1. Bmi-1 and Mel-18 immunostaining pattern in two different breast tumor samples in which normal and tumor tissues could be visualized side by side. Immunostaining was done as described in the main text (materials and methods).

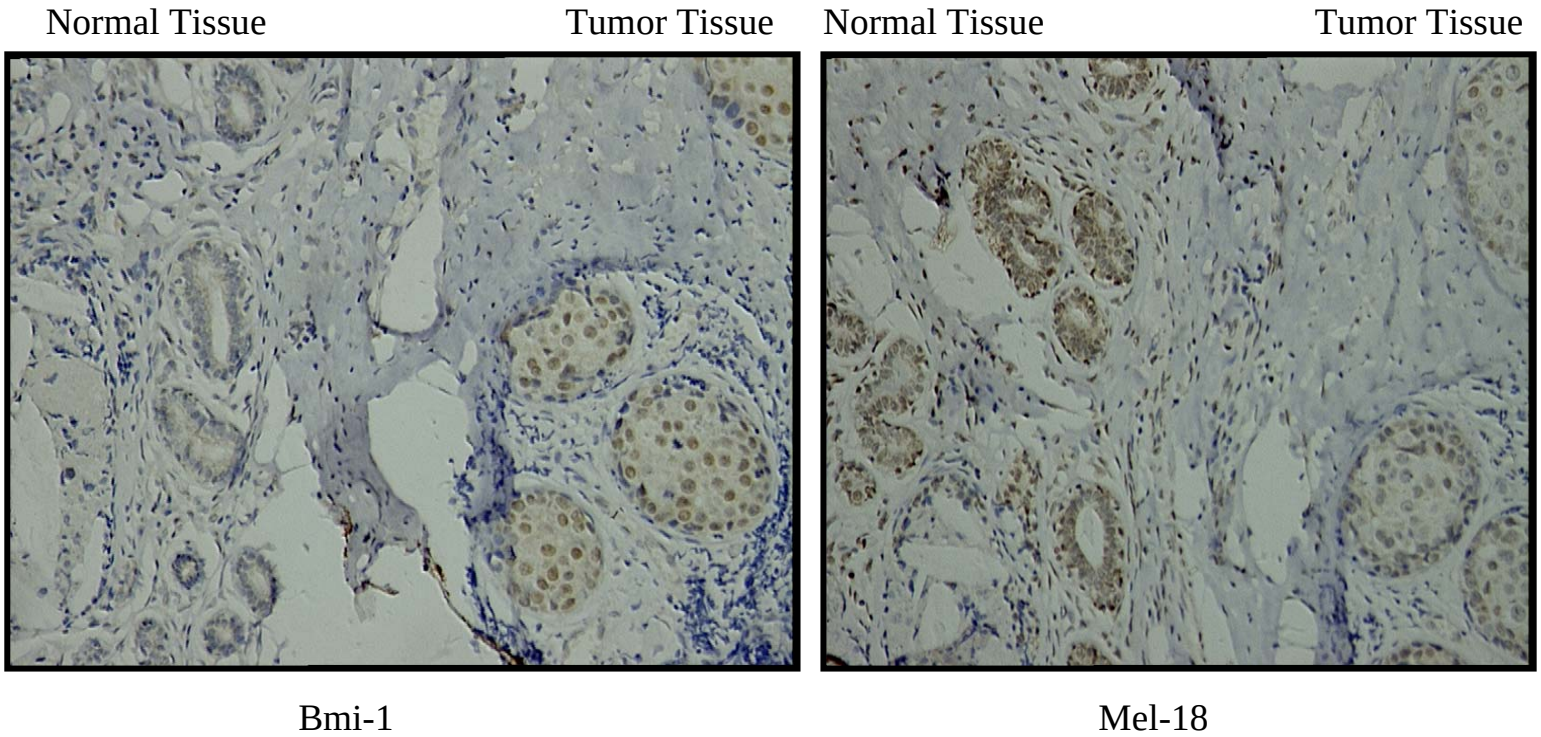
Fig. S2. Mel-18 overexpression or Bmi-1 knockdown using Bmi-1 shRNA (Bmi-1 i#2) does not result in altered expression of p53 and pRb. However, overexpression of wild type Mel-18 but not the ring finger mutant of Mel-18 results in downregulation of Bmi-1 and Akt activity. Bmi-1 knockdown also results in downregulation of Akt activity.

Fig. S3. Regulation of Akt activity by Mel-18 and Bmi-1 in MCF7 depends on estradiol (E2) present in fetal calf serum (FCS). Charcoal stripped serum does not induce Akt activity, while regular FCS induces Akt activity in control cells but the induction of activated Akt is attenuated in Mel-18 overexpressing and Bmi-1 knockdown cells. * indicate a non-specific band.

Fig. S4. Bmi-1 regulates Akt activity via activation of PI3K pathway. Vector control or Bmi-1 overexpressing MCF10A cells (as indicated) were treated with PI3K inhibitors (LY294002 and Wortmannin) and analyzed for Akt activation as described in the main text (materials and methods). Bmi-1 induces Akt only in vehicle (DMSO) treated cells. Results also indicate that PI3K inhibitors do not modulate Bmi-1 expression.

Fig. S5. Restoration of Akt activity by overexpressing mAkt overcomes inhibition of anchorage-independent growth by knockdown of Bmi-1 expression. mAkt was overexpressed using a retroviral vector in MCF7 cells expressing Bmi-1 shRNA (Bmi-1 i). Control cells (Ctrl i), and Bmi-1 i, Ctrl i + mAkt and Bmi-1 i + mAkt cells were analyzed for the expression of activated Akt by western blot analysis (Fig. S5*A*) and colony formation using soft agar assay (Fig. S5*B*, and S5*C*) as described in the main text and Fig. 4 legend.

Fig. S1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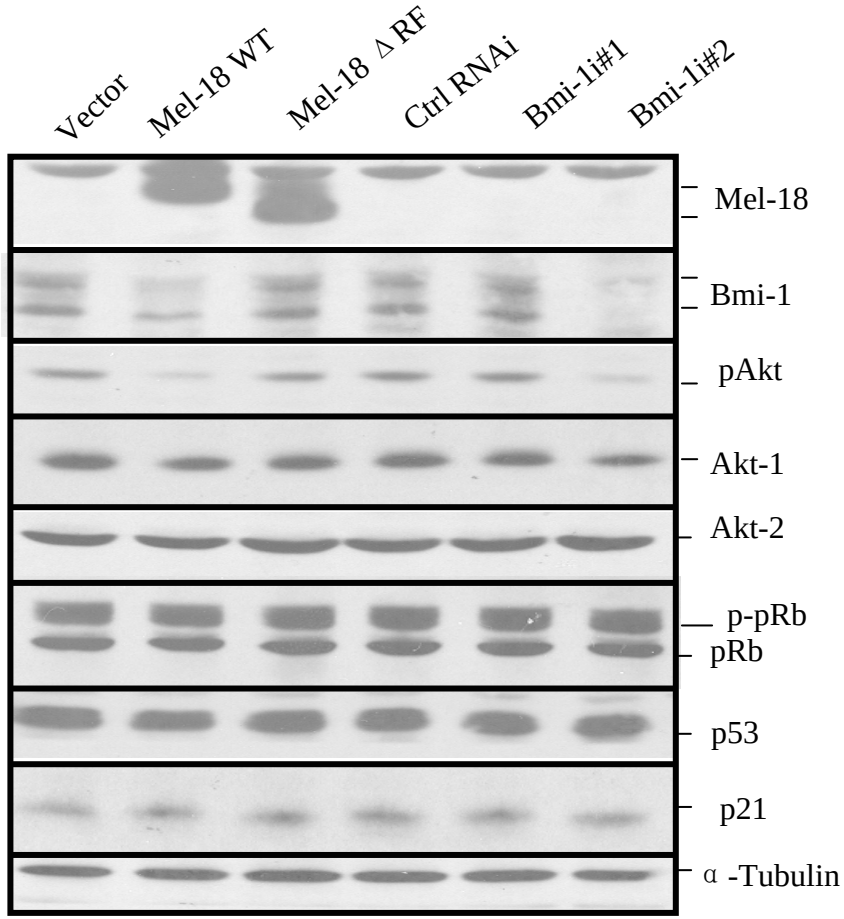
Normal Tissue
Tumor Tissue
Bmi-1
Mel-18

Fig. S2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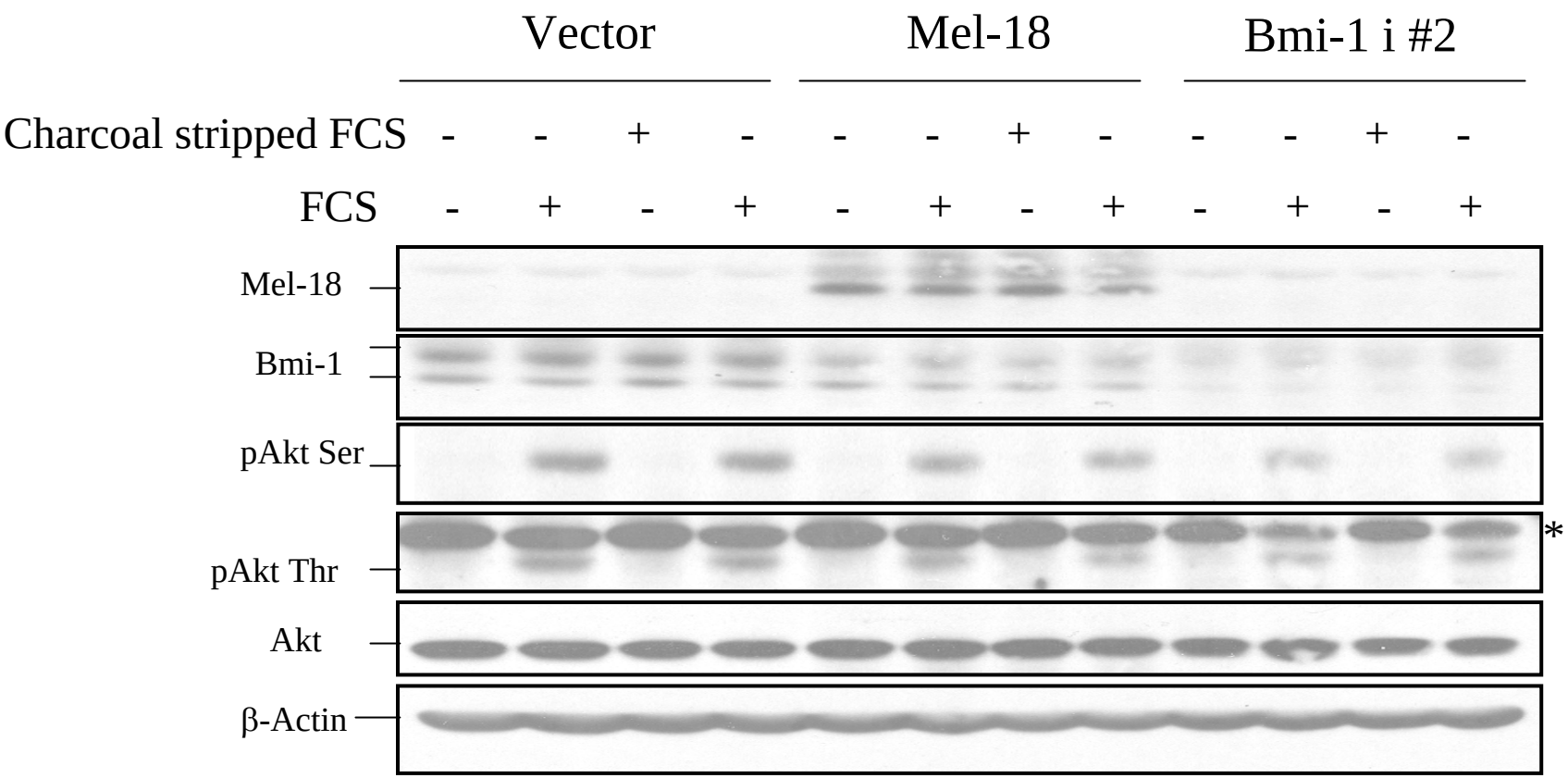
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Fig. S4

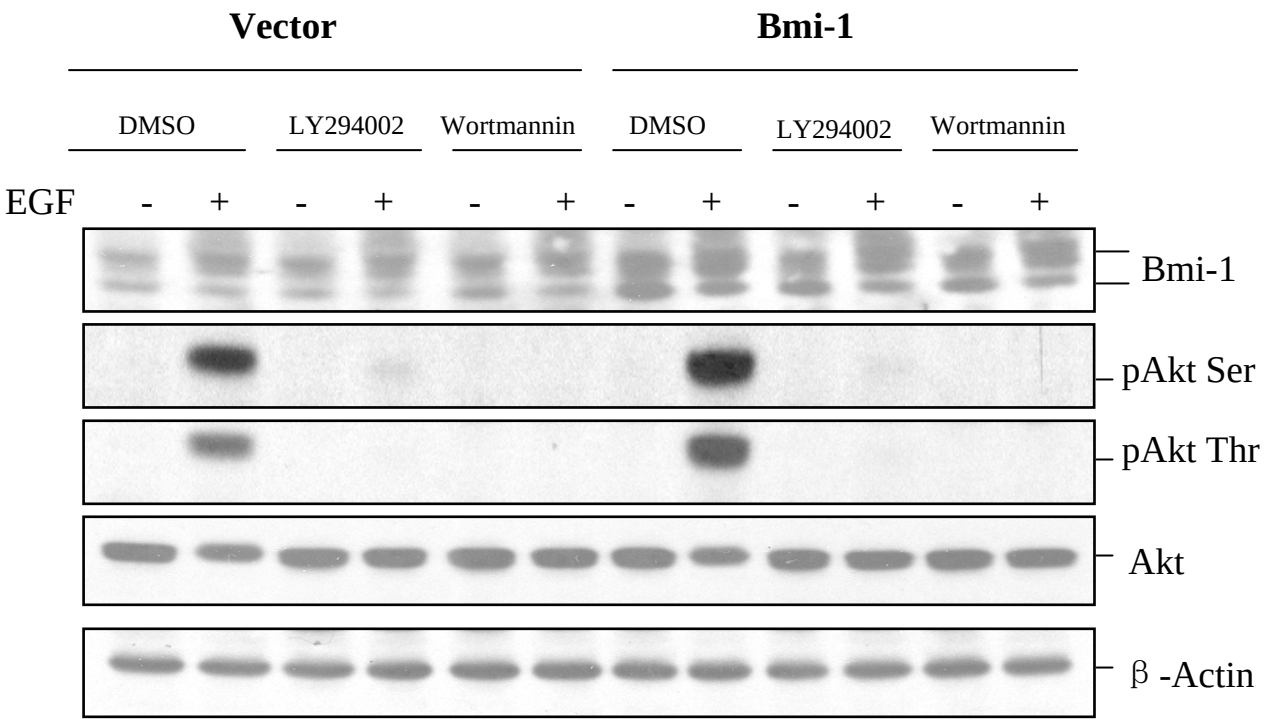
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Fig. S5

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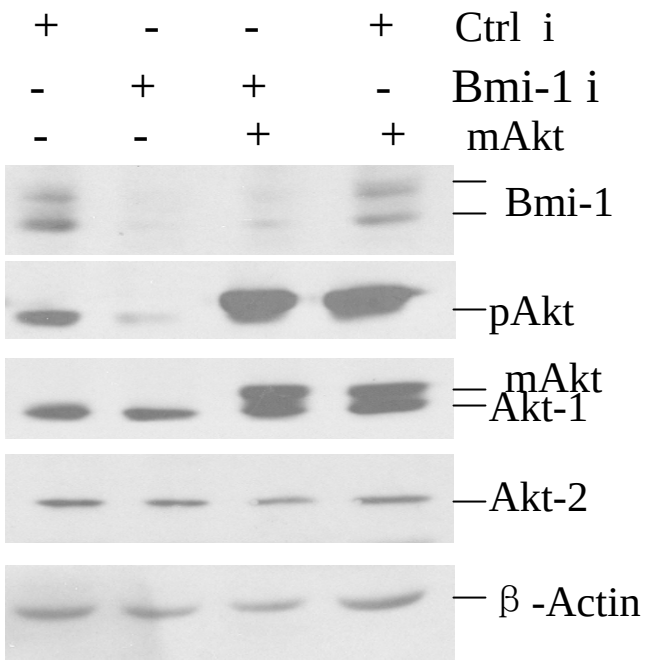
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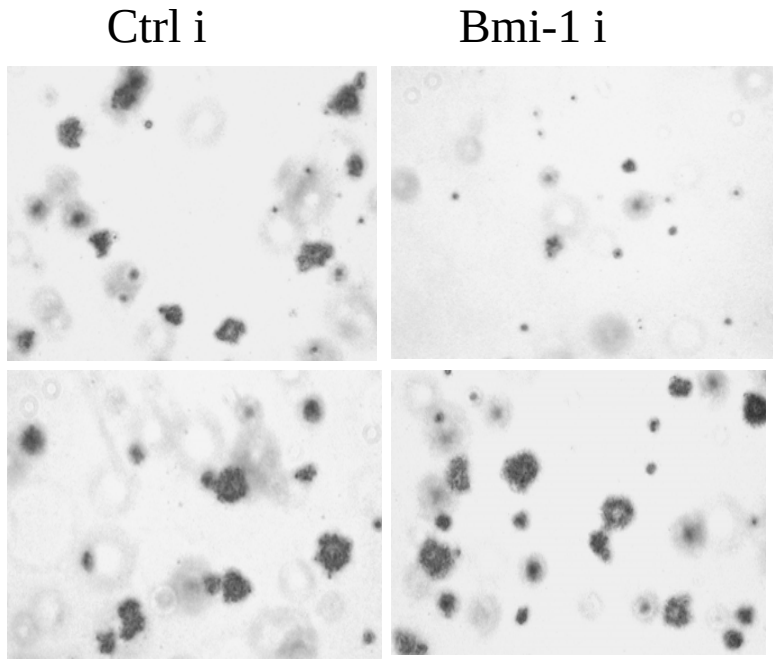
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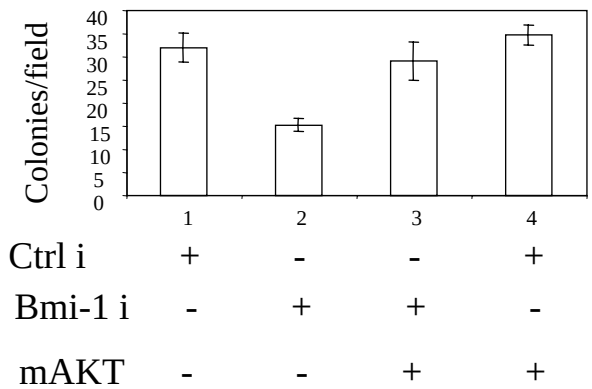
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Research Article

Bmi-1 Cooperates with H-Ras to Transform Human Mammary Epithelial Cells via Dysregulation of Multiple Growth-Regulatory Pathways

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Abstract

Elevated expression of Bmi-1 is associated with many cancers, including breast cancer. Here, we examined the oncogenic potential of Bmi-1 in MCF10A cells, a spontaneously immortalized, nontransformed strain of human mammary epithelial cells (HMEC). Bmi-1 overexpression alone in MCF10A cells did not result in oncogenic transformation. However, Bmi-1 co-overexpression with activated H-Ras (RasG12V) resulted in efficient transformation of MCF10A cells *in vitro*. Although early-passage H-Ras–expressing MCF10A cells were not transformed, late-passage H-Ras–expressing cells exhibited features of transformation *in vitro*. Early- and late-passage H-Ras–expressing cells also differed in levels of expression of H-Ras and Ki-67, a marker of proliferation. Subsets of early-passage H-Ras–expressing cells exhibited high Ras expression and were negative for Ki-67, whereas most late-passage H-Ras–expressing cells expressed low levels of Ras and were Ki-67 positive. Injection of late-passage H-Ras–expressing cells in severe combined immunodeficient mice formed carcinomas with leiomatous, hemangiomatous, and mast cell components; these tumors were quite distinct from those induced by late-passage cells co-overexpressing Bmi-1 and H-Ras, which formed poorly differentiated carcinomas with spindle cell features. Bmi-1 and H-Ras co-overexpression in MCF10A cells also induced features of epithelial-to-mesenchymal transition. Importantly, Bmi-1 inhibited senescence and permitted proliferation of cells expressing high levels of Ras. Examination of various growth-regulatory pathways suggested that Bmi-1 overexpression together with H-Ras promotes HMEC transformation and breast oncogenesis by deregulation of multiple growth-regulatory pathways by $p16^{INK4a}$-independent mechanisms. [Cancer Res 2007;67(21):1–10]

Introduction

Proteins of the polycomb group (PcG) play an important role as epigenetic gene silencers during development (1). In addition to their role in development, these proteins were recently reported to be overexpressed in various human cancers such as malignant lymphomas and various solid tumors (2). In particular, *Bmi-1* oncogene is overexpressed in a number of malignancies such as mantle cell lymphoma (3), B-cell non–Hodgkin's lymphoma (4), myeloid leukemia (5), non–small cell lung cancer (6), colorectal cancer (7), breast cancer (8), prostate cancer (9), and head and neck cancers (10, 11). Apart from its role in oncogenesis, Bmi-1 has been shown to be required for self-renewal of hematopoietic stem cells and neuronal stem cells (12–15). In addition, it was recently shown that Bmi-1 regulates self-renewal of normal and cancer stem cells in breast, and that modulation of Bmi-1 expression in mammosphere-initiating cells alters mammary development in a humanized nonobese diabetic–severe combined immunodeficient (SCID) mouse model (16, 17).

Recent studies using *in vivo* mouse and *in vitro* cell culture models have shown that Bmi-1 regulates the expression of *INK4A/ARF* locus, which encodes two important tumor suppressors $p16^{INK4A}$ and $p19^{ARF}$ ($p14^{ARF}$ in human; refs. 18, 19). By down-regulating $p16^{INK4A}$ and ARF, Bmi-1 can potentially regulate p16-pRb and p53-p21 pathways of senescence (20). Indeed, overexpression of Bmi-1 bypasses senescence in human and rodent fibroblasts, human mammary epithelial cells (HMEC), nasopharyngeal epithelial cells, and normal oral keratinocytes (11, 18, 19, 21, 22). Along these lines, we have recently reported that Bmi-1 down-regulation by another PcG protein Mel-18, and Bmi-1 knockdown using an RNA interference approach induces premature senescence via up-regulation of $p16^{INK4A}$ (23). Apart from regulating *INK4a/ARF* locus, Bmi-1 can also regulate cell proliferation and oncogenesis via INK4a/ARF–independent pathways. For example, Bmi-1 overexpression leads to immortalization of the 76N strain of HMECs via activation of telomerase (21). In addition, we recently reported that in normal human oral keratinocytes, and skin keratinocytes, Bmi-1 does not down-regulate $p16^{INK4A}$, suggesting the possible role of other unidentified targets of Bmi-1 that are involved in cell proliferation (10, 24).

Our recent data suggests that independent of its effect on $p16^{INK4A}$, Bmi-1 regulates AKT activity in MCF10A and MCF7 cells (25). It is thought that the precursor cells for breast cancer are $p16^{INK4A}$ negative due to promoter methylation and silencing (26), suggesting that overexpression of Bmi-1 in $p16^{INK4A}$-negative tumors may contribute to oncogenesis via 16^{INK4A}-independent mechanisms. Here, we examined the oncogenic potential of Bmi-1 in an immortal but untransformed HMEC line MCF10A, which does not express $p16^{INK4A}$, $p14^{ARF}$, and $p15^{INK4B}$ (27, 28). Using *in vitro* cell culture and *in vivo* mouse model, we show that overexpression of Bmi-1 alone is not sufficient for oncogenic transformation of immortal Hems. However, the combined

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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overexpression of the G12V mutant of H-Ras and Bmi-1 was able to transform HMECs in culture as determined by transformation assays. Furthermore, orthotopic injection of cells co-overexpressing Bmi-1 and activated H-Ras resulted in the formation of poorly differentiated and invasive tumors in SCID mice.

Materials and Methods

Cells, cell culture, expression vectors, retrovirus production, and infection of HMECs. MCF10A and MCF10A-derived cell lines were cultured as described (21). A retroviral vector overexpressing Bmi-1 has been described earlier (21, 23). A retroviral vector pMSCV-Ras expressing H-Ras G12V mutant was constructed by subcloning cDNA of H-Ras from pcDNA3.1 obtained from UMR cDNA Resource Center (University of Missouri, Rolla, MO). Stable cell lines expressing gene(s) of interest were generated by infection of the retroviral vector(s) expressing a particular gene and selecting cells in either puromycin, G418, or hygromycin as described (21, 23). Q3

Antibodies, Western blot analysis, immunostaining, Matrigel, soft agar, and wound-healing assays. Bmi-1 was detected using either F6 mouse monoclonal antibody (mAb) from Upstate Cell Signaling Solutions, or 1H6B10G7 mAb from Zymed. Among other antibodies, phosphorylated AKT 1/2/3 (Ser-473), AKT-1 (B-1) and AKT-2 (F-7), CDK4 (C-22), cyclin D1 (A-12), H-Ras (F-235), p21 (F-5), p53 (DO-1), p53-Ser-15, PUMA (FL-193), Bax (6A7), extracellular signal-regulated kinase (ERK; C-16), phosphorylated ERK (E-4), p38-regulated/activated protein kinase (PRAK; A-7), and QM (C-17) antibodies were obtained from Santa Cruz Biotechnology. p53-Ser-37 rabbit polyclonal antibody was obtained from Cell Signaling Technology. Vimentin, fibronectin, and E-cadherin mAbs were obtained from BD Transduction Laboratories. β-Actin and α-smooth muscle actin (α-SMA) mAbs were obtained from Sigma-Aldrich. α-Tubulin mAb was obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). For Ki-67 and H-Ras co-immunostaining, Alexa Fluor 488–conjugated Ki-67 (BD Biosciences) and a Ras mAb (BD Biosciences) were used. Q4

Oncogene-induced senescence (OIS) was determined using staining for senescence-associated β-galactosidase marker as described (29). To determine the AKT and ERK activity in synchronized cells, MCF10A cells were growth factor deprived using D3 medium (30) for 48 h and stimulated for 40 min by addition of D medium, which contains 12.5 ng/mL epidermal growth factor (EGF; 30). Western blot analyses of total cell extracts using antibodies that detect various proteins were done as described (21, 23). Immunostaining for epithelial-to-mesenchymal transition (EMT) markers, such as E-cadherin, fibronectin, and vimentin, and soft agar, Matrigel, and wound-healing assays were done as described (25, 31).

Mice injections, necropsy, histopathology, histochemistry, and immunohistochemistry. For mammary fat pad injection experiments, four cohorts of 10 SCID mice each were used. Each cohort was injected in the right axillary mammary fat pad with 1×10^6 cells from each cell line. Tumor growth was measured weekly by caliper, and mice were euthanized by CO_2 asphyxiation once tumors reached 2 cm in diameter, or until mice became clinically ill. All animal work was done following NIH guidelines under an approved animal protocol. At necropsy examination, tumor tissue, brain, lung, heart, liver, spleen, and kidney were collected and fixed in 4% paraformaldehyde and routinely processed into paraffin blocks from which 4-μm sections were cut and stained with H&E, Masson's trichrome, and Giemsa. For immunohistochemical analysis, after deparaffinization, rehydration, antigen retrieval, and quenching of endogenous peroxidase activity, polyclonal and monoclonal primary antibodies were applied. Negative controls were obtained by substitution of the primary antibody with buffer solution.

Results

Bmi-1 overexpression does not lead to transformation of HMECs. To define the role of Bmi-1 in breast cancer progression, we overexpressed Bmi-1 in MCF10A, a nontumorigenic but immortal HMEC cell line (Fig. 1*A*). Next, we examined the oncogenic potential of MCF10A cells overexpressing Bmi-1. Consistent with recent observation that four or more oncogenic events are required for the *in vitro* transformation of HMECs (32), Bmi-1–overexpressing MCF10A cells did not form colonies in soft agar, indicating that Bmi-1 is insufficient to cause transformation of immortal $p16^{INK4A}$-negative HMECs. Similar results were obtained using Bmi-1–immortalized 76N HMECs (Supplementary Fig. S1), which also do not express $p16^{INK4A}$ (21). F1 SF1

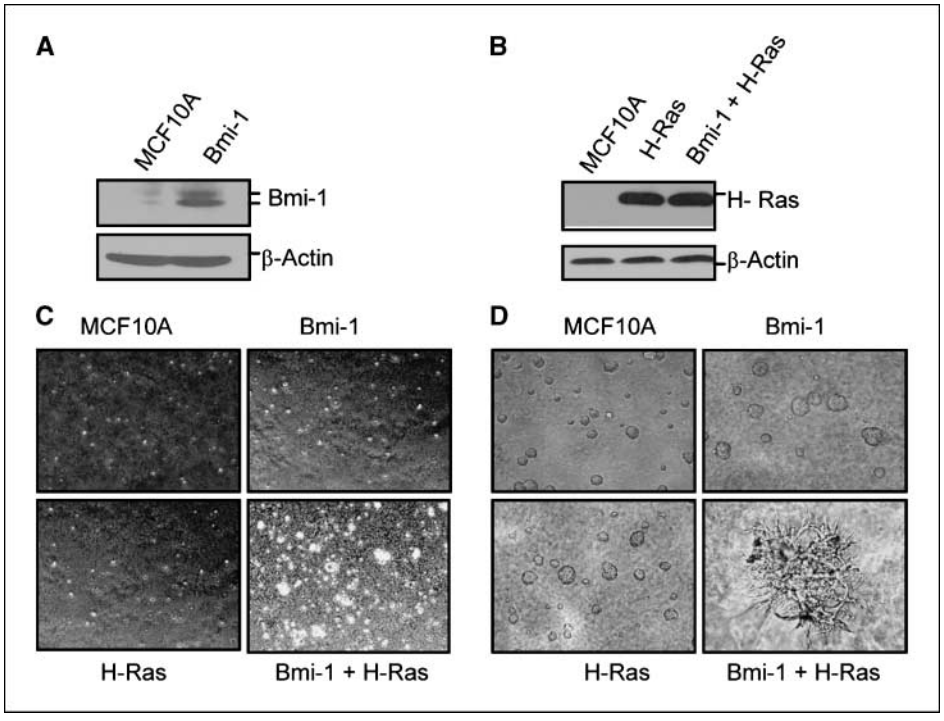

Figure 1. Bmi-1 and H-Ras co-overexpression transforms HMECs. *A,* Bmi-1–overexpressing MCF10A cells were generated by stable overexpression of Bmi-1, and cells (as indicated) were analyzed for Bmi-1 overexpression by Western blot analysis. *B,* H-Ras was introduced in control MCF10A and MCF10A–Bmi-1 cells, and cells were analyzed for H-Ras expression by Western blot analysis. Cells after Ras selection were considered at passage 1. *C,* MCF10A and MCF10A cells expressing H-Ras alone, Bmi-1 alone, or Bmi-1 together with H-Ras (as indicated) at passage 2 (after Ras selection) were analyzed under light microscope for anchorage-independent growth using soft agar assays, and photographed (×4). *D,* MCF10A and MCF10A-derived cells (as indicated) at passage 2 were analyzed for acini formation using Matrigel assays and photographed (×6).

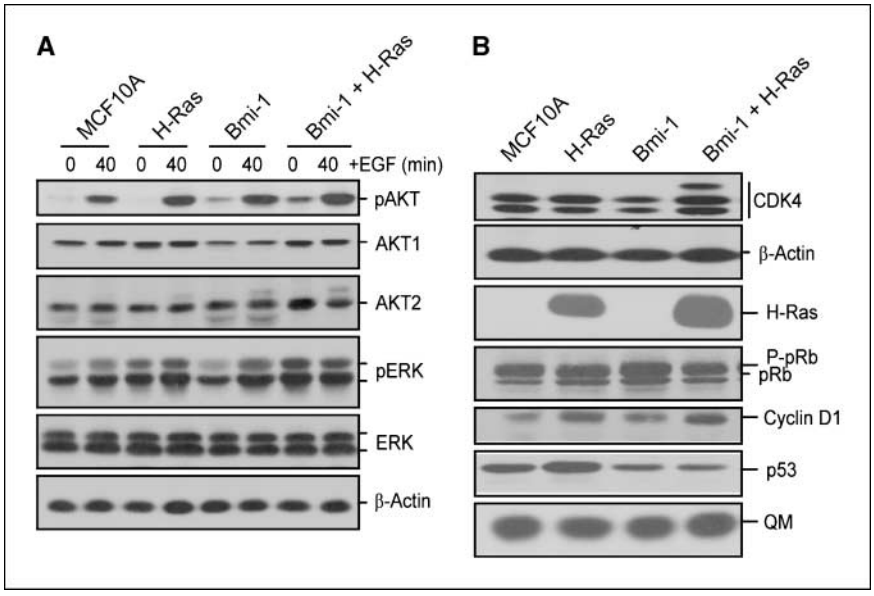

Figure 2. Various growth-regulatory pathways are dysregulated in cells co-overexpressing Bmi-1 and H-Ras. All cells were analyzed at passage 2 after Ras selection and/or mock infection. *A,* Western blot analysis of phosphorylated AKT, total AKT (AKT1 and AKT2), phosphorylated ERK, and total ERK in control MCF10A and MCF10A-derived cells (as indicated). Western blot analysis using β-actin served as a loading control. *B,* Western blot analysis of p53, pRb, CDK4, and cyclin D1 in asynchronously growing MCF10A and MCF10A-derived cells (as indicated). β-Actin and QM are loading controls.

Overexpression of H-Ras together with Bmi-1 transforms MCF10A cells via deregulation of multiple growth-regulatory pathways. Next, we overexpressed a constitutively active mutant G12V of H-Ras (33) in control MCF10A and Bmi-1–overexpressing MCF10A cells (Fig. 1*B*). The pool populations of cells expressing H-Ras (MCF10A–H-Ras), Bmi-1 (MCF10A–Bmi-1), or both Bmi-1 and H-Ras (MCF10A–Bmi-1+H-Ras) were studied for transformed phenotype using soft agar and Matrigel assays (Fig. 1*C* and *D*). The soft agar assay indicated that cells expressing either Bmi-1 or H-Ras alone did not exhibit anchorage-independent growth. However, cells co-overexpressing both Bmi-1 and H-Ras readily formed colonies in soft agar (Fig. 1*C*). Bmi-1 and H-Ras co-overexpression in 76N cells also led to colony formation in soft agar (Supplementary Fig. S1). To further confirm the *in vitro* transformation potential of MCF10A-derived cells, Bmi-1, H-Ras, and Bmi-1+H-Ras–expressing cells were seeded in Matrigel. The results indicated that control MCF10A, MCF10A–Bmi-1, and MCF10A–H-Ras cells formed normal spherical acini, whereas MCF10A–Bmi-1+H-Ras cells formed large irregular branched structures indicative of transformed phenotype of seeded cells (Fig. 1*D*).

To determine the mechanism of Bmi-1– and H-Ras–induced transformation of HMECS, we analyzed MCF10A and MCF10A-derived cells for the expression of Ras effectors such as AKT and ERK kinases. The results indicated that control MCF10A and MCF10A-Ras cells had very little or no basal phosphorylated AKT (pAKT) expression, whereas MCF10A–Bmi-1 and MCF10A–Bmi-1+H-Ras cells expressed significant amount of activated AKT (pAKT) even under EGF-starved conditions (Fig. 2*A*). AKT activity was induced in all cells after EGF addition; however, the induction of AKT activity was more noticeable in Bmi-1+H-Ras–expressing cells. On the other hand, ERK activity was constitutively high in H-Ras and Bmi-1+H-Ras–expressing cells regardless of EGF (Fig. 2*A*). These results suggest that Bmi-1 and H-Ras could transform HMECs by activating AKT and ERK kinases. F2

Next, we determined the expression of cyclin D1 and CDK4, as the overexpression of these two cell cycle–regulatory proteins has been linked to breast cancer progression (34, 35). Our results indicated that compared with control cells, Bmi-1 or H-Ras overexpression up-regulated cyclin D1, whereas Bmi-1 and H-Ras co-overexpression up-regulated CDK4 as well as cyclin D1 expression in MCF10A cells (Fig. 2*B*). We also determined the expression of pRb and p53 tumor suppressors in control and MCF10A-derived cells. Because MCF10A cells are $p16^{INK4A}$ negative and contained high hyperphosphorylated pRb, no significant differences were found between different forms of pRb in control and MCF10A-derived cells (Fig. 2*B*). On examining p53 expression, we found that MCF10A–H-Ras cells contained slightly higher p53 protein levels, whereas MCF10A–Bmi-1 and MCF10A–Bmi-1+H-Ras cells showed down-regulation of p53 (Fig. 2*B*). Collectively, our data indicate that Bmi-1 together with H-Ras overexpression leads to activation of ERK and AKT, up-regulation of cyclin D1 and CDK4 expression, and down-regulation of p53.

H-Ras–expressing late-passage HMEC cells exhibit a transformed phenotype. It has been reported in the literature that in some instances, H-Ras overexpression alone can lead to transformation of MCF10A cells, whereas other reports suggest the opposite (36–40). In our case, the H-Ras–expressing early-passage (EP) cells were clearly not transformed. These early-passage cultures of cells were also heterogeneous and exhibited mixed morphologies with some enlarged senescent cells and some small normal proliferating cells. The late-passage (LP; more than five passages) culture of H-Ras expressing cells, on the other hand, exhibited more uniform morphology with most cells proliferating. We considered whether these late-passage cells have undergone selection for rapidly proliferating cells and that during this selection may have acquired transformed properties.

To probe this hypothesis, MCF10A–Bmi-1+H-Ras (LP) and MCF10A–H-Ras (LP) cells were plated on soft agar and allowed to form colonies for 10 to 14 days. The results indicated that similar to Bmi-1 and H-Ras co-overexpressing cells, MCF10A–H-Ras (LP) cells formed colonies in soft agar, indicating that H-Ras (LP) cells have also undergone transformation (Fig. 3*A*). However, MCF10A–Bmi-1 (LP) cells still did not make colonies in soft agar, indicating that Bmi-1 expression alone is not sufficient to cause transformation even after extensive passaging of cells in culture. The transformed phenotype of H-Ras (LP) cells was also confirmed by Matrigel assay, which indicated that H-Ras (LP) and H-Ras+Bmi-1 (LP) cells form highly disorganized, branched, and sieve-like structures (Fig. 3*B*). F3

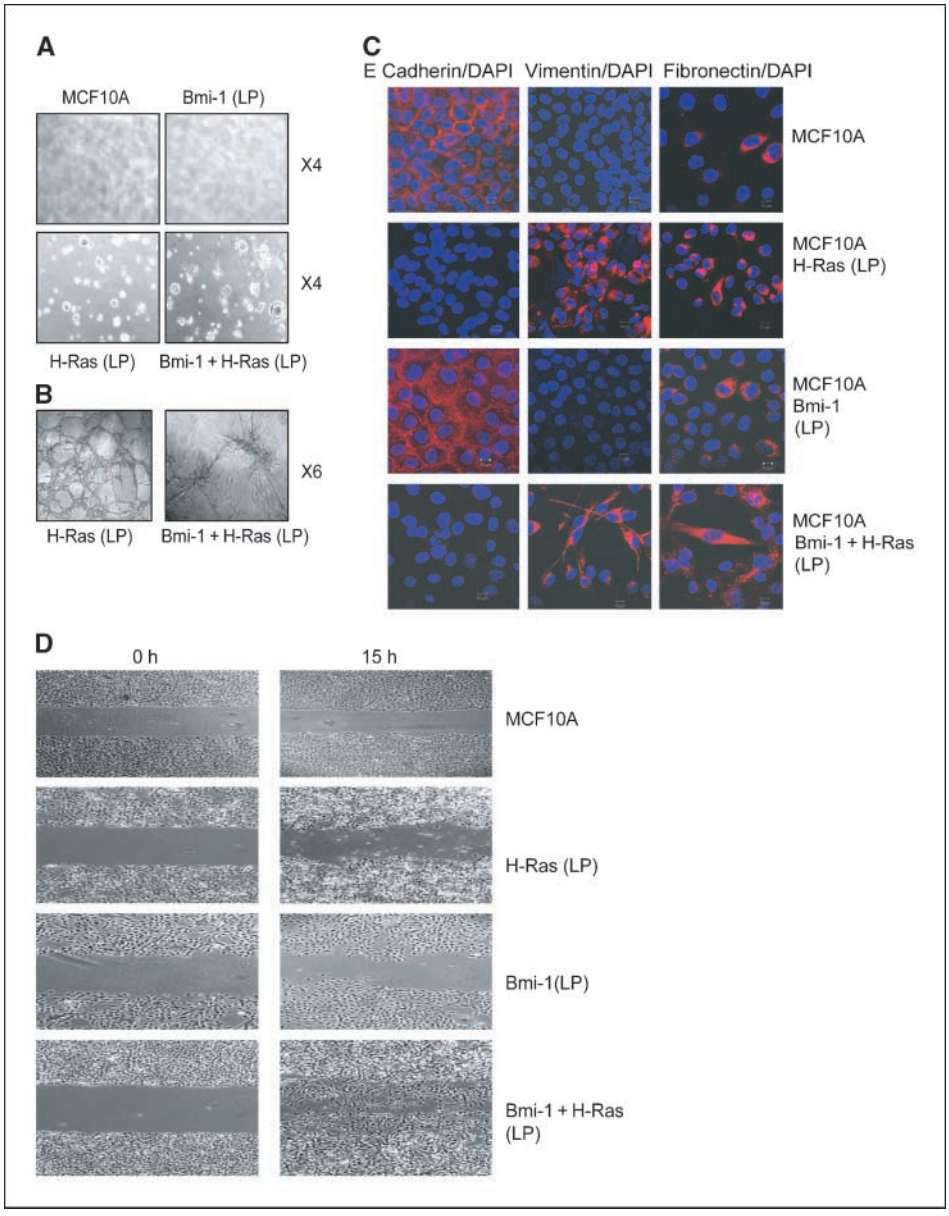

Figure 3. Late-passage H-Ras–expressing MCF10A cells exhibit transformed features. All MCF10A-derived cells were analyzed at passage 8. *A,* control MCF10A and MCF10A-derived late-passage cells (as indicated) were grown in soft agar to determine anchorage-independent growth potential of these cells. Cells were photographed (×4) at day 14. *B,* three-dimensional growth of MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) was analyzed using Matrigel assays as described in Materials and Methods. Cells in Matrigel were photographed (×10) at day 7. *C,* EMT phenotype of MCF10A and MCF10A-derived late-passage cells was analyzed by immunostaining using antibodies specific for E-cadherin, vimentin, and fibronectin (as indicated). To visualize nuclei, cells were stained with 4′,6-diamidino-2-phenylindole, and immunostained cells were visualized and photographed using Zeiss LSM510 UV META confocal microscope (×60). *D,* the migration potential of MCF10A and MCF10A-derived cells was determined by wound-healing assay. The control MCF10A, and Bmi-1–, H-Ras–, and Bmi-1+H-Ras–overexpressing MCF10A cells were grown to 80% confluence, starved in D3 medium for 48 h. A wound was made in the middle of culture dish containing near-confluent cells and the cells were stimulated with EGF-containing D medium for 15 h. Cells were photographed at 0 h, before adding D medium and at 15 h, after stimulating with D medium. Cells were photographed using a light microscope (×4).

Bmi-1 expression together with H-Ras induces EMT in HMECs. When examining the morphology of MCF10A-derived cells, we noticed that cells expressing both H-Ras and Bmi-1 exhibited fibroblastic morphology suggestive of EMT phenotype. To confirm this, we examined these cells for the presence of EMT markers by immunostaining (Fig. 3*D*). The results indicated that control MCF10A and MCF10A–Bmi-1 (LP) cells expressed E-cadherin, a cell junction protein characteristic of epithelial cells, whereas MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) lost the expression of E-cadherin. On the other hand, MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) cells expressed fibroblastic markers such as vimentin and fibronectin (Fig. 3*C*). Similar results were obtained using Western blot analysis (Supplementary Fig. S2). These data indicate that Bmi-1 and H-Ras co-overexpression induces a strong EMT phenotype.

As Bmi-1+H-Ras–expressing cells exhibited EMT phenotype, which is closely linked to migration and invasion, we did a wound-healing assay to determine the migratory potential of these cells. The results indicated that MCF10A–Bmi-1+H-Ras cells have the highest migration potential and that these cells filled the wound quickly compared with other cells (Fig. 3*D*). H-Ras–expressing MCF10A cells also showed a moderate migratory potential (Fig. 3*D*). These cells tend to undergo cell death during migration. Control MCF10A cells showed no migration, whereas Bmi-1–expressing cells only exhibited a minimal migration (Fig. 3*D*). Thus, our data suggest that Bmi-1 and H-Ras co-overexpressing cells have acquired migration and invasion potential typical of highly transformed HMECs.

Expression level of H-Ras determines proliferation in H-Ras–expressing MCF10A cells. The differential ability of MCF10A–H-Ras (EP) and MCF10A–H-Ras (LP) cells to undergo transformation could be related to the different levels of Ras, which in turn may determine the proliferation in these cells. To examine this possibility, we determined expression of H-Ras by Western blot analysis in control MCF10A, and MCF10A-derived early- and late-passage cells, and did Ras and Ki-67 coimmunostaining in these

F4 cells (Fig. 4). The Western blot analysis of control, early-, and late-passage cells indicated that H-Ras (EP) cells expressed a high level of Ras, whereas H-Ras (LP) cells expressed a low level of Ras (Fig. 4*A*). On the other hand, Bmi-1+H-Ras (LP) cells expressed a high level of Ras (Fig. 4*A* and *B*). Bmi-1+H-Ras (EP) cells and H-Ras (EP) cells expressed similar levels of Ras (Fig. 4*A* and *B*). Because early-passage cultures are heterogeneous with cells expressing variable levels of Ras, it is possible that cells expressing Ras above a certain threshold level are not proliferating. At increasing number of population doublings, there may be selection for cells expressing a lower level of Ras, which permits continued proliferation. Accordingly, H-Ras (LP) cells will have low expression of Ras. Consistent with this hypothesis, on a single-cell basis, we observed that in H-Ras (EP) cultures, most cells with high Ras stained negative for Ki-67, a proliferation marker, whereas cells with low Ras stained positive for Ki-67 (Fig. 4*C* and *D*). On the other hand, H-Ras (LP) culture mostly contained cells with low Ras, which stained positive with Ki-67 (Fig. 4*C* and *D*). The percentage of low Ras–expressing cells, which were Ki-67 positive, was also high in MCF10A–Bmi-1+H-Ras (EP) culture, although some cells in this culture also expressed high Ras, which were positive for Ki-67 (Fig. 4*C* and *D*). Importantly, most Bmi-1+H-Ras (LP) cells expressed high Ras and stained positive for Ki-67, indicating that Bmi-1 permits proliferation of these cells despite high Ras (Fig. 4*C* and *D*). In all cultures, variable percentages of low Ras–expressing cells were Ki-67 negative. Because of growth asynchrony in culture, such cells may not be proliferating at the time of staining.

MCF10A cells expressing H-Ras, and Bmi-1+H-Ras form histologically distinct tumors *in vivo*. To address the contributory role of Bmi-1 on tumor progression, MCF10A, MCF10A–Bmi-1 (LP), MCF10A–H-Ras (LP), and MCF10A–Bmi-1+H-Ras (LP) cells were injected into the mammary fat pad. As expected, MCF10A control cells did not produce tumors *in vivo*. Injection of MCF10A+Bmi-1 cells also did not result in tumor formation even after 60 days, indicating that overexpression of Bmi-1 alone is not sufficient for neoplastic transformation of HMECs *in vivo*. In contrast, MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) cells produced progressively enlarging tumors in the mammary fat pad. Grossly, these tumors were strikingly different (Fig. 5*A*); F5 MCF10A–Bmi-1+H-Ras (LP) cells formed tumors that were solid,

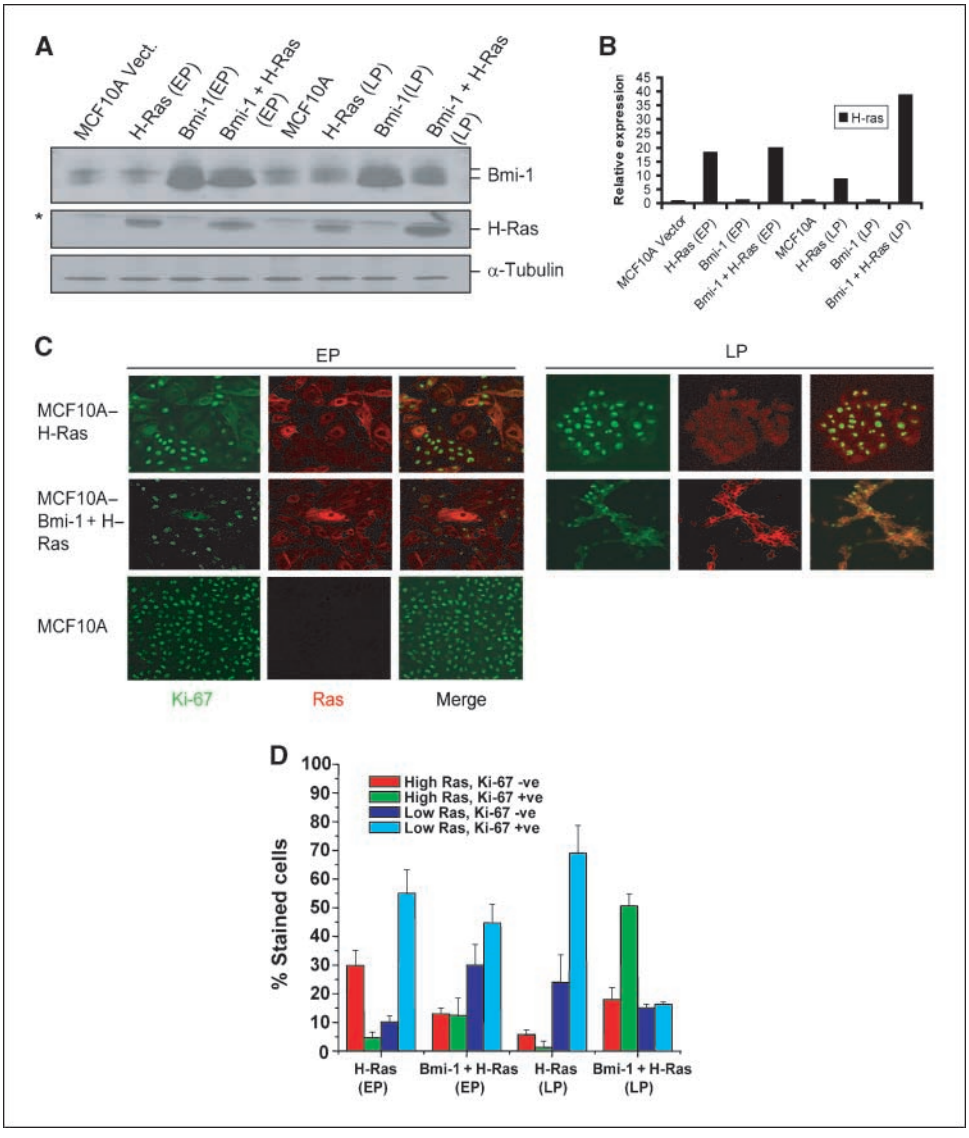

Figure 4. Expression level of H-Ras determines proliferation in MCF10A cells overexpressing H-Ras. *A,* H-Ras expression in MCF10A control and MCF10A-derived early-passage (passage 2 after Ras selection) and late-passage cells (passage 8) was determined by Western blot analysis as described in Materials and Methods. *B,* to determine the relative expression of H-Ras in MCF10A and MCF10A-derived cells, its signal in each lane was quantified by densitometric analysis using ImageJ1.3 software (NIH) and normalized to α-tubulin signal. *C,* H-Ras and Ki-67 coimmunostaining was done to determine proliferation in MCF10A-derived early-passage (passage 2) and late-passage (passage 8) cells. MCF10A cells were used as control, which do not express detectable Ras but are Ki-67 positive under our experimental conditions. Representative photos (×60) of costaining in each cell line (as indicated). *D,* quantification of Ras- and Ki-67–expressing cells in MCF10A-derived early-passage (passage 2) and late-passage (passage 8) culture of H-Ras and Bmi-1+H-Ras cells. Costaining was done in triplicates and a total of 100 to 200 stained cells were counted in multiple fields.

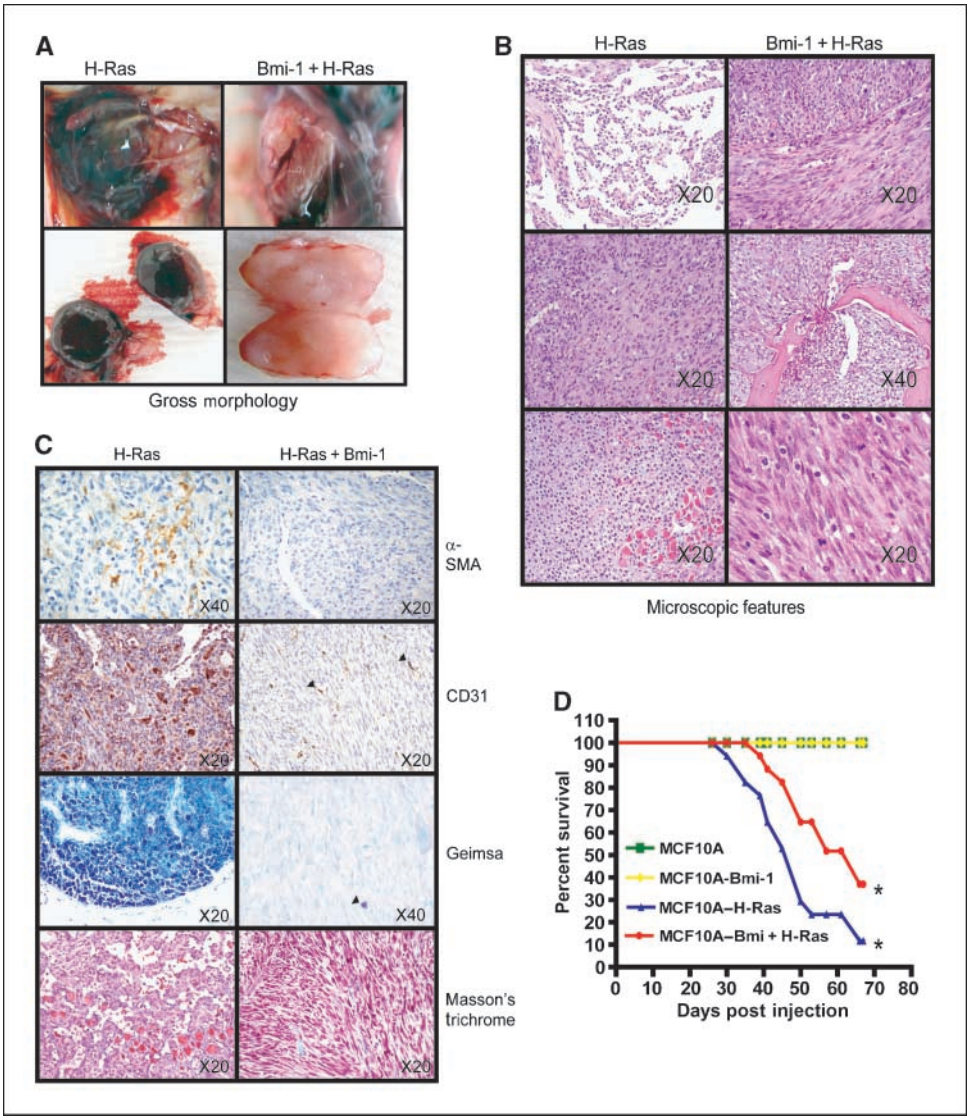

Figure 5. Gross morphology, histopathology, and immunohistochemistry of tumors originating from xenografts. *A,* gross morphology of tumors resulting from injection of MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras cells (LP; as indicated). *Left,* tumors induced by MCF10A–H-Ras. *Right,* tumors induced by MCF10A–Bmi-1+H-ras cells. *B,* histopathology of tumors resulting from injection of MCF10A–H-Ras (*left*) and MCF10A–Bmi-1+H-Ras (*right*) cells. *Left,* tumors induced by MCF10A–H-Ras cells were composed of variable populations of poorly differentiated to well-differentiated endothelial cells forming haphazard vascular channels, spindle-shaped cells resembling smooth muscle (middle), and multiple variable-sized clusters of poorly differentiated to well-differentiated mast cells (*bottom*). *Right,* tumors induced by MCF10A–Bmi-1+H-Ras cells were composed of a homogeneous population of sheets and intersecting bundles of poorly differentiated spindle cells (*top*) that infiltrated adjacent adipose tissue and bone (*middle*). Cells were poorly differentiated with large pleomorphic nuclei and frequent mitoses (*bottom*). *C,* histochemical and immunohistochemical staining of tumors induced by MCF10A–H-Ras (*left*) and MCF10A–Bmi-1+H-Ras (*right*) cells. *Left,* tumors induced by MCF10A–H-Ras were multifocally immunoreactive for antibodies against α-SMA and CD31; mast cell clusters were diffusely positive with Giemsa staining for mast cell granules, and tumors were diffusely negative for collagen by Masson's trichrome staining. *Right,* tumors induced by MCF10A–H-Ras+Bmi-1 cells were diffusely negative for α-SMA and CD31 except for the presence of intratumoral capillaries (*arrowheads*), diffusely negative with Giemsa staining except for occasional resident mast cells (*arrowhead*), and showed very little collagen production with Masson's trichrome stain. *D,* Kaplan-Meier survival curve. Whereas MCF10A and MCF10A–Bmi-1 xenografted mice did not develop tumors and survived throughout the course of the study, mice xenografted with MCF10A–H-Ras and MCF10A–Bmi-1+H-Ras had decreased survival after the development of palpable tumors. MCF10A–H-Ras xenografted mice had significantly decreased survival compared with MCF10A–Bmi-1+H-Ras mice ($P < 0.002$).

firm, and irregular, whitish-tan on cut surface with well-differentiated vasculature. In contrast, tumors formed by MCF10A–H-Ras (LP) cells were variably hemorrhagic and often cystic, composed predominantly of large thin cysts filled with clotted and/or unclotted blood (Fig. 5*A*).

Histologically, MCF10A–H-Ras tumors consisted of variable populations of poorly to fairly well-differentiated smooth muscle, variably cystic irregular vascular spaces lined by poorly to fairly well-differentiated endothelial cells, and multifocal clusters and nests of poorly to well-differentiated mast cells (Fig. 5*B*). In contrast, MCF10A–Bmi-1+H-Ras tumors were composed of streams and bundles of poorly differentiated spindle-shaped cells with scant, faintly eosinophilic fibrillar cytoplasm embedded in scant eosinophilic stroma, large round to oval hyperchromatic nuclei with multiple prominent nucleoli, and numerous mitotic figures (~2–3/hpf; Fig. 5*B*). These cells often infiltrated into the surrounding fat pad, effacing normal ducts and adipose tissue, and in one case infiltrating and destroying the cortical bone of a subjacent rib and invading and effacing the bone marrow (Fig. 5*B*).

MCF10A–H-Ras tumors were multifocally immunoreactive to antibodies to α-SMA and CD31 (PECAM), illustrating the smooth muscle and hemangiomatous components of these tumors (Fig. 5*C*). Giemsa staining for mast cell granules confirmed the multifocal mast cell clusters of varying differentiation in the MCF10A–H-Ras tumors, whereas Masson's trichrome staining showed no collagen production in these tumors (Fig. 5*C*). MCF10A–Bmi-1+H-Ras tumors were diffusely negative for α-SMA and CD31 except for preexisting intratumoral capillaries, supplying the tumors that were immunoreactive to CD31 (Fig. 5*C*). Giemsa staining confirmed the absence of mast cells in these tumors except for a rare mature resident mast cell, and Masson's trichrome staining confirmed that these tumors are composed of spindle cells with scant collagen production, more suggestive of a myogenic phenotype than a fibrosarcomatous one (Fig. 5*C*). Both MCF10A–H-Ras and MCF10A–Bmi-1+H-Ras tumors were diffusely immunoreactive to antibodies to cytokeratin and vimentin (Supplementary Fig. S3). Animals with tumors formed by MCF10A–H-Ras cells were often very hemorrhagic, resulting in early morbidity due to anemia rather than tumor burden in contrast to mice bearing tumors formed by MCF10A–H-Ras+Bmi-1 cells, which as a group lived longer with tumors than MCF10A–H-Ras tumor-bearing mice (Fig. 5*D*).

SF3

MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) cells display a partially defective p53 phosphorylation and attenuated induction of p53 target genes in response to DNA damage. H-Ras is known to cause OIS in primary cells, which is mediated by $p16^{INK4A}$ and p53 (41–43). Using senescence-associated β-galactosidase marker, we noticed senescence induction in a significant number (40–50%) of MCF10A cells by H-Ras overexpression at early passages (Supplementary Fig. S4*A*). Because MCF10A cells are $p16^{IKN4A}$ negative, the partial OIS in these cells may depend on p53 and its target genes. Consistent with partial OIS, early-passage MCF10A-Ras cells also showed slower growth compared with vector control MCF10A and MCF10A cells co-overexpressing H-Ras and Bmi-1 (Supplementary Fig. S2*B*).

SF4

The senescent cells in MCF10A–H-Ras and MCF10A–Bmi-1+H-Ras cells were progressively lost, and rapidly proliferating cells were selected in later passages. We hypothesized that the selection of rapidly proliferating cells in late-passage cultures of MCF10A–H-Ras and MCF10A–Bmi-1+H-Ras cells may depend on a defect in p53 pathway in these cells. To examine this hypothesis, we determined p53 expression in control MCF10A, MCF10A–Bmi-1 (LP), MCF10A–H-Ras (LP), and MCF10A–Bmi-1+H-Ras (LP) cells. The results indicated that unlike in MCF10A–H-Ras (EP) cells (Fig. 2*B*), p53 was down-regulated in MCF10A–H-Ras (LP) cells (Fig. 6*A*). To determine the mechanism of p53 down-regulation and its possible significance with respect to transformed phenotype of MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) cells, we further studied p53 pathway in these cells.

F6

MCF10A control and MCF10A-derived late-passage cells were treated with the DNA-damaging agent camptothecin (500 nmol/L) for the indicated amount of time, and expression of p53, phosphorylated p53, and p53 target genes was studied by Western blot analysis (Fig. 6*B*). The results indicated that although MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) cells had overall low p53 compared with control MCF10A and

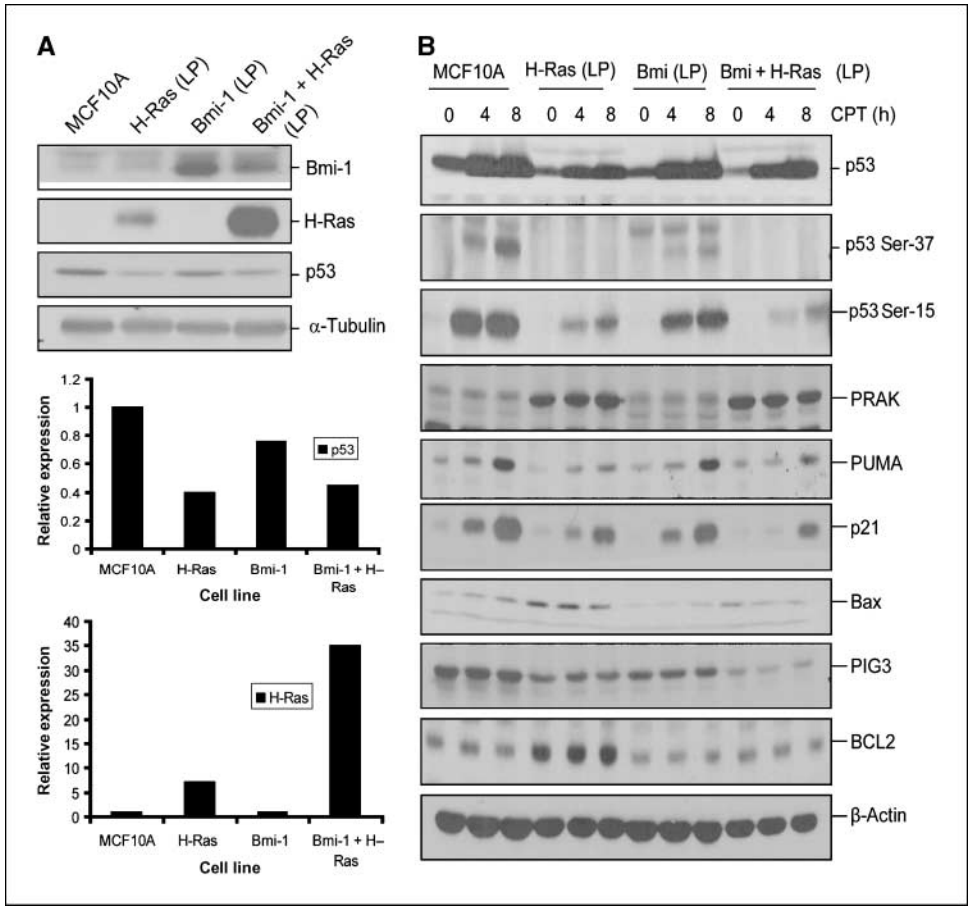

Figure 6. Analysis of p53 pathway in control MCF10A and MCF10A-derived late-passage cells. All cells except parental MCF10A cells, passage 9 cells, were used for the analysis. *A, top,* Western blot analysis of Bmi-1, H-Ras, and p53 in control MCF10A and MCF10A-derived (LP) cells (as indicated) was done as described in Fig. 2. *Bottom,* densitometric analysis of signals (of p53 and H-Ras) present in each lane was done, normalized to corresponding α-tubulin signal, and plotted to determine the expression levels of p53 and H-Ras as indicated. *B,* analysis of DNA damage response in MCF10A and MCF10A-derived late-passage cells. The cells were treated with camptothecin (*CPT*) for indicated amount of time, harvested, and analyzed by Western blot analysis for total p53, phosphorylated p53 (Ser-15 and Ser-37), p53 target genes (*p21, PUMA, Bax*, and *PIG3*), PRAK, and BCL2. β-Actin was used as a loading control.

MCF10A–Bmi-1 cells, p53 remained inducible by camptothecin in all four set of cells, although the induced levels of p53 was still low in MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) cells (Fig. 6*B*; Supplementary Fig. S5). Further analysis of phosphorylated p53 indicated that MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) cells were partially defective in phosphorylation of p53 at Ser-15 and Ser-37 residues (Fig. 6*B*; Supplementary Fig. S5). Quantification of Western blot data showed reduced phosphorylation of p53 at Ser-15 in both MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) cells at 4 and 8 h time points, whereas the basal levels of p53 Ser-15 were similar in all MCF10A-derived cells (Supplementary Fig. S5). Ser-37 phosphorylation was also compromised in MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras (LP) cells. Neither of these cell lines showed any induction of Ser-37 phosphorylation of p53 by camptothecin treatment (Supplementary Fig. S5).

SF5

Because it has been reported that PRAK mediates Ser-37 phosphorylation of p53 induced by H-Ras and that PRAK mediates Ras-induced OIS (42), we hypothesized that PRAK may be lost during selection of rapidly proliferating cells in H-Ras (LP) cells in culture. To examine this possibility, we determined PRAK expression in these cells by Western blot analysis. The results indicated that regardless of DNA damage, PRAK expression is not lost in control or H-Ras (LP) cells (Fig. 6*B*). Interestingly, PRAK expression was up-regulated in H-Ras (LP) cells (Fig. 6*B*). The up-regulation of PRAK is consistent with the notion that PRAK is an H-Ras target, which acts negatively to suppress H-Ras–induced proliferation (44). Nonetheless, it seems that this PRAK-mediated negative feedback regulation of H-Ras–mediated proliferation is lost in MCF10A–H-Ras (LP) cells, which may have allowed these cells to undergo transformation in culture.

Next, we studied the induction of p21 and PUMA (p53 up-regulated modulator of apoptosis), two well-known transcriptional targets of p53 (45, 46). Our results indicated that both p21 and PUMA induction by camptothecin is partially compromised in MCF10A–H-Ras (LP) cells (Fig. 6*B*; Supplementary Fig. S5), and p21 induction was more compromised in MCF10A–Bmi-1+H-Ras (LP) cells. Attenuated response of these targets of p53 is consistent with defective phosphorylation at Ser-15 and Ser-37 residues. We also examined expression of *Bax* and *PIG3* (p53-inducible gene 3), two other known targets of p53 (45). Analysis of these two genes indicated that *Bax* is expressed at very low levels and is inducible in control MCF10A cells. However, MCF10A–H-Ras (LP) cells had higher levels of Bax, which were not inducible by DNA damage (Fig. 6*B*; Supplementary Fig. S5). Interestingly, among all four cell types, MCF10A–H-Ras (LP) cells expressed high BCL2, which may be related to transformed properties of these cells. *PIG3*, which usually has a delayed kinetics of induction by p53 (47), was not inducible in any of the cell types within the time frame used in our experiment (Fig. 6*B*). Interestingly, compared with control MCF10A cells, MCF10A-derived (LP) cells showed significant down-regulation of *PIG3* (Fig. 6*B*).

Discussion

Several recent studies have suggested that PcG proteins, in particular EZH2 and Bmi-1, are overexpressed in human cancers. Recent elegant studies have clearly shown that oncogenic transformation of human cells is a multistep process (48). It is very likely that overexpression of a single PcG protein alone is not sufficient to cause transformation of human cells. To gain an insight into breast cancer progression, here we examined the transformation potential of Bmi-1 oncoprotein in immortalized HMECs. Although immortalized HMECs that we studied lack $p16^{INK4A}$, Bmi-1 expression still provides an oncogenic signal in these cells by the activation of phosphoinositide 3-kinase (PI3K)-AKT pathway (25). However, the oncogenic signal provided by Bmi-1 alone does not seem to be sufficient to cause transformation of HMECs, despite these cells being immortal and lacking $p16^{INK4A}$, $p14^{ARF}$, and $p15^{INK4B}$ (27). This observation underscores the stringency of transformation in HMECs. Nonetheless, Bmi-1 overexpression is frequently observed in invasive breast tumors (8, 9, 25), suggesting the involvement of additional oncogenic events during breast cancer progression in such tumors.

To understand the genetic basis of these presumptive additional oncogenic events, we overexpressed a constitutively active mutant G12V of H-Ras (33) in Bmi-1–overexpressing MCF10A cells. G12V mutant of H-Ras promotes proliferation and oncogenesis via activation of mitogen-activated protein kinase (MAPK) kinase (MEK)/MAPK and the PI3K/AKT pathways. However, the activation of these pathways and their outcome is cell-type specific. For example, in primary cells, activation of these pathways lead to induction of OIS, whereas in immortalized cells with compromised p53-p21 and/or $p16^{INK4A}$ pathways, H-Ras G12V promotes proliferation. Our reasoning behind using H-Ras G12V in these assays was based on its relevance to breast cancer and its reported use in oncogenic assays (32). Although the direct mutational activation of H-Ras is rare in breast cancer, its hyperactivation by persistent growth factor signaling caused by EGF receptor and HER2/neu overexpression occurs in a proportion of breast cancers (49, 50).

OIS caused by G12V mutant of H-Ras may require both functional $p16^{INK4A}$ and p53. In MCF10A cells, which have functional p53, we initially noticed the appearance of a heterogeneous culture with ~40% to 50% cells exhibiting senescent morphology upon H-Ras overexpression. Consistent with partial OIS, our Western blot data also indicated up-regulation of p53 protein in these cells. Senescence acts as a strong barrier to oncogenesis (20); hence, the initial OIS in a proportion of MCF10A cells by H-Ras indicates an antioncogenic response. As expected, these early-passage cells were not transformed by soft agar and Matrigel assays. However, late-passage culture, which were much more homogenous and did not contain cells with senescent morphology, displayed transformed phenotype in Matrigel and soft agar assays. Ras and Ki-67 costaining data also suggest that early-passage culture of MCF10A–H-Ras are more heterogeneous in terms of Ras expression, whereas the late-passage culture of these cells are homogenous in terms of Ras expression. Importantly, only low Ras–expressing cells tend to be Ki-67 positive, suggesting that low Ras permits proliferation, whereas high Ras blocks proliferation, possibly via OIS. This differential effect of Ras on proliferation explains the emergence of low Ras–expressing culture at late passages.

The H-Ras overexpression in Bmi-1–overexpressing MCF10A cells caused senescence only in a minority of cells and homogenous culture with proliferating cells appeared much more rapidly from MCF10A–Bmi-1+H-Ras cultures. These data indicate that to some extent, Bmi-1 can overcome H-Ras–induced OIS, even in $p16^{INK4A}$-negative cells, presumably via p16INK4a/ARF–independent targets of Bmi-1. The homogenous culture that rapidly emerged from Bmi-1+H-Ras–expressing cells continued to express high Ras. Most cells in this culture were Ki-67 positive despite expressing high Ras,

suggesting that Bmi-1 permits proliferation of cells despite high Ras, and thus there is no selection for cells expressing low Ras. The biochemical basis for proliferation of MCF10A–Bmi-1+H-Ras (LP) cells despite high Ras remains to be elucidated.

On examination of Ser-37 and Ser-15 phosphorylation of p53 in response to DNA damage, we found that Ser-37 phosphorylation of p53 is significantly low and not inducible in both late-passage H-Ras and Bmi-1+H-Ras–expressing cells. In addition, these cells also had much lower induction of Ser-15 phosphorylated p53, suggesting a possible defect in other p53-activating kinases such as ATM. A detailed analysis of various p53 phosphorylating kinases in late-passage MCF10A–H-Ras and MCF10A–Bmi-1+H-Ras remains to be elucidated. Nevertheless, our data clearly indicate that these late-passage H-Ras– and Bmi-1+H-Ras–expressing cells have defects in p53 phosphorylating pathways, which results in attenuation of induction of p53 targets such as p21 and PUMA. This compromised induction of p53 targets may contribute to a transformed phenotype of MCF10A cells expressing Bmi-1 and H-Ras.

The differential behavior of early- and late-passage H-Ras–overexpressing MCF10A cells with respect to the transformed phenotype explains the different results that are reported in the literature (36–40). Our data suggest that in cases where H-Ras–expressing MCF10A cells showed a transformed phenotype and gave rise to tumors in nude mice assays, late-passage H-Ras–expressing cells with defective p53 regulation may have been used. In other studies, where transformation of H-Ras–expressing MCF10A cells was not reported, early-passage H-Ras–expressing MCF10A cells may have been used. Alternatively, the transforming potential of H-Ras cells could be correlated with the level of expression of H-Ras. In studies where H-Ras alone was reported to be transforming, the expression of H-Ras may be low, which permits proliferation. On the other hand, in cases where Ras was reported to be insufficient for transformation, the expression of Ras may be very high, which causes proliferation arrest and OIS. Neither of these possibilities is mutually exclusive and both possibilities are likely to contribute to transformation of HMECs by H-Ras. Recently, it was shown that low levels of K-Ras induce proliferation and mammary epithelial cell hyperplasias, whereas high expression of K-Ras induces proliferation arrest and OIS in doxycycline-inducible *K-Ras* transgenic mice (51). In this report, it was also shown that inactivation of p53 permits transformation of mammary epithelial cells and tumor formation by high expression of Ras (51). Our *in vitro* data are consistent with this report.

The results of histopathology, including special stains and immunohistochemistry, confirm that the MCF10A+H-Ras tumors are composed of multiple different populations of varying phenotypes (smooth muscle, hemangiomatous, and mast cells), suggesting that these populations may be in part an *in vivo* response to the xenografted tumor population rather than original components of the neoplastic population that have undergone dedifferentiation and redifferentiation along multiple lines. The MCF10A–Bmi-1+H-Ras tumors, on the other hand, represent a pure population of highly atypical, poorly differentiated, and infiltrative spindle cells consistent with a mesenchymal phenotype. Although the α-SMA immunohistochemistry was negative in these tumors, Masson's trichrome stain along with positive immunohistochemistry for vimentin would suggest that these cells may represent a myoepithelial phenotype consistent with EMT.

Although MCF10A–Bmi-1+H-Ras (LP) and MCF10A–H-Ras (LP) cells give rise to histologically distinct type of tumors, biochemically these cells show only minor differences in regulation of growth-regulatory pathways. The only significant difference between these two cell lines is that H-Ras (LP) cells expressed higher levels of BCL2, which may contribute to the oncogenicity of these cells. In any case, we did not observe tumor formation by MCF10A–Bmi-1 cells, suggesting the involvement of additional oncogenic events such as down-regulation of p53, overexpression of CDK4 and cyclin D1, and up-regulation of AKT and ERK activities in the transformation of HMECs and breast cancer progression. Our data also indicate that Bmi-1 may cooperate with Ras in transformation by simply allowing high Ras–expressing cells to proliferate. The additional oncogenic events then may be largely contributed by H-Ras in the experiments described here. It remains to be determined which of these oncogenic lesions, together with Bmi-1, are sufficient to transform HMECs and form tumors *in vivo*.

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Mel-18, a Polycomb Group Protein, Regulates Cell Proliferation and Senescence via Transcriptional Repression of Bmi-1 and c-Myc Oncoproteins[D]

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Polycomb group (PcG) protein Bmi-1 is an important regulator of cell proliferation. It regulates cellular senescence and proliferation of cells via the transcriptional repression of INK4a/ARF locus and other target genes. Here, we report that Mel-18, a PcG ring finger protein (PCGF) transcriptionally down-regulates Bmi-1. Furthermore, the expression of Bmi-1 and Mel-18 inversely correlates in proliferating and senescent human fibroblasts. Bmi-1 down-regulation by Mel-18 results in accelerated senescence and shortening of the replicative life span in normal human cells. Importantly, using promoter-reporter, chromatin immunoprecipitation, and quantitative real-time primary transcript RT-PCR assays, and an RNA interference approach, we demonstrate that Bmi-1 is a bona fide target of c-Myc oncoprotein. Finally, our data suggest that Mel-18 regulates Bmi-1 expression during senescence via down-regulation of c-Myc. These studies link c-Myc and polycomb function in cell proliferation and senescence.

INTRODUCTION

After a finite number of cell divisions, most normal human cells undergo cellular senescence, whereby cells cease to divide (reviewed in Campisi, 2005; Dimri, 2005). Cellular senescence constitutes a tumor suppressor mechanism (Campisi, 2005; Dimri, 2005), and bypass of senescence is required for tumorigenesis (Dimri, 2005). It is regulated by an array of growth regulators including polycomb group (PcG) proteins (reviewed in Itahana *et al.*, 2004). PcG proteins are chromatin-modifying proteins, which play an important role in development (reviewed in Ringrose and Paro, 2004). Besides their role in development, these proteins also regulate cell proliferation, senescence, and tumorigenesis (reviewed in Valk-Lingbeek *et al.*, 2004; Gil *et al.*, 2005). In particular, EZH2 and Bmi-1 overexpression has been linked to invasive breast and prostate cancers (Varambally *et al.*, 2002; Kleer *et al.*, 2003; Kim *et al.*, 2004; Glinsky *et al.*, 2005). In addition to its role in oncogenesis, recent work from several laboratories indicates that Bmi-1 is required for self-renewal of hematopoietic stem cells (HSCs) and neural stem cells in murine models (Lessard and Sauvageau, 2003; Molofsky *et al.*, 2003; Park *et al.*, 2003; Iwama *et al.*, 2004). Bmi-1 is also involved in the maintenance and proliferation of breast stem cells (Liu *et al.*, 2006).

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The exact role of PcG proteins in tumorigenesis is still unclear. However, some of the polycomb proteins, such as Bmi-1 and EZH2, are known to regulate senescence and proliferation via well-known growth regulatory pathways (Jacobs *et al.*, 1999; Bracken *et al.*, 2003; Itahana *et al.*, 2003). For example, Bmi-1 negatively regulates INK4a/ARF locus (Jacobs *et al.*, 1999), which may impact both p16-pRb and ARF-p53-p21 pathways of cellular senescence (Dimri, 2005). Indeed, Bmi-1 has been shown to regulate cellular senescence in murine and human cells (Jacobs *et al.*, 1999; Itahana *et al.*, 2003). Bmi-1 is also thought to prevent premature senescence of neural stem cells by repressing INK4a/ARF locus (Bruggeman *et al.*, 2005; Molofsky *et al.*, 2005). Premature senescence of cells may contribute to organismic aging (Campisi, 2005). If so, the regulators of senescence are likely to play a role in aging. Indeed, down-regulation of Bmi-1 by the disruption of the SNF2-like gene PASG was shown to result in growth retardation and premature aging in a murine model (Sun *et al.*, 2004).

Bmi-1 is a particularly interesting oncoprotein; it not only regulates the INK4a/ARF locus, but can also immortalize human mammary epithelial cells (HMECs) (Dimri *et al.*, 2002). We recently reported that Bmi-1 expression is down-regulated during cellular senescence (Itahana *et al.*, 2003). Molecular pathways that regulate Bmi-1 expression during cellular senescence are unknown. Identification of such regulatory pathways is important for our understanding of the role of Bmi-1 and other PcG proteins in cell proliferation, oncogenesis, stem cell biology, and aging.

In addition to Bmi-1, mammalian cells also express Mel-18 (also known as polycomb group ring finger 2 or PCGF2), a closely related PcG protein (Ishida *et al.*, 1993). The Mel-18 gene product is structurally highly similar to Bmi-1. Its N-terminal region, which contains a RING finger domain, is

93% homologous to the similar region of Bmi-1 (Ishida *et al.*, 1993). The homology toward the C-terminal region, which contains a nuclear localization signal (NLS) and a proline-serine–rich (PS) domain, is less conspicuous than the N-terminal region (Ishida *et al.*, 1993). Bmi-1 and Mel-18 are known to interact and are thought to be the constituents of PRC1 (polycomb repressive complex 1; Alkema *et al.*, 1997; Ringrose and Paro, 2004). However, a recent study suggests that Mel-18 may not be part of PRC1, although it could structurally but not functionally replace Bmi-1 in the PRC1 complex (Cao *et al.*, 2005).

It is thought that Bmi-1 and Mel-18 regulate overlapping and unique sets of genes (Kanno *et al.*, 1995; Tetsu *et al.*, 1998, Akasaka *et al.*, 2001). However, unlike Bmi-1, it has been reported that Mel-18 can bind to a well-defined nucleotide sequence 5′-GACTNGACT-3′ present in the promoter region of certain genes (Kanno *et al.*, 1995). One of the unique target genes of Mel-18 is c-Myc, which is transcriptionally repressed by Mel-18 (Kanno *et al.*, 1995; Tetsu *et al.*, 1998). The exact role of Mel-18 in senescence, proliferation, and oncogenesis is unclear. Although its structural similarities to Bmi-1 suggest it to be an oncoprotein, a few studies have indicated that Mel-18 may in fact function as a tumor suppressor (Kanno *et al.*, 1995; Tetsu *et al.*, 1998) and that it might negatively regulate self-renewal of HSCs (Kajiume *et al.*, 2004).

Despite the high similarity between Bmi-1 and Mel-18, we found that Mel-18 overexpression leads to accelerated or premature senescence in proliferating fibroblasts and that it is overexpressed in senescent fibroblasts. We also report that Mel-18 functions as a transcriptional repressor of Bmi-1 expression in human cells. Importantly, we found that the Bmi-1 promoter region contains a functional E-box through which c-Myc and Mel-18 regulate expression of Bmi-1. Because Mel-18 down-regulates c-Myc expression and Bmi-1 is a c-Myc target, our data suggest that Mel-18 regulates expression of Bmi-1 via repression of c-Myc during cellular senescence.

MATERIALS AND METHODS

Cellular Reagents and Methods

WI-38 and BJ fibroblasts were obtained from J. Campisi (Lawrence Berkeley National Laboratory, Berkeley, CA). The MRC-5 fibroblast strain was obtained from the NIA Aging Cell Repository (Coriell Institute for Medical Research, Camden, NJ). The fibroblasts strains were grown and serially passaged in DMEM supplemented with 10% fetal calf serum, and the onset of senescence in fibroblasts was determined using Senescence-associated beta galactosidase (SA-β-gal) assay as described (Dimri *et al.*, 1995; Itahana *et al.*, 2003). MCF10A and MCF7 cells were cultured as described in Dimri *et al.* (2002). Stable cell lines expressing Mel-18 or other genes of interest were generated by infection of the retroviral vectors expressing the particular gene as described (Dimri *et al.*, 2000). The retroviruses were produced by transient transfection of the retroviral vector together with pIK packaging plasmid into tsa 54 packaging cell line as described (Dimri *et al.*, 2000).

Molecular Reagents and Methods: Retroviral Expression and Short-Hairpin RNA Vectors

The vector containing cDNAs of Mel-18 and c-Myc were obtained from ATCC (American Type Culture Collection, Manassas, VA). Mel-18 cDNA was amplified and cloned either in pLPC retroviral vector obtained from Dr. J. Campisi (originally from Dr. T. deLange, Rockefeller University, New York) or in pBabe-puro vector (Dimri *et al.*, 2000). Bmi-1 and Mel-18 short-hairpin RNAs (shRNAs) were designed and cloned in the retroviral vector pRS (retro-super) obtained from Oligoengine (Seattle, WA). The sequences of shRNA were as follows: Mel-18 no. 1: CGACGCCACCACUAUCGUG; no. 2: AGACCAACAAAUACUGCCC; and Bmi-1 shRNA no. 1 GUUCACAAGACCAGACCAC and no. 2 GACCAGACCACUACUGAAU. A retroviral vector expressing c-Myc shRNA (clone no. SH2236-B-10) was obtained from Open Biosystems (Huntsville, AL).

Promoter-Reporter Vectors and Luciferase Assays

The promoter region of Bmi-1 was identified by BLAST comparison of the untranslated region of Bmi-1 cDNA with human genomic clones and analyzing the region further upstream of it. The putative promoter region was amplified using a BAC clone RP11-573G6 obtained from the Children's Hospital Oakland Research Institute, Oakland, CA. The promoter regions of different sizes were amplified by PCR and cloned in the pGL3 luciferase reporter vector (Promega, Madison, WI). The reporter assays were performed using a luciferase assay kit (Promega) as described (Dimri *et al.*, 2002).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) assays were performed using a kit from Upstate Cell Signaling Solutions (Charlottesville, VA). Briefly, chromatin that was cross-linked to transcription factors was immunoprecipitated using antibodies against c-Myc or Mel-18 (obtained from Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitated chromatin was amplified using 5′ACGGGCCTGACTACACCGACACT3′ and 5′CTGAAGGCAGAGTGGAAACTGACAC3′ primers, which flank the c-Myc binding site of the Bmi-1 promoter. The primers- 5′TTCAAAGGCATCTTCTGCAG3′ and 5′CTTAACCGCCCAGATACATC3′, which amplify a non-Myc binding region of the Bmi-1 promoter were used as a negative control.

Quantitative Real-Time RT-PCR Assays

The real-time RT-PCR (QRT-PCR) was carried out using Brilliant SYBR Green QRT-PCR Master Mix, 2-Step kit (Stratagene, La Jolla, CA). Briefly, total RNA was isolated using TRIzol reagent as described by manufacturer (Invitrogen, Carlsbad, CA), and treated with DNase (Promega) to remove any contaminating genomic DNA. The cDNA was generated using oligo dT primer mix and 2.0 μg of DNase treated total RNA. The cDNA was PCR-amplified using primers specific for GAPDH, c-Myc, and Bmi-1. The PCR amplification was carried out using Mx 3000P QPCR system (Stratagene). The PCR conditions consisted of an initial activation of SureStart Taq DNA polymerase at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min. The Ct (threshold cycle) value of Bmi-1 or c-Myc amplification was normalized to that of GAPDH control. The primers for QRT-PCR were as follows: GAPDH forward (F), 5′ GCTGAACGGGAAGCTCACTG 3′; GAPDH reverse (R), 5′GTGCTCAGTGTAGCCCAGGA 3′; Bmi-1 F, 5′ TGGAGAAGGAATGGTCCACTTC 3′; Bmi-1 R, 5′ GTGAGGAAACTGTGGATGAGGA 3′; and c-Myc F, 5′ TACATCCTGTCCGTCCAAGCA 3′; and c-Myc R, 5′ TCAGCCAAGGTTGTGAGGTTG 3′.

Quantitative real-time PCR to detect primary transcription, referred to as PT RT-PCR (primary transcript real-time RT-PCR) was carried out as described (Murray, 2005). Briefly, DNase-treated RNA was reverse-transcribed using random primer mix and amplified using primers that amplify a region of ~200 base pairs of reverse-transcribed unspliced RNAs. The primers for PT RT-PCR were as follows: Bmi-1 F, 5′ CGTGTATTGTTCGTTACCTGGA3′ (present in Exon 2); Bmi-1 R, 5′ GGCAAGAAATTAAACGGCTACC3′ (present in Intron 3); c-Myc F, 5′GTCCAGAGACCTTTCTAACGTA3′ (present in Intron 2); and c-Myc R, 5′AGAAGGTGATCCAGACTCTGAC3′ (present in Exon 3).

Immunological Reagents, Western Blot Analysis, and Determination of Protein Half-Life

Bmi-1 was detected using either F6 mouse mAb from Upstate Cell Signaling Solutions or 1H6B10G7 mAb from Zymed (South San Francisco, CA). Mel-18 was detected by a rabbit polyclonal H-115 (Santa Cruz Biotechnology). The 9E10 mAb (Santa Cruz Biotechnology) against c-Myc was used to detect the expression of c-Myc tag in exogenously expressed proteins. p14ARF was detected using a rabbit polyclonal H-132 Ab (Santa Cruz Biotechnology). Western blot analyses to detect the expression of various proteins were performed as described (Dimri *et al.*, 2000; Itahana *et al.*, 2003). Protein half-life was determined using cyclohexamide (CHX) treatment to block the synthesis of new protein or by pulse-chase immunoprecipitation (IP) experiment using in vivo labeling of proteins with ^{35}S-Express labeling mix (cat. no. NE-072, PerkinElmer Life and Analytical Sciences, Wellesley, MA) followed by chase with cold methionine/cysteine mix for different time points and IP using a specific antibody as described (Boyer *et al.*, 1996).

RESULTS

Mel-18 Induces Premature Senescence in Normal Human Diploid Fibroblasts

To understand the role of Bmi-1–related PcG proteins in cellular senescence and proliferation, we cloned the cDNA of Mel-18 into a retroviral expression vector pLPC. Using this vector, we overexpressed Mel-18 in MRC-5, a normal strain of human diploid fibroblasts (HDFs; Figure 1). In contrast to Bmi-1, which enhances proliferation and extends replicative life span (Itahana *et al.*, 2003), Mel-18 overexpres-

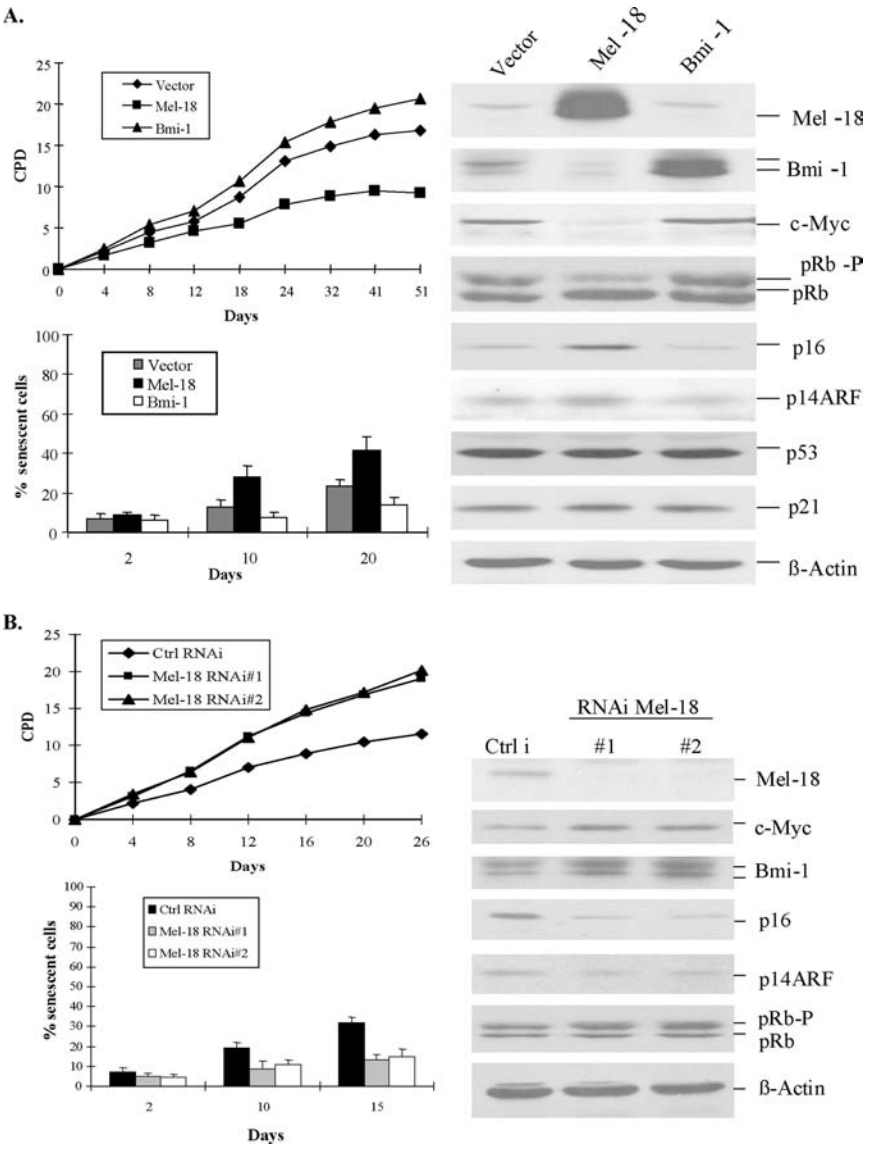

Figure 1. Mel-18 regulates cellular senescence in human fibroblasts. (A) Overexpression of Mel-18 induces premature senescence in proliferating fibroblasts. MRC-5 fibroblasts overexpressing Mel-18 or Bmi-1 and vector-infected control cells were serially passaged in culture to determine the replicative life span (top left panel). Premature induction of senescence was determined using SA-β-gal staining (Dimri *et al.*, 1995) of Mel-18– or Bmi-1–overexpressing and control cells (bottom left panel). % senescent cells, the percentage of SA-β-gal positive cells as determined by counting 200 cells in four different fields. Western blot analysis of various regulators of senescence (right panel) was done as described (Dimri *et al.*, 2002; Itahana *et al.*, 2003). CPD denotes cumulative population doublings. (B) Knockdown of Mel-18 expression leads to the extension of replicative life span in MRC-5 fibroblasts. Mel-18–expressing shRNAs (RNAi Mel-18 no. 1, RNAi Mel-18 no. 2) and control cells (Ctrl i) were passaged in culture to determine replicative life span, and SA-β-gal staining was done to determine the onset of senescence as described above. Western blot analyses (right panel) of Bmi-1, p16, pRb, and Mel-18 were done as described in *Materials and Methods.*

sion led to inhibition of cellular proliferation (Figure 1A) and induction of premature senescence in MRC-5 fibroblasts as determined by SA-β-gal staining (Figure 1A, bottom panel, and Supplementary Figure 1). To further explore the mechanism of induction of premature senescence by Mel-18, we examined the expression of various senescence-associated genes in control and Mel-18– and Bmi-1–overexpressing MRC-5 fibroblasts (Figure 1A, right panel). Because, c-Myc has been reported to be down-regulated by Mel-18 (Tetsu *et al.*, 1998), we also examined c-Myc expression in Mel-18–overexpressing cells. Consistent with published findings, significant down-regulation of c-Myc was noticed in Mel-18–overexpressing cells.

Interestingly, however, we found that Mel-18 overexpression leads to down-regulation of endogenous Bmi-1 and up-regulation of p16 in MRC-5 fibroblasts (Figure 1A). This finding suggests that Mel-18 may induce premature senescence via down-regulation of Bmi-1, leading to up-regulation of p16 and reduction in pRb phosphorylation. We also examined the expression of p14ARF in Mel-18– and Bmi-1–overexpressing cells. Consistent with published data, Bmi-1 modestly down-regulated p14ARF, whereas Mel-18–expressing cells showed a modest up-regulation of p14ARF. However, no induction of p53 or its target p21 was evident in Mel-18–overexpressing cells, suggesting that a modest up-regulation of p14ARF does not impact p53 and p21 expression in these cells. These data are consistent with our earlier finding that Bmi-1 does not significantly alter p53 and p21 expression in human fibroblasts (Itahana *et al.*, 2003). Nonetheless, a modest up-regulation of p14ARF in Mel-18–overexpressing cells may still contribute to growth inhibition by p53-independent mechanisms (reviewed in Sherr *et al.*, 2005).

To confirm the results of overexpression studies, we further determined if knockdown of Mel-18 expression by RNA interference (RNAi) approach up-regulates Bmi-1 and extends the replicative life span. Indeed, stable overexpression of two different Mel-18 shRNAs extended the replicative life span in MRC-5 fibroblasts (Figure 1B). Mel-18 shRNA-expressing cells also exhibited considerably less numbers of senescent cells (Figure 1B). Consistent with overexpression studies, Western blot analysis of Mel-18 knockdown cells showed up-regulation of Bmi-1, down-regulation of p16, and a consequent increase in pRb phosphorylation (Figure

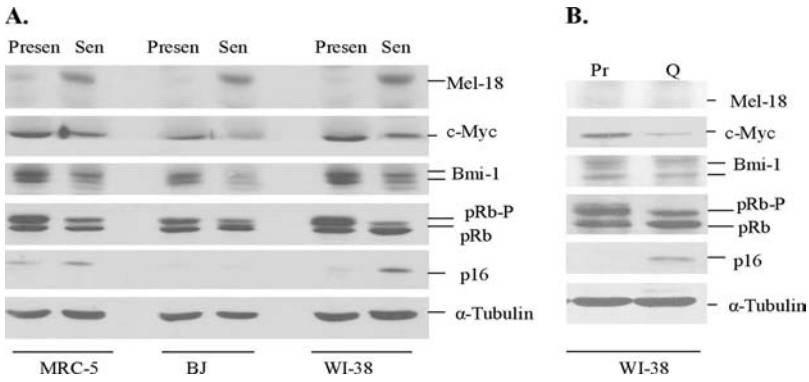

Figure 2. Mel-18 is overexpressed in senescent human fibroblasts. (A) MRC-5, BJ, and WI-38 strains of human fibroblast were serially passaged in culture until senescence as determined by measuring the SA-β-gal index. Mel-18, Bmi-1, pRb, p16, and α-tubulin in total cell lysates from proliferating presenescent (Presen) and senescent (Sen) cultures were detected by Western blot analysis. (B) Mel-18 is not up-regulated during quiescence in WI-38 fibroblasts. Proliferating presenescent (Pr) cells were made quiescent (Q) by incubating cells in 0.1% serum for 5 d. Total cell lysates were prepared from proliferating (Pr) and quiescent (Q) cells, and the expression of Mel-18, c-Myc, Bmi-1, pRb, p16, and α-tubulin was determined by Western blot analysis.

1B). We also used stable expression of two different shRNAs (Bmi-1 RNAi no. 1 and Bmi-1 RNAi no. 2) against Bmi-1 and determined the replicative life span of MRC-5 fibroblasts. Western blot analysis indicated that only Bmi-1 RNAi no. 2 was effective in down-regulating Bmi-1 expression (Supplementary Figure 2). Furthermore, our results indicated that similar to Mel-18 overexpression, Bmi-1 knockdown by RNAi no. 2 accelerates the entry of cells into senescence by up-regulating p16 and increasing the growth inhibitory form of pRb (Supplementary Figure 2).

Mel-18 Expression Is Up-regulated during Cellular Senescence in HDFs

We have previously reported that Bmi-1 expression is down-regulated during cellular or replicative senescence in HDFs (Itahana *et al.,* 2003). The molecular basis of Bmi-1 down-regulation during cellular senescence is not known. On the basis of our data, we surmised that Mel-18 expression might be up-regulated during senescence, which would result in down-regulation of Bmi-1 expression. Conversely, low levels of Mel-18 or absence of its expression in presenescent cells may permit high Bmi-1 expression in these cells. To address these hypotheses, we prepared total cell extract from presenescent (Presen), and senescent (Sen) cells of MRC5, BJ, and WI-38 fibroblast strains and examined Mel-18, Bmi-1, c-Myc, pRb, and p16 expression by Western blot analysis. The onset of senescence in these fibroblast strains was determined using SA-β-gal marker (Dimri *et al.,* 1995). Consistent with our previous results (Itahana *et al.,* 2003), Bmi-1 was down-regulated during cellular senescence (Figure 2A). Importantly, down-regulation of Bmi-1 also correlated with reduced c-Myc expression and a marked increase in Mel-18 expression in senescent cells (Figure 2A).

Our data indicate that Mel-18 expression is virtually undetectable in presenescent (Presen) cells and is up-regulated in senescent (Sen) fibroblasts (Figure 2A). Consistent with previously published literature, senescent cells contained high levels of hypophosphorylated pRb (Figure 2A). Our results also indicated that p16 was conspicuously up-regulated in senescent MRC-5 fibroblasts, but not in senescent BJ fibroblasts, which expresses much lower levels of p16 even during senescence (Itahana *et al.,* 2003). Up-regulation of Mel-18 in senescent cells could result because of the growth-arrested stage of these cells and not necessarily because of senescence. To rule out this possibility, we also examined Mel-18 expression in quiescent cells, which were growth arrested by serum starvation as described (Dimri *et al.,* 1995). The results indicated that growth arrest due to quiescence does not increase Mel-18 expression, suggesting that up-regulation of Mel-18 is senescence-specific and is not due to growth arrest per se (Figure 2B). We also examined c-Myc and Bmi-1 expression under quiescence condition. Consistent with published literature (Waters *et al.,* 1991), c-Myc expression was significantly reduced in quiescent fibroblasts. Our data also suggested a correlation between c-Myc and Bmi-1 expression in growing and senescent but not in quiescent fibroblasts. Importantly, Bmi-1 expression inversely correlated with Mel-18 expression during all three growth conditions: senescence (Sen), quiescence (Q), and proliferation (Pr; Figure 2, A and B).

Mel-18 Regulates Bmi-1 and c-Myc Expression

To further confirm that Mel-18 regulates Bmi-1 expression and to gain insight into its mechanisms, we carried out Mel-18 overexpression and Mel-18 knockdown studies in multiple cell types. Our results indicated that similar to MRC-5 fibroblasts, stable overexpression of Mel-18 results in Bmi-1 down-regulation in MCF10A and MCF7 cells (Figure 3A). We also examined the expression of c-Myc in Mel-18–overexpressing cells. Consistent with the published report (Tetsu *et al.,* 1998), Mel-18 overexpression led to down-regulation of c-Myc in multiple cell types (Figures 1A and 3A).

To rule out the possibility of unknown genetic changes contributing to Bmi-1 down-regulation during selection of the stable expression of Mel-18, we also performed transient transfection assays in 293T cells. Increasing concentrations of transiently transfected Mel-18 resulted in a corresponding down-regulation of endogenous Bmi-1 and c-Myc in these cells (Figure 3B). The regulation of Bmi-1 by Mel-18 was further confirmed by the RNAi approach in multiple types of normal cells. MCF10A, MRC-5, WI-38, and BJ cells expressing two different Mel-18 shRNAs were generated. Western blot analysis of cells expressing Mel-18 shRNAs showed significant down-regulation of Mel-18 and up-regulation of Bmi-1 in these multiple cell types (Figures 1B and 3C). However, we notice that the knockdown effect of Mel-18 are more pronounced in MCF10A cells than in MRC-5 and WI-38 fibroblasts, suggesting that Mel-18 may more tightly regulate Bmi-1 in epithelial cell types. As expected, knockdown of Mel-18 also up-regulated c-Myc expression (Figure 3C). These results strongly suggest that Bmi-1 and c-Myc are physiological targets of Mel-18.

RING Finger of Mel-18 Is Required for the Down-Regulation of Bmi-1

To identify the structural domain(s) of Mel-18 required for Bmi-1 down-regulation, we generated ΔRF (lacks RING finger domain), ΔRFNLS (lacks RING finger and nuclear localization signal), and ΔPS (lacks a PS region) mutants (Figure

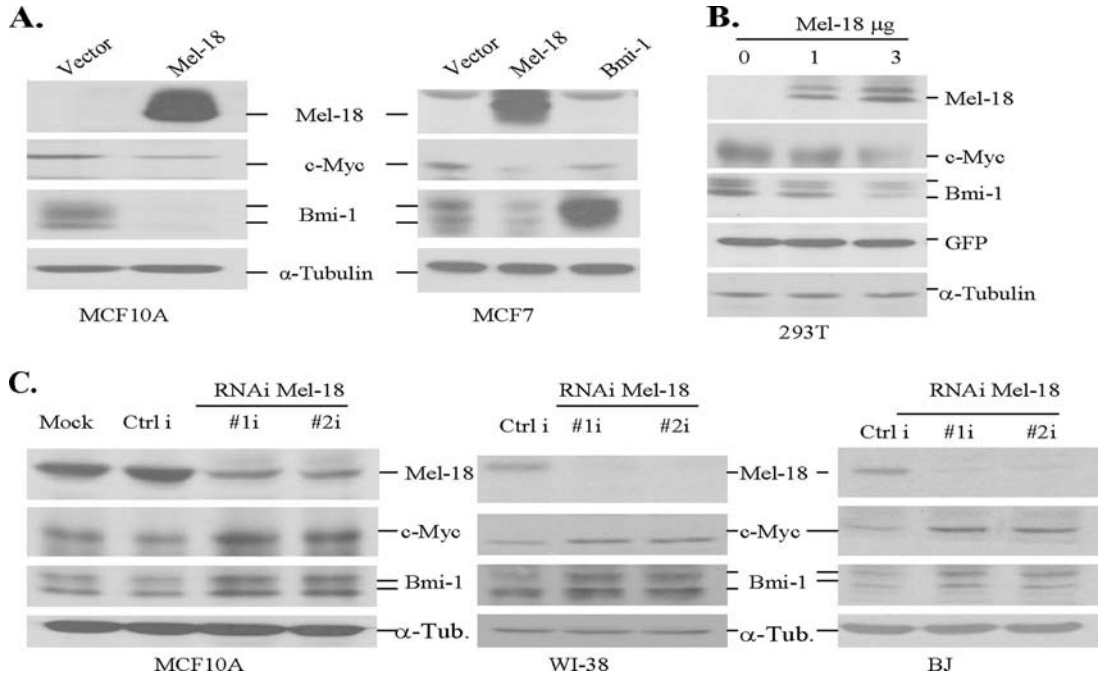

Figure 3. Mel-18 regulates Bmi-1 in human cells. (A) Stable expression of Mel-18 in MCF10A and MCF7 cells leads to down-regulation of Bmi-1 and c-Myc oncoprotein. Total cell lysate from indicated cells was analyzed by Western blot analysis using antibody against Bmi-1, Mel-18, c-Myc, and α-tubulin (loading control). (B) Transient overexpression of Mel-18 in 293T cells leads to the down-regulation of c-Myc and Bmi-1 in a dose-dependent manner. 293T cells were transiently transfected with increasing amounts pLPC-Mel-18, and 48 h after transfection total cell lysate was analyzed by Western blot analysis using antibody against Mel-18, Bmi-1, GFP (transfection control), and α-tubulin (loading control). (C) Stable knockdown of Mel-18 expression using the RNAi approach in MCF10A, WI-38, and BJ cells leads to up-regulation of c-Myc and Bmi-1 expression. MCF10A (left panel), WI-38 (middle panel), and BJ (right panel) cells were infected with pRS vector expressing either Mel-18 shRNA no. 1 (#1i), Mel-18 shRNA no. 2 (#2i), or an irrelevant control shRNA (Ctrl i), selected in puromycin and analyzed for the expression of Mel-18, c-Myc, and Bmi-1 by Western blot analysis.

4A). These mutants were stably overexpressed in MCF10A cells (Figure 4B). Next, we examined the expression of c-Myc and Bmi-1 in cells stably overexpressing wild type or different mutants of Mel-18. The results (Figure 4B) indicated that wild-type Mel-18 and the ΔPS mutant, both of which contained intact RING finger domain down-regulated Bmi-1 expression, suggesting that the RING finger domain of Mel-18 is required for down-regulation of Bmi-1. As expected, overexpression of wild type and the ΔPS mutant also led to c-Myc down-regulation. Interestingly, ΔRF and ΔRFNLS mutants of Mel-18 up-regulated Bmi-1 and c-Myc expression (Figure 4B).

Mel-18 Transcriptionally Down-Regulates Bmi-1 Gene Expression

Next, we determined the mechanism of down-regulation of Bmi-1 by Mel-18. Because Mel-18 contains a RING finger domain, and RING finger proteins can function as an E3 ubiquitin ligase and promote protein degradation via proteosome pathway (Pickart, 2001), we hypothesized that Mel-18 may down-regulate Bmi-1 at the protein level. To examine this possibility, we subjected Mel-18–overexpressing and control cells to treatment with proteosome inhibitor MG-132 and determined Bmi-1 protein levels by Western blot analysis. The results indicated that MG-132 treatment did not significantly increase Bmi-1 protein levels, suggesting that Mel-18 does not regulate Bmi-1 by promoting its degradation via proteosomal pathway (Figure 5A).

To further confirm the above result, we determined the half-life of Bmi-1 and p53 proteins in control and Mel-18–overexpressing cells using CHX treatment (Figure 5B). The p53 protein was used as a control, which is known to have

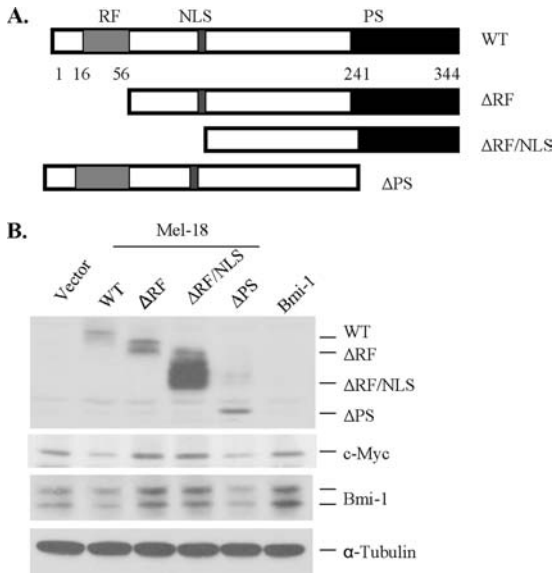

Figure 4. Structural analysis of Mel-18. (A) Schematic representation of mutants of Mel-18 depicting various domains. These mutants were generated by PCR and cloned in the pLPC retroviral vector. (B) Stable overexpression of wild type (WT) and the mutants of Mel-18 in MCF10A cells; WT and the PS mutant down-regulated Bmi-1 and c-Myc expression, whereas overexpression of ΔRF and ΔRFNLS mutants led to up-regulation of Bmi-1 and c-Myc. WT or mutants of Mel-18 were stably expressed using retroviral expression, and Bmi-1, c-Myc, Mel-18, and α-tubulin were detected by Western blot analysis as described in *Materials and Methods.*

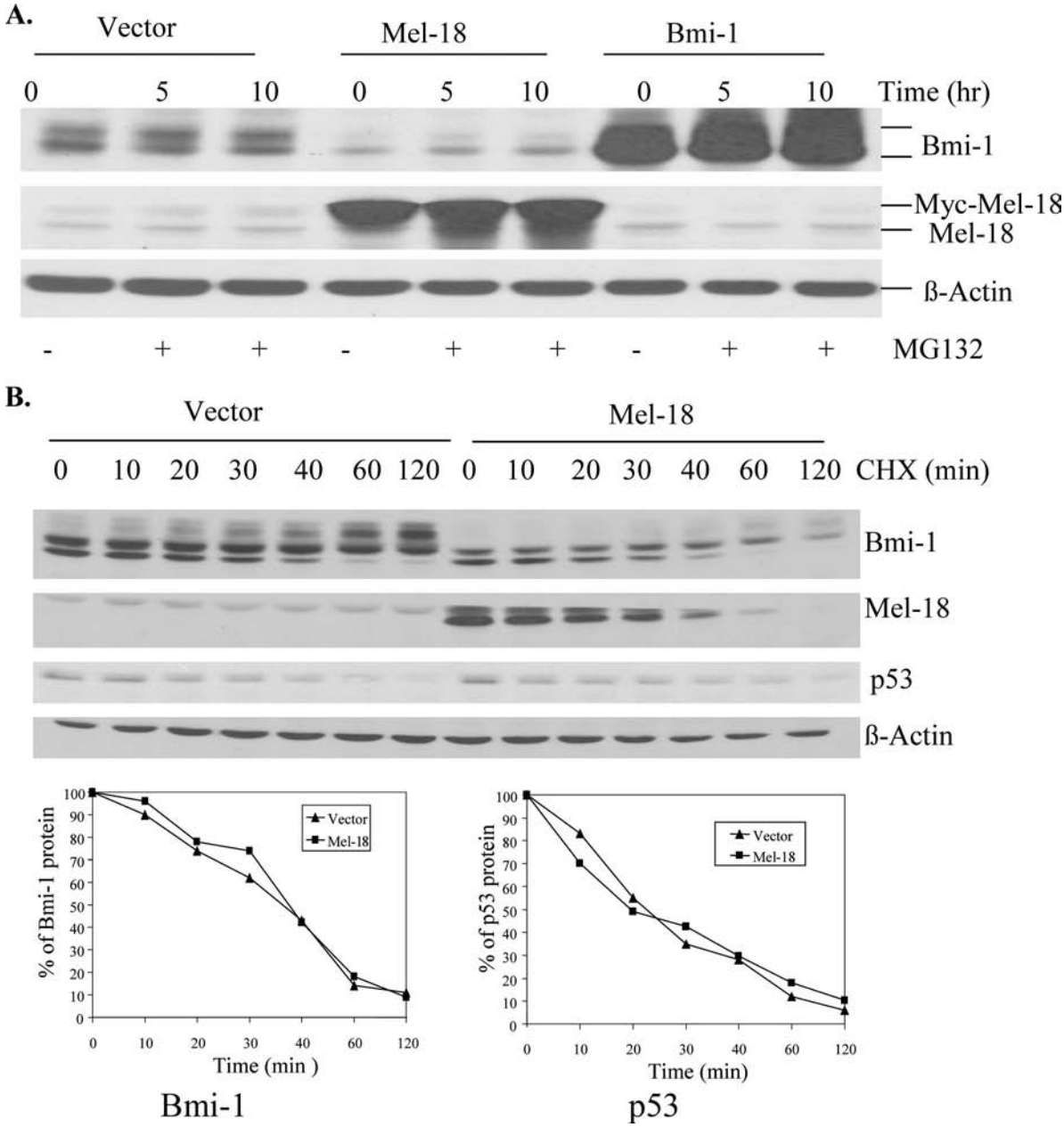

Figure 5. Mel-18 does not regulate Bmi-1 protein level. (A) Treatment with MG132, a proteosome inhibitor does not restore Bmi-1 expression in Mel-18–overexpressing cells. Control, Mel-18– and Bmi-1–overexpressing cells were treated with 10 μM MG132 for the indicted time period and analyzed by Western blot analysis for the expression of Bmi-1, Mel-18, and α-tubulin as described in Figure 1. (B) Bmi-1 half-life is similar in control and Mel-18–overexpressing cells. Vector control and Mel-18–overexpressing cells were treated with 100 μg/ml cyclohexamide (CHX) for the indicated amounts of time and analyzed for the expression of Bmi-1, Mel-18, p53, and β-actin. The percent remaining Bmi-1 (bottom left panel) or p53 (bottom right panel) protein was calculated by densitometry of the Bmi-1 signal present in different lanes and by normalizing it with the β-actin control signal present in the corresponding lanes. Only the lower band of Bmi-1 Western analysis, which clearly showed time-dependent degradation, was used to calculate half-life of Bmi-1.

a short half-life of 20–25 min in these cells. We did not find any significant difference in the half-life of Bmi-1 in control and Mel-18–overexpressing cells, further indicating that Bmi-1 is not regulated at the protein level by Mel-18 (Figure 5B). As expected, p53 half-life in vector and Mel-18–overexpressing cells was ~20 min (Figure 5B). Because CHX treatment leads to the generation of multiple bands of Bmi-1, which appear to have different half-lives, we confirmed the half-life of newly synthesized Bmi-1 by a pulse-chase IP experiment (Supplementary Figure 3). The results indicated that Bmi-1 has a half-life of ~30 min in both vector control and Mel-18–overexpressing cells. Collectively, these data indicate that Mel-18 does not significantly alter Bmi-1 protein stability.

Because Mel-18 did not appear to regulate Bmi-1 expression via protein stability, we determined whether Mel-18 could regulate the transcription of the *Bmi-1* gene. To examine this possibility, we first performed a QRT-PCR to determine the mRNA levels of Bmi-1 and c-Myc in control and Mel-18–overexpressing MCF 10A and MCF7 cells. Our data

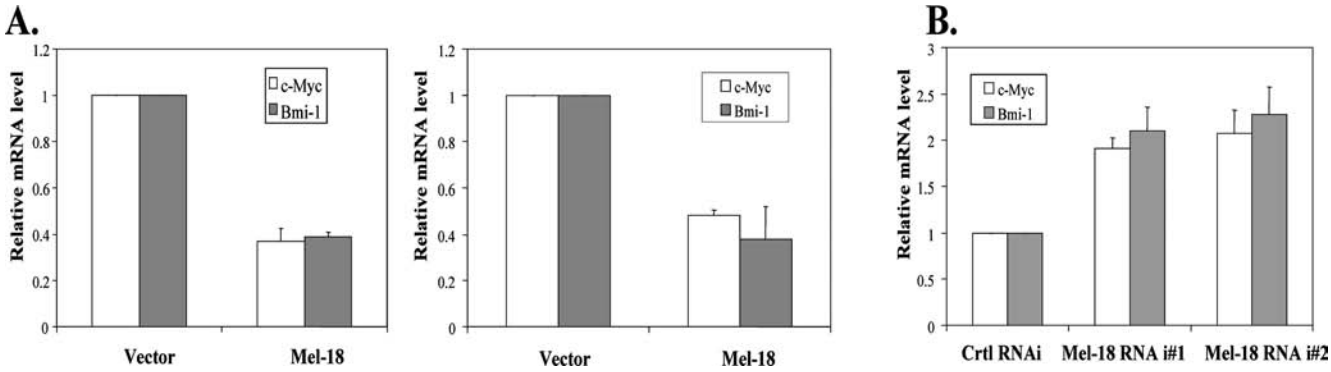

Figure 6. Mel-18 regulates mRNA levels of c-Myc and Bmi-1 as determined by QRT-PCR analysis. (A) The mRNA levels of Bmi-1 and c-Myc in Mel-18–overexpressing and control MCF10A and MCF7 cells were quantified by QRT-PCR and normalized to GAPDH mRNA levels as described in *Materials and Methods.* (B) Using QRT-PCR assay, the mRNA levels of c-Myc and Bmi-1 were quantified and normalized to GAPDH mRNA levels in control (Ctrl RNAi) and Mel-18 knockdown cells (Mel-18 i no. 1 and Mel-18 i no. 2). The QRT-PCR assays were performed in triplicates.

indicated that Mel-18 down-regulates both Bmi-1 and c-Myc at the mRNA level (Figure 6A). Using QRT-PCR, we also determined whether knockdown of Mel-18 up-regulates mRNA levels of Bmi-1 and c-Myc in MCF10A cells. Our data indicated that knockdown of Mel-18 expression indeed leads to up-regulation of c-Myc and Bmi-1 at the mRNA level (Figure 6B). Thus, our results suggest that Mel-18 possibly regulates transcription of *Bmi-1,* perhaps via down-regulation of c-Myc at the mRNA level.

Mel-18 and c-Myc Regulate Bmi-1 Transcription via the c-Myc Binding Site Present in Its Promoter

To examine the possibility of Mel-18 regulating Bmi-1 transcription, we analyzed 400 base pairs of 5′ untranslated region (UTR) containing the *Bmi-1* promoter. The analysis of binding sites for various transcription factors was done using TFSEARCH, version 1.3 (www.cbrc.jp/research/db/TFSEARCH.html). This analysis showed that the *Bmi-1* promoter is a GC-rich promoter without a well-defined TATA sequence and that it contains numerous potential SP-1 binding sites (Supplementary Figure 4). We did not find any potential binding sites (GACTNGACT) for Mel-18. However, the sequence analysis showed the presence of a perfect E-box sequence (CACGTG), which is a potential binding site for the Myc family of transcription factors (Adhikary and Eilers, 2005). The importance of Myc binding sites in the *Bmi-1* promoter was further underscored by the fact that this site is also present in the mouse *Bmi-1* promoter (data not shown).

To determine if Bmi-1 is regulated by c-Myc via the E-box present in the *Bmi-1* promoter, we first performed ChIP assay using vector control and Mel-18–overexpressing MCF7 and MCF10A cells. The cross-linked chromatin was immunoprecipitated (IPed) using a rabbit polyclonal Ab against c-Myc and the control rabbit IgG, and the PCR was performed using primers (c-Myc primer set) that flank c-Myc binding sites in the *Bmi-1* promoter. A primer set derived from further upstream sequences that does not flank the c-Myc binding site was used as a control primer set. The results indicated that c-Myc primer set was specifically able to amplify the PCR product of an expected size (200 base pairs) from the vector control cells (Figure 7A). The yield of the PCR product was much less in Mel-18–overexpressing cells, indicating the down-regulation of c-Myc in Mel-18–overexpressing cells (Figure 7A). The control primer set using c-Myc and IgG IPed extracts did not yield any PCR product indicating the specificity of binding of c-Myc to the E-box present in the *Bmi-1* promoter (Figure 7A).

We further cloned the E-box region (150 base pairs) of the *Bmi-1* promoter in the pLuc vector (Stratagene), which contains a minimal promoter and studied c-Myc regulation of the reconstituted promoter (pLuc-Myc). The results strongly indicated that the E-box present in the *Bmi-1* promoter is functional. Transient cotransfection of c-Myc increased the activity of the reconstituted promoter, whereas knockdown

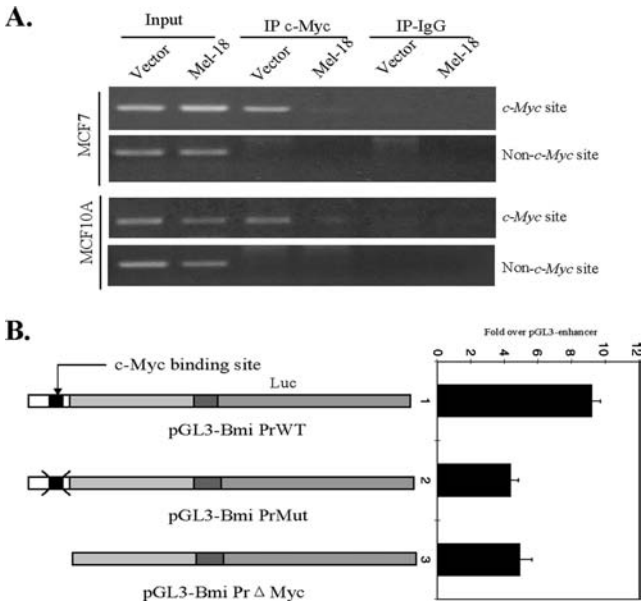

Figure 7. c-Myc binds to the Bmi-1 promoter and regulates its activity. (A) c-Myc binds to the E-box sequences in the Bmi-1 promoter as shown by the ChIP analysis. The ChIP analysis was performed using vector control or Mel-18–overexpressing MCF10A and MCF7 cells as indicated. The cell lysates were IPed using c-Myc antibody or control IgG and a primer set that either amplifies the c-Myc binding flanking region in the Bmi-1 promoter (c-Myc site) or a region further upstream that does not contain a c-Myc binding site (Non-Myc site). (B) Detailed analysis of Bmi-1 promoter activity. The pGL-Bmi PrWT, pGL-Bmi PrMut, and pGL-Bmi PrΔMyc reporters (described in the text) were analyzed for the luciferase activity in 293T cells by transient transfection as described in *Materials and Methods.*

of c-Myc using a c-Myc shRNA resulted in inhibition of pLuc-Myc promoter activity (Supplementary Figure 5).

Next, three different Bmi-1 promoter-reporter constructs based on the pGL3 vector were generated (Figure 7B). pGL3-Bmi PrWT contained the +45 to −233 region of the *Bmi-1* promoter and untranslated region of *Bmi-1* mRNA. The second construct pGL3-Bmi PrMut contained a mutation in the Myc binding sequences (CACGTG changed to CGCGTG). The third construct pGL3-Bmi PrΔMyc contained a complete deletion of the c-Myc binding site. We determined the luciferase activity driven by wild-type or mutant promoters. The results indicated that wild-type promoter displays robust promoter activity, whereas the mutant promoters exhibited 50% less activity than the wild-type promoter (Figure 7B).

We further studied the regulation of the *Bmi-1* promoter by c-Myc and Mel-18 (Figure 8, A–C). The promoter-reporter constructs were cotransfected with increasing amounts of Mel-18–overexpressing plasmid (Figure 8A), c-Myc overexpressing plasmid (Figure 8B), or a plasmid expressing c-Myc shRNA (Figure 8C). Analysis of the luciferase activity of these promoter-reporter constructs suggested that Mel-18 negatively regulates the *Bmi-1* promoter through the c-Myc binding site, because the promoter that lacked the E-box or contained mutant c-Myc binding site did not respond to increasing concentrations of the Mel-18 expressing plasmid (Figure 8A). Furthermore, the transient cotransfection of c-Myc–overexpressing plasmid led to the up-regulation of activity of wild-type but not mutant promoters (Figure 8B). Similarly, knockdown of c-Myc expression by transfection of a plasmid expressing c-Myc shRNA down-regulated *Bmi-1* promoter activity of the promoter that contained the wild-type c-Myc binding site (Figure 8C).

Our promoter-reporter analysis suggested that c-Myc positively regulates the expression of Bmi-1 and that Mel-18 negatively regulates Bmi-1 expression via repression of c-Myc. To further confirm these results, we performed a real-time PT RT-PCR assay, which accurately determines the regulation of a particular gene in its native state at the level of primary transcription (Murray, 2005). Our data indicated that Mel-18 indeed down-regulates Bmi-1 and c-Myc at the level of primary transcription (Figure 9).

Although our data indicate that Mel-18 and c-Myc regulate the expression of the *Bmi-1* promoter via the E-box and Mel-18 acts via c-Myc repression, it is possible that Mel-18 directly binds to the E-box binding site and represses *Bmi-1* promoter activity independent of c-Myc. To exclude this possibility, we performed ChIP assay using Mel-18 antibody. Because the E-box region is sufficient for Mel-18–mediated regulation of the *Bmi-1* promoter, PCR primers in this region were chosen for the ChIP assay. Our results (Supplementary Figure 7) indicate that Mel-18 does not bind to the E-box present in the promoter region of Bmi-1; hence, Mel-18 does not directly regulate Bmi-1 expression. On the other hand, c-Myc was clearly able to bind the E-box as determined by ChIP assay (Figure 7A and Supplementary Figure 6).

Bmi-1 Is a Bona Fide Target of c-Myc, and c-Myc Overexpression Rescues Mel-18–mediated Repression of Bmi-1 Expression

To further confirm that the endogenous promoter of Bmi-1 is regulated by c-Myc, we studied the expression of Bmi-1 in MCF10A cells, which stably overexpress c-Myc under a retroviral promoter (Figure 10A), and in MCF10A cells where the expression of c-Myc was stably knockdown by the RNAi approach (Figure 10B). Our data suggest that the stable overexpression of c-Myc results in up-regulation of

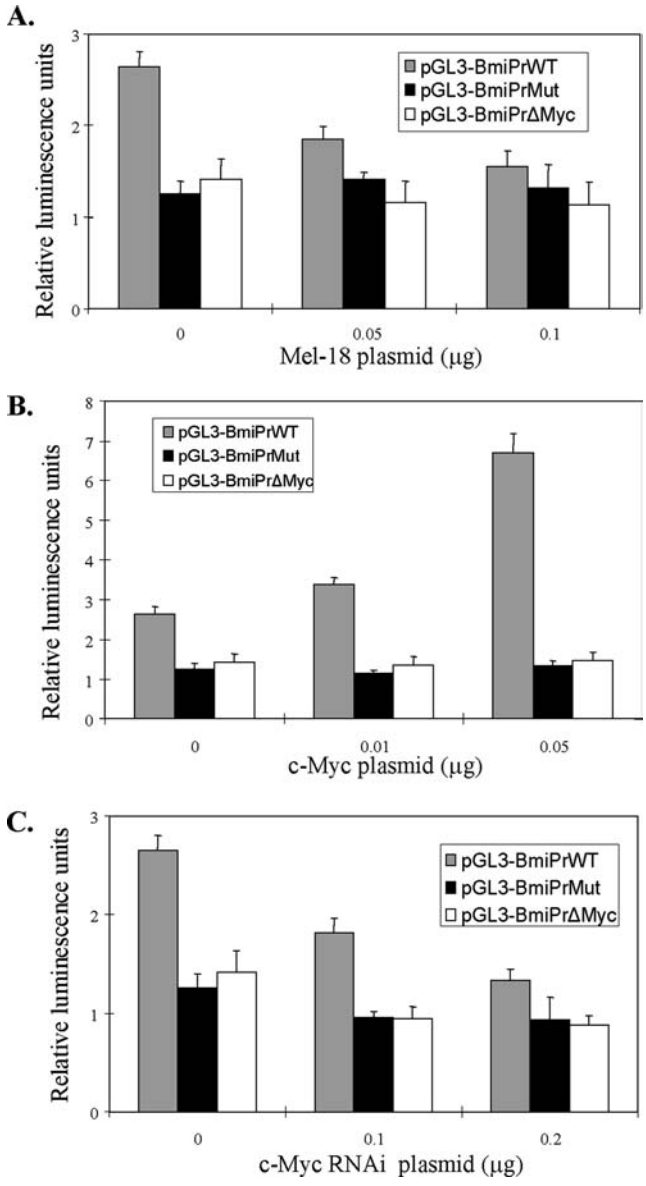

Figure 8. Mel-18 and c-Myc regulate Bmi-1 promoter activity. (A) Overexpression of Mel-18 down-regulates only the wild-type Bmi-1 promoter. pGL3-Bmi-1 PrWT, pGL3-Bmi-1PrMut, and pGL3- Bmi-1PrΔMyc plasmids were transiently transfected into 293T cells together with an increasing amount of Mel-18–overexpressing plasmid (pLPC-Mel-18) and a plasmid expressing renilla luciferase. Forty-eight hours after transfection luciferase activity was determined as described in *Materials and Methods.* (B) Transient overexpression of c-Myc up-regulates wild-type Bmi-1 promoter activity through the c-Myc binding site. Different promoter-reporter constructs (as indicated) were transiently transfected into 293T cells with an increasing amount of pCMV-Myc expression plasmid together with a plasmid expressing renilla luciferase, and luciferase activity was determined as described in *Materials and Methods.* (C) c-Myc knockdown using transient transfection of a plasmid containing c-Myc shRNA down-regulates activity of the Bmi-1 promoter, which contains an intact c-Myc binding site. The promoter activity of various promoter-reporter constructs with the increasing amount of a plasmid expressing c-Myc shRNA was analyzed in 293T cells as described in *Materials and Methods.*

Bmi-1 expression (Figure 10B). Accordingly, we also found that knockdown of c-Myc expression using the RNAi ap-

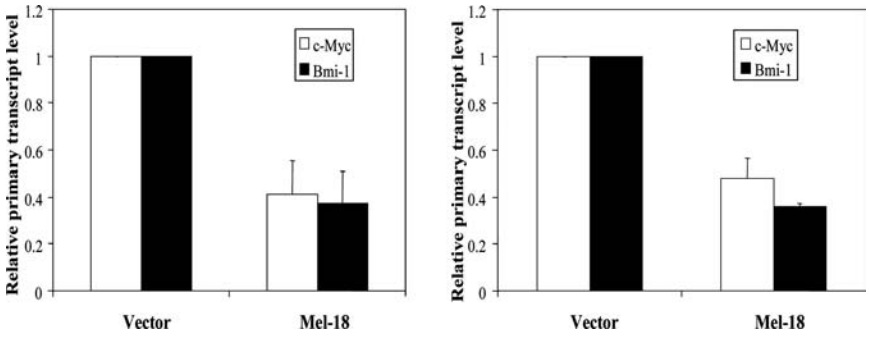

Figure 9. Mel-18 regulates c-Myc and Bmi-1 transcription. Quantitative PT RT-PCR analysis of primary transcripts of c-Myc and Bmi-1 in control and Mel-18–overexpressing MCF7 (left panel) and MCF10A cells (right panel). PT RT-PCR analysis was performed in triplicate as described in *Materials and Methods.*

proach results in a substantial down-regulation of endogenous Bmi-1 expression (Figure 10B).

Next, we carried out a c-Myc rescue experiment. Because Mel-18 represses c-Myc expression by binding to its native promoter, we reasoned that c-Myc overexpression using a heterologous promoter should rescue Bmi-1 repression caused by Mel-18 overexpression. To test this hypothesis, we transiently transfected pLPC-Mel-18 together with pCMV-Myc. Our results indicated that indeed c-Myc overexpression using the CMV promoter rescues Mel-18–mediated repression of endogenous Bmi-1 (Figure 10C). Thus, our data strongly suggest that Mel-18 down-regulates Bmi-1 expression at the transcriptional level via c-Myc repression and that c-Myc acts as a positive regulator of Bmi-1 expression.

DISCUSSION

Various PcG proteins form higher order complexes such as PRC1 and PRC2 in cells (Ringrose and Paro, 2004). These complexes are thought to regulate expression of target genes such as members of the Hox family (Ringrose and Paro, 2004). When over- or underexpressed, individual polycomb proteins such as Bmi-1 can also regulate expression of specific target genes that are involved in proliferation and senescence. Virtually nothing is known about the regulation of the expression of various PcG proteins. Here, we report a novel observation that Bmi-1 is specifically regulated by another PcG protein Mel-18.

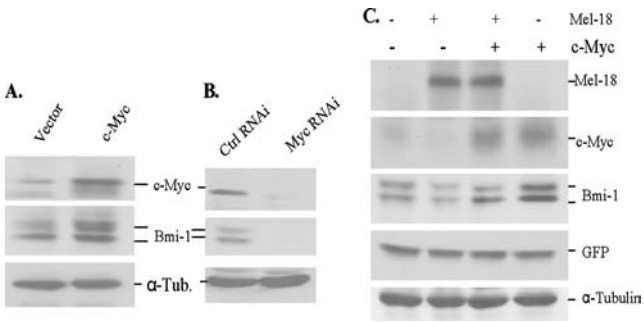

Figure 10. c-Myc regulates endogenous Bmi-1 expression and transient expression of c-Myc in Mel-18–overexpressing cells restores Bmi-1 expression. (A) Stable overexpression of c-Myc leads to Bmi-1 up-regulation. MCF10A cells were infected with a c-Myc expressing retrovirus (pLNCX2-Myc), selected in G418, and the expression of c-Myc, Bmi-1, and α-tubulin was determined by Western blot analysis. (B) Knockdown of c-Myc expression by RNAi approach leads to down-regulation of endogenous Bmi-1. MCF10A cells expressing c-Myc shRNA (Myc RNAi) or a control shRNA (Ctrl. RNAi) were generated and analyzed for the expression of c-Myc, Bmi-1, and α-tubulin by Western blot analysis. (C) Restoration of c-Myc in Mel-18–overexpressing cells by its transient overexpression leads to the reversal of Bmi-1 repression by Mel-18. 293T cells were transfected with either Mel-18, c-Myc, or both and a GFP expressing plasmid. The total cell lysate from each set was analyzed for the expression of Mel-18, c-Myc, Bmi-1, GFP, and α-tubulin by Western blot analysis.

Our novel observation suggests that Mel-18 is an upstream negative regulator of Bmi-1 function, which promotes proliferation, oncogenesis, and stem "cell-ness." Consistent with such an observation, Bmi-1 knockdown by RNAi as well as its down-regulation by Mel-18 overexpression in cells resulted in accelerated senescence. As senescence constitutes a tumor suppressor mechanism and is regulated by various tumor suppressors (Dimri, 2005), our results clearly place Mel-18 in the tumor suppressor category. It is known that various tumor suppressors are either up-regulated (for example, p16) or the physiological activity of tumor suppressors is up-regulated during senescence (Dimri, 2005). For example, DNA binding activity of p53 is up-regulated, and there is a relative increase in hypophosphorylated pRb compared with hyperphosphorylated pRb during senescence (Dimri, 2005). Forced expression of p16, p14ARF, and other tumor suppressors has been shown to accelerate senescence in human cells (Dimri, 2005). Consistent with these properties of tumor suppressors, we found that Mel-18 is up-regulated during senescence in human fibroblasts, which contributes to down-regulation of c-Myc and Bmi-1 oncoproteins. Accelerated senescence in Mel-18–overexpressing fibroblasts is accompanied by down-regulation of Bmi-1, robust p16 up-regulation, and an increase in hypophosphorylated pRb.

In contrast to wild-type Mel-18, RING finger mutants up-regulate Bmi-1, suggesting potential dominant negative activity (DN) of these mutants. RING finger mutants may bind to the promoter of presumptive target(s), which may be Bmi-1 itself or the other target(s) that regulate Bmi-1, and inhibit the function of nuclear Mel-18. However, if this was the case, ΔRFNLS should not have exhibited a DN activity because it lacks the nuclear localization signal. We speculate that RING finger mutants may simply up-regulate Bmi-1 by inhibiting the function of endogenous Mel-18, by binding it and sequestering it in the cytoplasm. Detailed mechanism of Bmi-1 up-regulation by RING mutants remains to be studied.

Although, Mel-18 can regulate its target genes by binding to the promoter regions, the Bmi-1 promoter does not contain presumptive Mel-18 binding sequences. However, it remains possible that Mel-18 regulates Bmi-1 expression by repressing a positive regulator of Bmi-1. Indeed, we found that the Bmi-1 promoter contains an E-box to which a positive or negative regulator of Bmi-1 can bind. Identification

of an E-box in the Bmi-1 promoter is very intriguing from an oncogenesis point of view. A number of E-box binding proteins are known, some of which act as repressors, whereas others act as activators of transcription. The c-Myc family of transcription factors, which bind to the E-box, are clearly implicated in oncogenesis. The c-Myc oncogene is amplified and/or overexpressed in a variety of malignancies (see Myc Cancer Gene web site: http://www.myccancergene.org). It acts as a transcription factor and regulates the expression of a number of genes (Zeller *et al.*, 2003; Adhikary and Eilers, 2005). However, it is still unclear what the cancer-relevant bona fide targets of c-Myc are. Here, we identified Bmi-1 oncogene as an important target of c-Myc oncoprotein. Similar to our results, a very recent report has also implicated c-Myc in regulation of Bmi-1 expression and induction of telomere-independent senescence by reduced c-Myc levels and a consequent increase in p16 (Guney *et al.*, 2006).

c-Myc oncoprotein dimerizes with Max, which is usually in excess. Myc-Max complexes positively regulate expression of Myc target genes. Myc and Max also dimerizes with Mad1, Mxi-1, Mad3, Mad4, and Mnt (Adhikary and Eilers, 2005). Heterodimers of these proteins also bind to the E-box and often negatively regulate the expression of the target genes. It is very likely that Bmi-1 is negatively regulated by Max-Mad and Mnt-Max complexes. Thus, our studies suggest that c-Myc and other E-box binding proteins may positively or negatively regulate Bmi-1, which in turn regulates proliferation, senescence, oncogenesis, and stem cell-ness. Besides E-box binding proteins, other transcription factors may also regulate the expression of Bmi-1. Indeed, a recent report suggests that E2F 1 also regulates Bmi-1 expression (Nowak *et al.*, 2006). Moreover, we did not find any positive correlation between Bmi-1 and c-Myc expression during quiescence, suggesting that under quiescence condition, transcription factors other than c-Myc regulate the expression of Bmi-1. Such regulators of Bmi-1 remain to be identified.

Our studies suggest that Mel-18 is a physiological regulator of Bmi-1 expression in human fibroblasts and mammary epithelial cells. On the basis of our data, we suspect that this inverse correlation between Bmi-1 and Mel-18 expression may persist with other cell types and various cancers. Indeed, our preliminary data suggest a strong negative correlation between Bmi-1 and Mel-18 expression in a significant number of breast tumors. It has been suggested that Bmi-1 may be a cancer stem cell marker (Lessard and Sauvageau, 2003; Glinsky *et al.*, 2005); it will be interesting to explore whether Mel-18 down-regulation in certain specific cell types makes them susceptible to cancer stem cell conversion.

In summary, our studies suggest that the Mel-18-c-Myc-Bmi-1-p16-pRb pathway regulates cellular senescence and proliferation in human cells. Additionally, Mel-18 and Bmi-1 can also regulate p14ARF expression, which may contribute to the regulation of proliferation and senescence via p53-independent mechanisms. Although there has already been considerable interest in c-Myc, p16, p14ARF, and pRb, our data suggest that PcG protein Mel-18 and Bmi-1 are also valid targets for cancer therapy. For example, restoration of Mel-18 expression or ablation of Bmi-1 expression in tumors by various therapeutic approaches might help in cancer treatment. Lastly, because stem cell defect has been linked to various age-related pathologies (reviewed in Ho *et al.*, 2005; Rosenthal, 2005), we speculate that Mel-18 may play an important role in the development of age-related ailments by virtue of down-regulating Bmi-1, a known regulator of stem cell-ness.

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