AD_____

AWARD NUMBER: DAMD17-02-1-0509

TITLE: Role of p53 in Mammary Epithelial Cell Senescence

PRINCIPAL INVESTIGATOR: Goberdhan P. Dimri, Ph.D.

CONTRACTING ORGANIZATION: Evanston Northwest Research Institute
Evanston, IL 60201

REPORT DATE: May 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 May 2009	Annual Summary	1 May 2002 – 30 Apr 2009

4. TITLE AND SUBTITLE

Role of p53 in Mammary Epithelial Cell Senescence

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0509

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Goberdhan P. Dimri, Ph.D

E-Mail: GDimri@northshore.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Evanston Northwest Research Institute
Evanston, IL 60201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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, senescence or autophagy. In this career development award, we studied the role of p53 in human mammary epithelial cell (HMEC) senescence and the requirement of p53 inactivation in transformation of HMECs. First, we determined that p53 binding activity increases with senescence in post-selection HMECs, and expression of its targets such as p21 is increased in post-selection senescent cells. We also found that there were no major differences in acetylation or phosphorylation of p53 in pre-selection and post-selection HMECs. However, there is a modest increase in acetylated p53 in post-selection senescent HMECs. Next, we studied the role of p53 in HMEC senescence using RNAi approach. Our studies suggested that p53 or p21 knockdown results in bypass of senescence in HMECs. We also carried out an array analysis of p53 targets in early passage (proliferating) and late passage (senescent) post-selection HMECs. Next, we studied the role of additional targets of p53 in HMEC senescence using RT-PCR analysis and identified additional targets of p53 using ChIP assay. Finally, we studied p53/p21 pathway in BMI1+H-RAS transformed HMECs, and the role of p53 in oncogene-induced senescence (OIS) in HMECs.

15. SUBJECT TERMS
P53, p21, senescence, HMECs, Cell proliferation, BMI1

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	71	USAMRMC

Table of Contents

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]. Two important tumor suppressor pRb and p53 are required for the maintenance and genesis of senescent phenotype.

The p53-p21 pathway is an important mediator of cellular senescence as well as senescence induced by non-telomeric signals such as oncogene-induced senescence (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 domain [3, 4] . Transcriptionally active p53 binds to a consensus site 5' -RRRCA/TA/TYYY-3', often present in pairs in p53 regulated genes [5, 6] . Tumor derived mutants of p53 are always defective in sequence-specific transactivation, thus attesting the importance of transcription activation function of p53 [7]. Activation of these transcriptional targets of p53 results in apoptosis, G1 and G2 cell cycle arrest or senescence [8, 9]. Mutations in p53 and genes of p53 pathway are also of common occurrence in breast cancer [10].

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 undergo senescence. Regular feeding of these cells 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]. These post-selection cells are widely used to study senescence and model breast cancer in vitro. The post-selection cells are p16 negative but still undergo senescence and never spontaneously immortalize [1]. Because cells need to overcome senescence in order to become transformed, and p53 and p53 targets regulate senescence, it is important to study the role of p53 and identify p53 targets genes that are involved in senescence induction in HMECs.

BODY:

The broad objective of this career development award (CDA) was to study the role of p53 in HMEC senescence and gain expertise in the area of breast cancer research. The first aim was to examine p53 binding activity and study posttranslational modifications in senescent HMECs. The second aim was to study the role of known p53 target genes using chromatin IP and RT-PCR method, and the third aim was to identify novel targets of senescence-relevant targets p53 using ChIP and array analysis. In following sections, we describe our results with respect to these aims and additional related studies that we carried out during this CDA.

Aim 1: The aim 1 of the grant was accomplished in year 1. The results are briefly described below.

In this aim, post-selection HMECs were serially passaged in culture and frozen at different passages. SA-β-gal index and %LN of HMECs were determined at each passage as described [11, 12]. At passage 17, these HMECs appear to be senescent as determined by %LN (<15%) and SA-β-gal index (>70%). Similarly, cells at passage 11 were considered early passage by determining %LN (>80%) and SA-β-gal index (<5%). Nuclear extracts were prepared from cells at passage 11, passage 15 and passage 17, and p53 DNA binding assay was performed as described [9]. The results (Fig. 1A) suggest that p53-binding activity modestly increases in senescent HMECs compared to early passage cells. Binding specificity of p53 was confirmed using competition with wild type and mutant (containing mutant p53 binding site) oligos (Fig. 1A).

Next, we studied posttranslational modifications of p53 during senescence in HMECs by western blot analysis using antibodies that detects total p53 or phosphorylated and acetylated forms of p53. Total cell extract was prepared from early passage and senescent pre-selection and post-selection HMECs. Equal amount of extract (40 μg) was run on 5-15% gradient polyacrylamide gel, transferred to PVDF membrane and probed with various antibodies as described [12]. The results (Fig. 1B) suggest that p53 is downregulated in senescent pre-selection cells but is upregulated in senescent post-selection cells. Furthermore, expression of p21, a p53 target gene, which is a CDK inhibitor, and known to be upregulated during senescence in fibroblasts correlates well with p53 level. Senescent post-selection but not pre-selection HMECs contained increased amount of p21 protein. As expected, post-selection cells contained undetectable p16; however, p16 was upregulated in senescent pre-selection HMECs. When probed with antibodies specific for acetylated p53

(Lys 320, and Lys-373, Lys-382), results showed no significant difference in steady state level of acetylated p53 (Fig. 1B). Although, there appears to be modest increase in acetylated p53 in senescent post-selection HMECs compared to early post-selection HMECs when probed with antibody specific for Lys-320. Western blot analysis using antibody specific for Ser-15 and Ser-37 showed that p53 phosphorylated at Ser-37 is increased in post-selection HMECs, but its levels do not increase with senescence. On the other hand, Ser-15 phosphorylation remained mostly unchanged in pre-and post-selection cells but senescent post-selection HMECs exhibited slight increase when compared to early passage cells (Fig.1B).

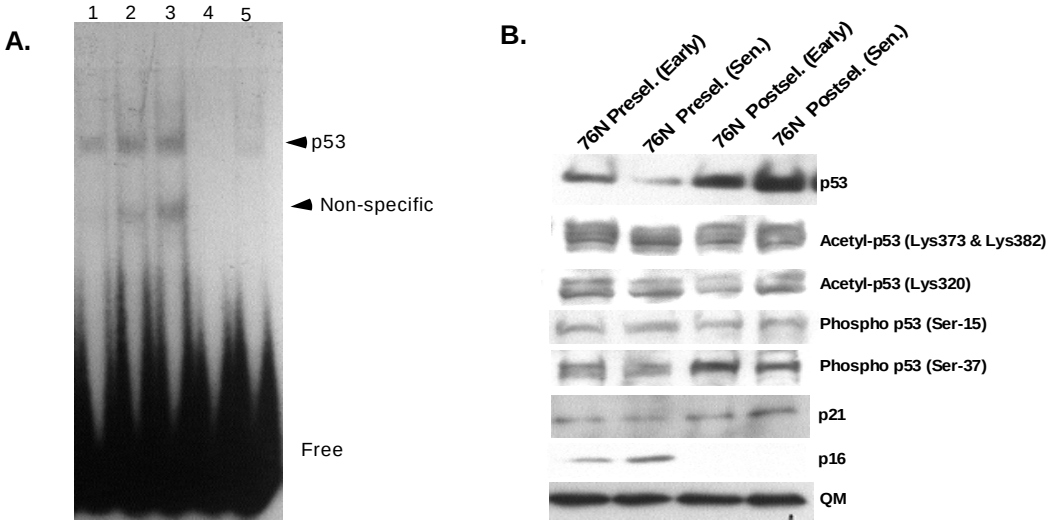


Figure 1: Analysis of p53 in senescent HMECs. A. p53 DNA binding activity increases with senescence in post-selection HMECs. Lane 1, 2 and 3 represent nuclear extract from HMECs at passage 11 (early passage), 15 (mid passage) and 17 (senescent) respectively. Lane 4 is competition using 100-fold excess wild type oligo, while lane 5 is competition using 100-fold excess mutant oligo (containing mutant p53 binding site). In competition assays, nuclear extract from passage 11 was used. Arrows indicate p53 specific and non-specific band (as indicated). Free is the labeled probe that did not bind p53. **B.** Western-blot analysis using indicated antibodies to determine the expression of various proteins (p53, p16, p21 and QM), and posttranslational modifications of p53 (acetyl and phospho p53) was performed as described in the text. QM is a loading control.

Aim 2: In this aim, we studied the role of known p53 targets in HMEC senescence using array, RT-PCR and ChIP,assays.

1. To analyze p53 target genes in senescent HMECs, we obtained "Human TranSignal p53 Target Gene Array" from Panomics Inc., Redwood City, CA. The array contains 146 human p53 target genes chosen from published literature. Total RNA was isolated from early passage growing (76N Early) and senescent (76N Late) HMECs, labeled with biotin-UTP using reverse transcriptase, and hybridized to individual array membranes overnight. The hybridization pattern was detected by enhanced chemiluminescence, and autoradiogram developed. The intensity of signal corresponding to various target genes by densitometry, and normalized to control spots in arrays. Our array data suggest that in general, p53 target genes are upregulated during senescence in HMECs (Figure 2). Results indicated p53 regulated genes falls into four categories of genes in HMECs (Figure 2). Category A- Genes that are showed the highest upregulation in senescent cells compared to early passage proliferating cells. This category include are well-characterized p53 target genes such as *TGFα, p63, WIG1* and *TSP-1*. Other gene that are also expressed at high level compared to early passage cells were- *Jun, RB1, MAD1, TP53INP1, PRG1, HGFL1, Slac19A, 15-LO, P2RXL1, RGS14, THBS2, TOP2A, TYR AFP,AR* and *TST* (Figure 1B). Category B- Genes in this category are moderately overexpressed in senescent HMECs. These genes include *IL-6, IGF-BP3,* and *BCL-6*. Category C- In the third category, genes showed similar level of expression in proliferating as well as senescent HMECs. These genes are

PTEN, CK8, PPM1D, MCG9 and *Killer /DR5*. Category C- Finally, genes that were downregulated in senescent HMECs are- *Cyclin B* and *c-myc*.

Several p53 target genes that were also present in array were not detected; this could be due to cell type specific expression of these target genes or relatively low level of expression of genes that were not detected in array analysis.

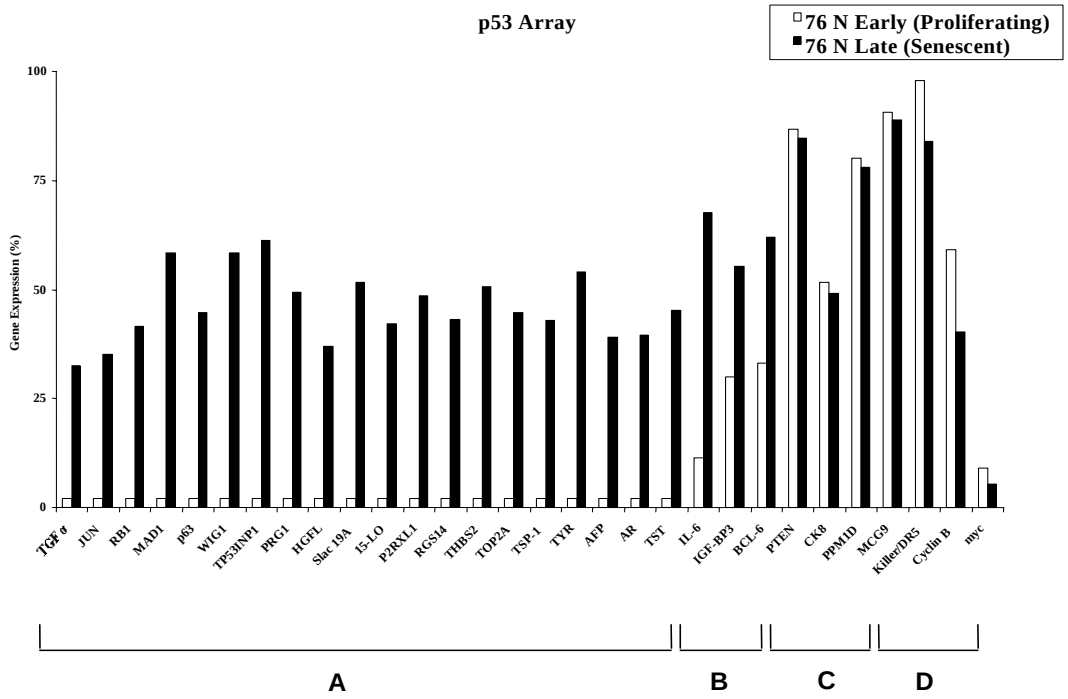


Figure 2. Quantitative analysis of various p53 target genes present in p53 array. Genes were divided into 4 categories (A-D) as described in the text.

2. Next, we analyzed several known p53 target genes and genes selected from array in senescent HMECs by RT-PCR analysis. Total RNA was prepared from early (growing) and late (senescent) HMECs and RT PCR analysis was done as described. The primer sequences and expected product sizes are given in table 1.

Table 1

Gene	Forward Primer	Reverse Primer	Product size (bp)
DDB2	TTACTCTGCTTCCCAGTG	GCTCCAGATGAGAATGTG	298 bp
UBTD1	CCATCTACTGCCTGTCAC	GATGATGACCTGGATGAC	293 bp
TMEM30A	GGTACAACAAAGCCTGTG	CAGCGATGTAAGCAATCC	292 bp
RPS27L	TACATCCGTCCTTGGAAG	TGAACACCCTTCTGTGAG	194 bp
TP53AP1	GCCTGACCCAGGATCTAG	CACTGGTGTAAGTGTTCG	183 bp
MDM4	GGCTCCTGTCGTTAGAC	CCCCAGCCTTCTTTAGTC	296 bp
ANKRD2	GAGGGATAAGCTGCTGAG	CAGCCCGTTATGCTCAG	298 bp

GADD45A	ATCACTGTCGGGGTGTAC	CTTAAGGCAGGATCCTTC	299 bp
BTG2	CAAACACCACTGGTTTCC	ACTGCCATCACGTAGTTC	300 bp
ANXA4	ACCGAAATCACCTGTTGC	AGTCTCCAGATGTGTCAC	294 bp
IGF-BP3	TAAAGACAGCCAGCGCTA	CTGCCCATACTTATCCACA	252 bp
TGF-α	TCAGTTCTGCTTCCATGG	TTTCTGAGTGGCAGCAAG	299 bp
Wig1	CGGAAGCTCAGAGTAACTC	CTCCATCTCATTCCTGTACC	300 bp
HGFL	GAAGGAGCAGTGGATACTGAC	GGACTGTGTCATTACCCGTAC	299 bp
BCL6	CAGTGACAAACCCTACAAG	GCTCTTCAGAGTCTGAAGG	300 bp
RGS 14	GTGTGAAGATCTCCAAAGC	CTGCTGATTTGGTCTGTG	297 bp
PPM1D	CTCGAGAGAATGTCCAAGG	GCTGAGCACCACTACTTC	300 bp
TSP-1	GGACAACTGTCCCTATGTG	CCAGTTAGGGTCATTTTGG	300 bp

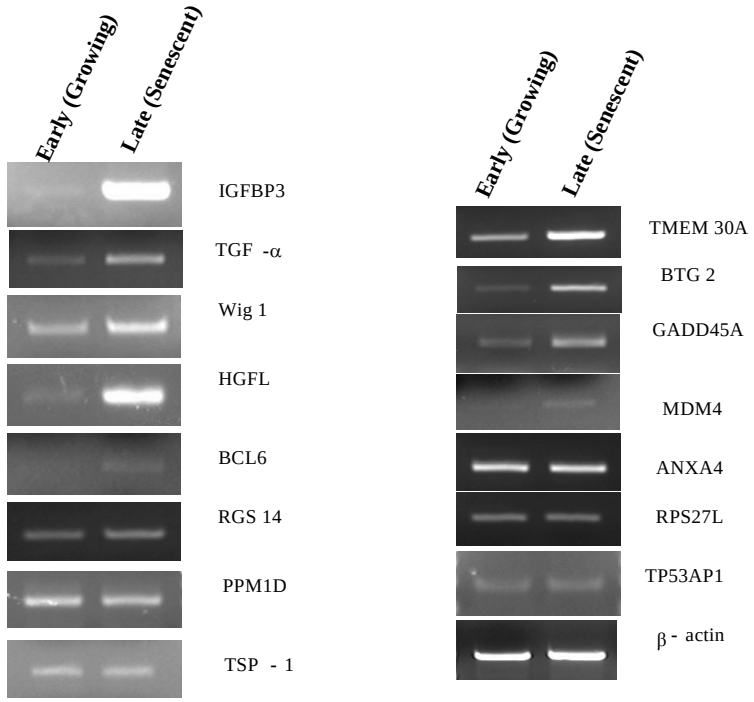


Figure 3: RT PCR analysis of selected p53 target genes in growing and senescent HMECs. ß-Actin was used as a loading control.

3. Next, ChIP assay was done using chromatin Immunoprecipitation kit from Upstate Cell Signaling Solutions (Charlottesville, Virgina) as described by manufacturer. The p53 immunoprecipitated DNA was PCR amplified for selected p53 target genes using primers flanking p53 binding sites. Immunoprecipitation using mouse IgG was used as a negative control. 10% of initial lysed and sonicated cell extract was used as input for PCR after phenol extraction and DNA precipitation. Initially, we compared ChIP assay pattern of

growing and senescent cells for p53 target genes p21 and PIG3 (Fig. 4A), and DDB2 and RPS27L using primers specific for these genes, which flank p53 binding sites in their promoter regions.

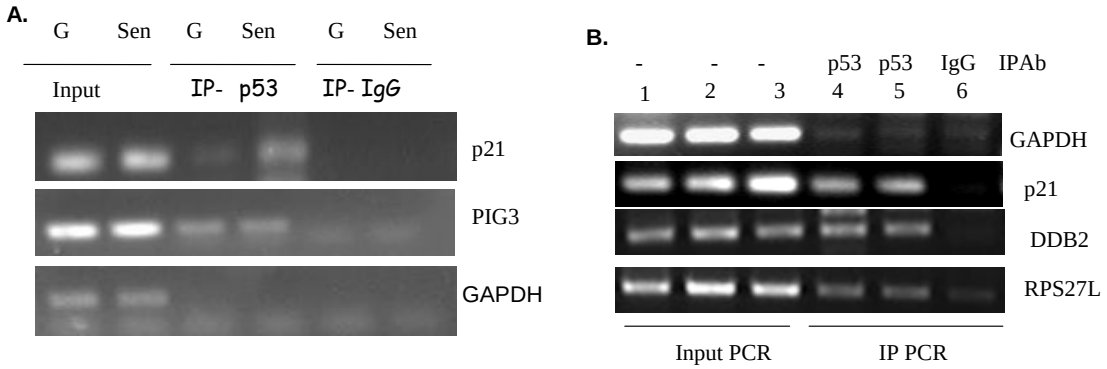


Figure 4: ChIP analysis of p53 targets in senescent HMECs. **A.** p53 targets p21 and PIG3 were analyzed by ChIP in growing (G) and senescent (Sen) cells. **B.** ChIP analysis of p53-IPed Chromatin using primers specific for p21, DDB2 and RPS27L. Lane 1 and 4 -76N senescent cells, lane 2, 3, 5 and 6- adriamycin treated (to induce p53) 76N hTERT cells. Lane 4 and 5 are IPed with p53 antibody. GAPDH is a negative control for ChIP PCR. IP-IgG is a negative control for ChIP assay.

Aim 3: Identification of potential p53 targets in senescent HMECs using ChIP cloning approach-
Next, the p53-IPed chromatin from senescent HMECs 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 putative p53 binding site that were identified are summarized in table 2.

Table 2

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

Aim 4: During the no cost extension period of this CDA, we added aim 4 and studied the role of p53/p21 pathway in oncogene-induced senescence (OIS) in HMECs and showed that BMI1 can overcome H-Ras induced OIS that partially depends on p53. To understand the role of p53 in OIS in HMECs, we overexpressed H-Ras in MCF10A cells. We also overexpressed BMI1 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. 5A and 5B). 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. 6A and 6B). Bmi-1 co-overexpression with H-Ras resulted in bypass of H-Ras-mediated OIS (Fig. 6A and 6B) 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. 7).

We further analyzed expression of p53 targets in H-Ras, BMI1 and H-Ras+BMI1 overexpressing cells to determine the role of p53 pathway in transformation of HMECs and cooperation between H-Ras and BMI1 in transforming HMECs via downregulation of p53 and inhibition of OIS. For the detailed analysis of the p53 pathway, MCF10A control and MCF10A- derived cells were treated with the DNA damaging agent Camptothecin (CPT) (500 nM) for the indicated amount of time (Fig. 8). Next, the expression of p53, phospho-p53 and p53 target genes was studied by Western blot analysis. The results indicated that p53 induction by CPT was compromised in H-Ras and BMI1+H-Ras expressing late passage MCF10A cells (Fig. 8). Further analysis of phospho-p53 indicated that MCF10A-H-Ras (LP) and MCF10A-Bmi-1+H-Ras (LP) cells were defective in phosphorylation of p53 at Ser-15 and Ser-37 residues (Fig. 8). MCF10A-BMI1 cells also had a reduced phosphorylation of p53 at Ser-15 (Fig. 8).

Next, we studied the induction of p21 and PUMA (p53 upregulated modulator of apoptosis), two well known transcriptional targets of p53, which are associated with p53 function such as apoptosis and senescence. Our results indicated that both p21 and PUMA induction by CPT is severally compromised in H-Ras overexpressing MCF10A cells (Fig. 8). We also examined expression of Bax and PIG3 (p53 inducible gene 3), two other known targets of p53. Analysis of these two genes indicated that Bax is expressed at very low levels and is inducible in control MCF10A cells. However, H-Ras expressing MCF10A cells had higher levels of Bax, which were not inducible by DNA damage (Fig. 8). Among all four cell types, MCF10A-H-Ras (LP) cells also expressed high BCL2, which may be related to transformed properties of these cells. PIG3, which usually have a delayed kinetics of induction by p53, was not inducible within the time frame used in our experiments in any of the cell type. Interestingly, compared to MCF10A control cells, PIG3 was downregulated in MCF10A-H-Ras, MCF10A-Bmi-1 and MCF10A-Bmi-1+H-Ras cells, with the later cells showing the most dramatic downregulation of PIG3 (Fig. 8). These findings are published in Datta et al. [13].

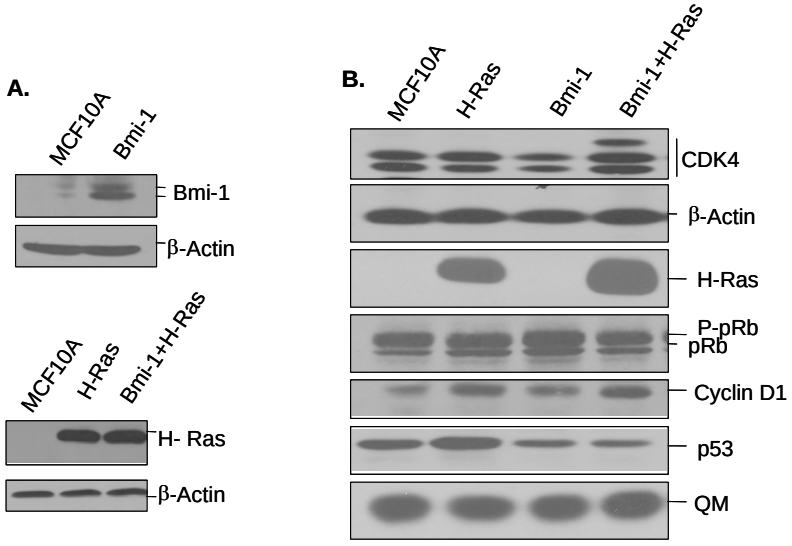


Figure 5: Generation of MCF10A cells overexpressing Bmi-1 (A, upper panel) and overexpressing H-Ras and Bmi-1+H-Ras (A, lower panel). MCF10A control and MCF10A-derived cells were studied for p53, pRb, CDK4, And Cyclin D1. Results show upregulation of p53 in H-Ras expressing cells, while Bmi-1 and Bmi-1+H-Ras cells showed p53 downregulation suggesting that Bmi-1 downregulates p53. QM is a loading control.

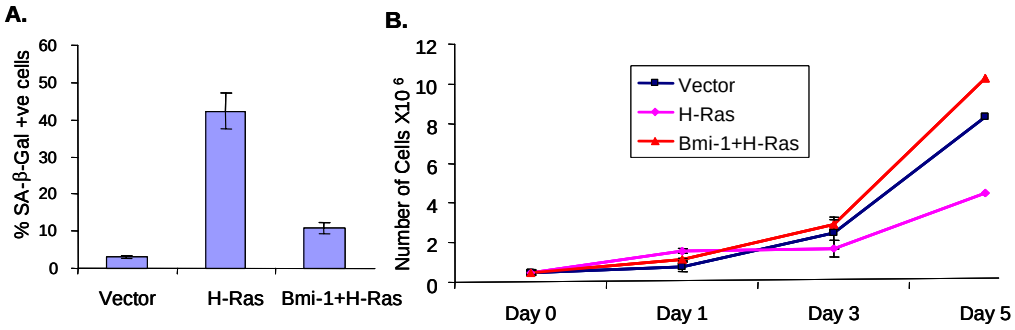


Figure 6: H-Ras overexpression in MCF10A leads to partial OIS (Oncogene Induced Senescence), which is compromised by Bmi -1 overexpression. **(A)** vector control or MCF10A cells expressing H -Ras alone or co-overexpressing Bmi-1 and H-Ras at passage 2 after selection were plated in multiwell plates, 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.

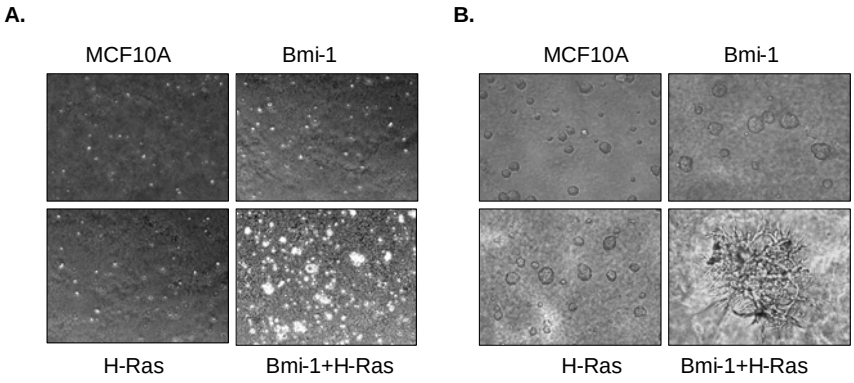


Figure 7: Transformed phenotype of MCF10A cells expressing Bmi-1+H-Ras. (A) MCF10A and MCF10A cells expressing H-Ras alone, Bmi-1 alone or Bmi-1 together with H-Ras (as indicated), at passage two (after Ras selection) were analyzed under light microscope for anchorage-independent growth using soft-agar 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).

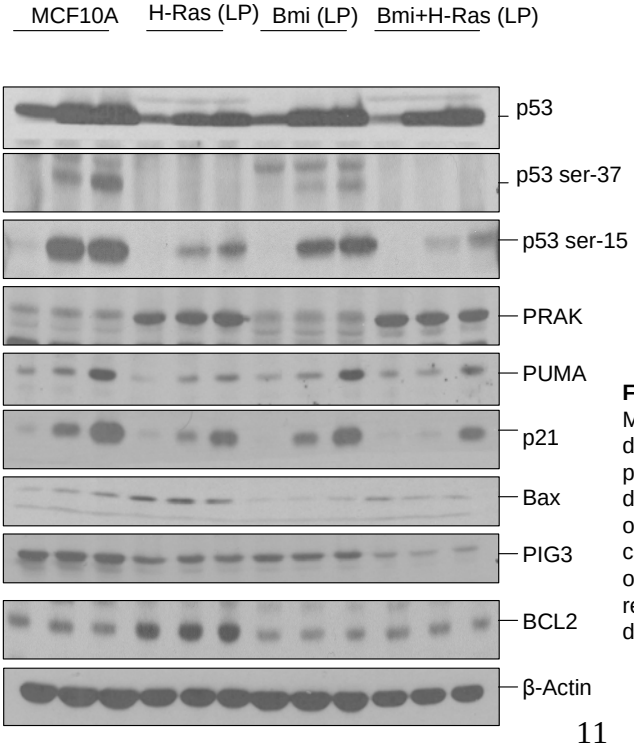


Figure 8: MCF10A-H-Ras and MCF10A-Bmi-1+H-Ras (LP) cells have defect in Ras-mediated p53 inducing pathway, and exhibit attenuated DNA damage response. Western blot analysis of p53 target genes after treatment with camptothecin (CPT) for indicated amount of time was performed to determine p53 response in control MCF10A and MCF10A derived cells (as indicated)

KEY RESEARCH ACCOMPLISHMENTS:

The Career Development Award "DAMD17-02-1-0509" from USAMRMC has been instrumental in advancing my academic career. The key research accomplishments and findings during the entire period of the CDA are as following-

- p53 DNA binding activity increases with senescence in post-selection HMECs.
- p53 levels and its transcription activity as determined by examining the level of its target gene p21 increases with senescence in post- but not pre-selection HMECs.
- There are no significant posttranslational changes in p53 during senescence in HMECs as determined by a limited set of antibodies.
- Stable downregulation of p53 and/or p21 using RNAi approach significantly extends replicative life span of HMECs.
- Stable downregulation of p53 is more effective than p21 downregulation in extending the replicative life span of HMECs.
- Several p53 target genes are differentially expressed in senescent verses proliferating HMECs. In general, p53 targets are overexpressed in senescent HMECs.
- p53 mediates H-Ras induced premature senescence (OIS) in HMECs.
- Bmi-1 overexpression overcomes p53-medaited OIS induced by H-Ras.
- 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:

Following publications resulted during the course of this CDA, which were partially supported by DAMD17-02-1-0509 award-

Peer Reviewed Publications:

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

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

3. **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.

4. 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.

5. 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.

6. 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.** 67:10286-10295.

7. Hoenerhoff, M.J., Chu, I., Datta, S., **Dimri, G.P.** and J.E. Green (2009) BMI1 Cooperates with H-RAS to Induce an Aggressive and Metastatic Phenotype with Spontaneous Brain Metastases. **Oncogene** 28: 3022-3032.

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.
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.

Editorial:
Dimri, G.P. (2008) In Search of Breast Cancer Culprits: Suspecting the Suspected and the Unsuspected. **Breast Cancer: Basic and Clinical Research.** 2008:1 1-5.

Abstract:
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.

CONCLUSIONS:
p53 an important mediator of cellular senescence, which plays a role in telomere length dependent senescence in post-selection HMECs. DNA binding and transcription activation activity og p53 increases with passage number in post-selection HMECs. Several of p53 target genes are upregulated in post-selection senescent HMECs. Accordingly, p53 knockdown bypasses senescence and extends replicative life span of post-selection HMECs. p53 is also induced during oncogene induced senescence (OIS) as an anti-onoogenic response. In order to become transformed, HMECs 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, and its abrogation is required for full transformation of HMECs and development of breast cancer.

REFRENCES:

1. Dimri, G., H. Band, and V. Band, *Mammary epithelial cell transformation: insights from cell culture and mouse models.* Breast Cancer Res, 2005. **7**(4): p. 171-9.
2. Dimri, G.P., *What has senescence got to do with cancer?* Cancer Cell, 2005. **7**(6): p. 505-12.
3. Hofseth, L.J., S.P. Hussain, and C.C. Harris, *p53: 25 years after its discovery.* Trends Pharmacol Sci, 2004. **25**(4): p. 177-81.
4. Levine, A.J., W. Hu, and Z. Feng, *The P53 pathway: what questions remain to be explored?* Cell Death Differ, 2006. **13**(6): p. 1027-36.
5. el-Deiry, W.S., *Regulation of p53 downstream genes.* Semin Cancer Biol, 1998. **8**(5): p. 345-57.
6. Rozan, L.M. and W.S. El-Deiry, *p53 downstream target genes and tumor suppression: a classical view in evolution.* Cell Death Differ, 2007. **14**(1): p. 3-9.
7. Lim, Y.P., et al., *The p53 knowledgebase: an integrated information resource for p53 research.* Oncogene, 2007. **26**(11): p. 1517-21.

8. Itahana, K., G. Dimri, and J. Campisi, *Regulation of cellular senescence by p53.* Eur J Biochem, 2001. **268**(10): p. 2784-91.
9. Itahana, K., et al., *A role for p53 in maintaining and establishing the quiescence growth arrest in human cells.* J Biol Chem, 2002. **277**(20): p. 18206-14.
10. Lacroix, M., R.A. Toillon, and G. Leclercq, *p53 and breast cancer, an update.* Endocr Relat Cancer, 2006. **13**(2): p. 293-325.
11. Dimri, G.P., et al., *A biomarker that identifies senescent human cells in culture and in aging skin in vivo.* Proc Natl Acad Sci U S A, 1995. **92**(20): p. 9363-7.
12. 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.
13. Datta, S., et al., *Bmi-1 cooperates with H-Ras to transform human mammary epithelial cells via dysregulation of multiple growth-regulatory pathways.* Cancer Res, 2007. **67**(21): p. 10286-95.

CURRICULUM VITAE

GENERAL INFORMATION

Name: Goberdhan P. Dimri, Ph.D.

Office Address: Department of Medicine, Room 304,
NorthShore University HealthSystem Research Institute
1001 University Place, Evanston, IL 60201

Phone: (224) 364-7521

Fax: (224) 364-7402

E-mail: Office- gdimri@northshore.org; g-dimri@northwestern.edu; gdimri@bsd.uchicago.edu;

EDUCATION AND TRAINING

Year	Degree (Field)	Institution
1981:	B.Sc. (Chemistry, Botany, Zoology)	University of Garhwal, Srinagar, India
1984:	M.Sc. (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.)

PROFESSIONAL APPOINTMENTS, SERVICES AND ACHEIVMENTS

ACADEMIC APPOINTMENTS:

Year	**Academic Title**	**Institution**
1995-1999:	Scientist	Lawrence Berkeley National Laboratory, Berkeley, CA (Mentor, Judith Campisi, Ph.D.)
1999-2003:	Assistant Professor of Radiation Oncology	Tufts University-New England Medical Center (NEMC) Boston, MA
2003-2009:	Assistant Professor of Medicine	Division of Hematology and Oncology, Feinberg School of Medicine, Northwestern University, Chicago, IL
2003-Present:	Senior Scientist	Divisi on of Cancer Biology, Department of Medicine, NorthShore University HealthSystem Research Institute, (formerly Evanston Northwestern Healthcare Research Institute), Evanston, IL
2008- Present:	Adjunct Professor	Depa rtment of Chemical and Biological Engineering, McCormick School of Engineering and Applied Science, Northwestern University, Evanston, IL

HOSPITAL AND AFFILIATED INSTITUTION SERVICE RESPONSIBILITIES:

Year	**Role**	**Institution**
1999-2003:	Special and Scientific Staff	Tufts-New England Medical Center, Boston, MA
2003-Present	Senior Scientist	NorthShore University Healthsystem Research Institute, Evanston, IL
2003-Present	Invention Disclosure and Patent Review Committee	NorthShore University Healthsystem, Evanston, IL

PEER REVIEW SERVICES:

2005-2006:	Ad-hoc Member	NIH "Cell Biology Fellowship" Study Section FO5
2005:	Member	DOD BCRP (Breast Cancer Research Program) Cell Biology 3 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 Genetics of	Pilot Research Projects for "Center for and Molecular Medicine', Univ. Louisville, KY
2006:	Mail Reviewer	The Italian Association for Cancer Research" (AIRC), Milan, Italy
2006:	Mail Reviewer Research Council)	Netherlands Organization for Scientific (NOW, the Dutch Research
2006:	External Evaluator Assistant UCLA	Faculty Appointments (Tenure-Track and Associate Professors), School of Dentistry, Los Angeles, CA
2007:	Mail Reviewer DOD	Ovarian Cancer Research Program, Concept Grants
2007:	Mem ber DOD	BCRP Training #4 Review Panel
2007:	Teleconference Reviewer	DOD BCRP Epidemiology Review Panel
2007:	Mail Reviewer Research	Association for International Cancer (AICR), St. Andrews, UK
2007:	Mail Reviewer	Medical Research Council (MRC), UK
2008:	Mem ber (BDP)	Biomarker-based Diagnosis & Prognosis Review Panel, Susan G. Komen for the Cure Grants (Breast Cancer)
2008:	Ad-hoc Member	NIH Study Section "ZRG1 F05, Cell Biology Fellowships"
2008:	Mail Reviewer	Medical Research Council (MRC), UK

2008: Member USAMRMC BCRP (Breast Cancer Research Program) Training #4 Review Panel

2008: Online Reviewer USAMRMC BCRP Concept- Molecular Biology and Genetics #3 grants

2008: Mail Reviewer Research Grant Council (RGC), Hong Kong, China

2008: Mail Reviewer Cancer Research UK, London, UK

2008: Mail Reviewer The Willy Gepts Research Foundation (UZ Brussels), Brussels, Belgium.

2008: Ad-hoc Member Special Emphasis Panel (NIAID/NIH): ZAI1-BDP-I(J2), RFA-AI-08-012 (Rejuvenating the Aged Immune System)

2009: Online Reviewer USAMRMC BCRP Concept- Molecular Biology and Genetics #3 grants

2009: Member USAMRMC BCRP Postdoctoral Fellowship Review Panel #9

2009: Mail Reviewer Health Research Board (HRB), Health Research Awards 2009, Dublin, Ireland

2009: Reviewer Pilot Grants, NorthShore University HealthSystem, Evanston, IL

2009: Mail Reviewer Research Grant Council (RGC), Hong Kong, China

2009: Mail Reviewer NIH Special Emphasis panel ZRG1 BDA-A 52R (hESC Challenge Grant Review Panel)

EDITORIAL BOARDS AND JOURNAL PEER REVIEW SERVICES:

2001- Present: Editorial Board Biogerontology

2006-Present: Editorial Board Research & Reviews in BioSciences

2007-Present: Editorial Board The Open Geriatric Medicine Journal

2008-Present:	Editorial Board	The Open Cell Signaling Journal
2008-Present:	Editorial Board	Breast Cancer: Targets and Therapy
2007- Present:	Editor-in-Chief	Breast Cancer: Basic and Clinical Research (www.la-press.com)
2008-Present:	Editor-in-Chief	Clinical Medicine: Women's Health

Ad-hoc Reviewer- Exp. Cell Res., J. Geron., Cancer Res., Biogerontology, Cancer Letters, J. Biol. Chem., Mol. Biol. Cell, J. Mol. Cell Life Sciences, Mech., Aging and Develop., 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, Proc. Natl. Acad. Sci. USA, Clin. Cancer Res., BBA, J. Cell Mol. Med., Cell Death & Differentiation, Aging Cell, BMC series of Journals, Cancer Cell, etc.

PROFESSIONAL SOCIETIES:

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

AWARDS AND HONORS:

Year	**Award**	**Institution**
1982-1984:	Merit Scholarship	J. N. University, New Delhi, India
1984-1986:	Junior Research Fellowship New	University Grants Commission, Delhi, Commission India
1987-1989:	Senior Research F ellowship New	University Grants Commission, 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"

ADVISORY AND SUPERVISORY ROLES

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

2006-2009: Dr. Rachana Sainger, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

2006-Present: Dr. Anag Saharsabuddhe, Postdoctoral Fellow, Evanston Northwestern Healthcare Research Institute, Evanston, IL

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

2006-2007: Hema RamKumar, Pre-Med Undergraduate Student, Northwestern University

2007-2009: Hema RamKumar, M.D. Student, Feinberg School of Medicine Northwestern University

2007-2009: Rahul Kataria, Undergraduate (Biomedical Eng.), Northwestern University

2007-2008: Amanda Gawin, Undergraduate Student (Biological Sciences), Northwestern University

2008, 2009: Adam Gluskin, Summer Student, Univ. Illinois, Urbana/Champaign, IL

2009: Justin Ornatowski, Summer Student, Northwestern University. Evanston, IL

2008-2009: Prasad Rote, M.S. (Biotechnology) student, McCormick School of Engineering and Applied Science, Northwestern University, Evanston, IL

FUNDING INFORMATION:

Active grants

1. Title: Role of BMI in Breast Cancer
Role: PI
Funding Agency and Grant Number: NCI/NIH; 2RO1 CA094150-07

Duration: 10/01/09-09/30/14

2. Title: Deconstructing MYC-induced transformation of human breast epithelial cells
Role: PI
Funding Agency and Grant Number: USAMRMC/DoD; BC085511

Duration: 09/15/09-09/14/10

Pending:

1. Title: The role of Polycomb Group proteins in MYC-induced oncogenesis
Role: PI
Funding Agency and Grant Number: NIH, 1R01CA138452-01

Duration: 5 years (pending)

2. Title: Defining the role of Polycomb Group protein EZH2 in c-Myc-induced breast oncogenesis
Role: PI
Funding Agency and Grant Number: KG100183, Susan G. Komen for the Cure Foundation

Duration: 3 year (pending)

3. Title: Targeting polycomb group proteins to prevent and treat early lung cancer development
Role: PI
Funding Agency and Grant Number: USAMRMC, DoD; LC090032

Duration: 1 year (pending)

Past:

1. Title: Senescence-specific Promoter Vectors
Role: PI
Funding Agency and Grant Number: NIH, AG165851-01

Duration: 03/01/99-02/28/01

2. Title: Role of Bmi-1 in Telomerase Regulation and Breast Cancer
Role: PI
Funding Agency: The Charlotte Geyer Foundation

Duration: 10/01/02-06/30/03

3. Title: Polycomb Proteins and Breast Epithelial Cell Transformation
Role: PI
Funding Agency and Grant Number: USAMRMC, DOD- BC032256

Duration: 07/01/04-06/30/05

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

Duration: 05/01/02-04/30/07

5. Title: Role of Bmi-1 in Telomerase Regulation and Breast Cancer
Role: PI
Funding Agency and Grant Number: NCI/NIH, 1RO1 CA094150-01

Duration: 09/17/03-06/30/08

6. Northwestern University Summer (2008) Undergraduate Research Grant () for Mr. Rahul Kataria

7. Northwestern University Summer (2008) Undergraduate Research Grant () for Ms. Amanda Gawin

8. Northwestern University Summer (2009) Undergraduate Research Grant () for Mr. Justin Ornatowski

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

2008: "Cellular Senescence and Skin Aging" Skin Summit II Conference (organized by Pharmaceutical Associates, LLC), Feb 20-21, Park Hyatt, Philadelphia, PA

2008: "Polycomb Protein BMI1 in Aging and Cancer", Univ. of Illinois, Chicago, IL

2008: "Polycomb Group proteins in cell proliferation and senescence", Epithelial Cell Biology Seminars, The Robert H. Luire Cancer Center, Northwestern University, Chicago, IL

2009: Control of cell proliferation and oncogenesis by the Polycomb Group protein BMI1, Virginia Commonwealth University, Richmond, VA

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 p14ARFtumor 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.

20. Itahana, K., Ying , Z., Itahana, Y, Martinez, J. L., Beausejour, C., Jacobs, J. L., van Lohuizen, M., Band, V., Campisi, J. and **G. P. Dimri** (2003) Control of replicative senescence in human fibroblast by p16 and the polycomb protein Bmi-1. **Mol. Cell. Biol.** 23: 389-401.

21. Meng, G., Zhao, Y., Nag, A., Zeng, M., **Dimri, G.**, Gao, Q., Wazer, D.E., Kumar, R., Band, H., and V. Band, (2004) Human ADA3 binds to estrogen receptor (ER) and functions as a coactivator for ER-mediated transactivation. **J. Biol. Chem.** 279: 54230-5440.

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.

23. Song, L-B., Zeng, M.-S., Liao, W-T., Zhang, L., Mo, H-Y., Liu, W.-L., Shao, J-Y., Wu, Q-L., Li, M Z., Xia, Y-H., Fu, L-W., Huang, W.-L., **Dimri, G**., Band, V. and Zeng, Y -X. (2006). Bmi-1 is a novel molecular marker of nasopharyngeal carcinoma progression and immortalizes primary human nasopharyngeal epithelial cells. **Cancer Res.** 66: 6225-6232.

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.

28. Zhao, Y., Katzman, R.B., Delmolino, L.M., Bhat, I., Zhang, Y., Gurumurthy, C.B., Reddi, H.V., Solomon, A., Zeng, M.S., Kung, A., Ma, H., Gao, Q., **Dimri, G.**, Stanculescu, A., Miele, L., Wu, L., Griffin, J.D., Wazer, D.E., Band, H. and V. Band. (2007) The notch regulator MamL1 interacts with p53 and functions as a coactivator. **J. Biol. Chem.** 282(16):11969-11981.

29. Dimri, M., Naramura, M., Duan, L., Chen, J., Cesar, O-Cava, Gengsheng, C., Goswami, R., Fernandes, N., Gao, Q., **Dimri, G. P.**, Band, V. and H. Band (2007) 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.

30. 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.

31. Lee, k., Adhikary, G., Balasubramanian, S., Gopalakrishnan, R., McCormick, T., **Dimri, G. P.**, Eckert, R. L. and E. A. Rorke (2007) Expression of Bmi-1 in Epidermis Enhances Cell Survival by Altering Cell Cycle Regulatory Protein Expression and Inhibiting Apoptosis. **J. Invest. Dermatol.** 128: 9-17.

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.** 67:10286-10295.

33. Li, S.K.M., Leung, W. Y., Smith, D., Cheung, A. M. S., Lam, E. W.-F., **Dimri, G. P.** and K. -M., Yao (2008) FoxM1C counteracts oxidative stress-induced senescence in mouse embryonic fibroblasts by up- regulation of Bmi-1 expression. **J. Biol. Chem.** 283: 16545-16553.

34. Klimova, T.A., Bell, E.L., Shroff, E.H., Weinberg, F.D., Synder, C.M., **Dimri, G.P**., Schumacker, P.T. , Budinger, G.R. and N.S. Chandel (2009) Hyproxia-induced premature senescence requires p53

and pRb but not mitochondrial matrix ROS. **FASEB J.** 23: 783-94.

35. Hoenerhoff, M.J., Chu, I., Datta, S., **Dimri, G.P.** and J.E. Green (2009) BMI1 Cooperates with H-RAS to Induce an Aggressive and Metastatic Phenotype with Spontaneous Brain Metastases. **Oncogene** 28: 3022-3032.

36. Dimri, M., Bommi, P., Sahrsabuddhe, A.A, Khandekar, J.D. and **G.P. Dimri** (2009) Dietary omega-3 polyunsaturated fatty acids suppress expression of EZH2 in breast cancer cells. **Carcinogenesis** Dec 7 [Epub ahead of print]

37. Ho, C., Lee, S., Xu, C.-R., Bommi, P., Huang, S.-A., Cheung, S.T., **Dimri, G.P.** and X. Chen (2009) Bmi1 functions as an oncogene independent of Ink4A/Arf repression in hepatic carcinogenesis. **Mol. Cancer Res.** Nov 24 [Epub ahead of print]

38. Zhang, X.-W, Sheng, Y., Lu, Y., Li, Q., Qin, W., Liu, B., Li, J., Chen, Y. F., Zhang, F., Lin, J., **Dimri, G.P.** and W.-J. Guo (2009) BMI1 and Mel-18 oppositely regulate development and progression of gastric cancer. Submitted.

39. Bommi, P., Dimri, M., Sahrsabuddhe, A.A, Khandekar, J.D. and **G.P. Dimri** (2009) Polycomb group protein BMI1 is a target of HDAC inhibitors. Submitted.

Editorial

40. **Dimri, G.P.** (2008) In Search of Breast Cancer Culprits: Suspecting the Suspected and the Unsuspected. **Breast Cancer: Basic and Clinical Research.** 2008:1 1-5.

Peer Reviewed Reviews, Invited Reviews and News and Views

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

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

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

43. **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.

44. **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.

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

46. **Dimri, G.P.** (2009) c-Myc and Telomerase activation. **Cell Cycle** 8: 3075-3076.

Proceedings and Book Chapters:

47. 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.

48. **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.

49. 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.

50. 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.

51. Itahana, K. and **G. P**. **Dimri.** Senescence and Cancer. In **Encyclopedia of Public Health.** Elsevier Ltd, Oxford, UK, pp 391-39.

52. 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, pp 129-148.

Selected 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.

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

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

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

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

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

Review

Mammary epithelial cell transformation: insights from cell culture and mouse models

Goberdhan Dimri[1], Hamid Band[2] and Vimla Band[1]

[1]Division of Cancer Biology, Department of Medicine, ENH Research Institute, and Robert H Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Evanston, Illinois, USA
[2]Division of Molecular Oncology, Department of Medicine, ENH Research Institute, and Robert H Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Evanston, Illinois, USA

Corresponding author. Vimla Band, v-band@northwestern.edu

Published: 3 June 2005
This article is online at http://breast-cancer-research.com/content/7/4/171

Breast Cancer Research 2005, **7:**171-179 (DOI 10.1186/bcr1275)

Abstract

Normal human mammary epithelial cells (HMECs) have a finite life span and do not undergo spontaneous immortalization in culture. Critical to oncogenic transformation is the ability of cells to overcome the senescence checkpoints that define their replicative life span and to multiply indefinitely – a phenomenon referred to as immortalization. HMECs can be immortalized by exposing them to chemicals or radiation, or by causing them to overexpress certain cellular genes or viral oncogenes. However, the most efficient and reproducible model of HMEC immortalization remains expression of high-risk human papillomavirus (HPV) oncogenes E6 and E7. Cell culture models have defined the role of tumor suppressor proteins (pRb and p53), inhibitors of cyclin-dependent kinases ($p16^{INK4a}$, p21, p27 and p57), $p14^{ARF}$, telomerase, and small G proteins Rap, Rho and Ras in immortalization and transformation of HMECs. These cell culture models have also provided evidence that multiple epithelial cell subtypes with distinct patterns of susceptibility to oncogenesis exist in the normal mammary tissue. Coupled with information from distinct molecular portraits of primary breast cancers, these findings suggest that various subtypes of mammary cells may be precursors of different subtypes of breast cancers. Full oncogenic transformation of HMECs in culture requires the expression of multiple gene products, such as SV40 large T and small t, hTERT (catalytic subunit of human telomerase), Raf, phosphatidylinositol 3-kinase, and Ral-GEFs (Ral guanine nucleotide exchange factors). However, when implanted into nude mice these transformed cells typically produce poorly differentiated carcinomas and not adenocarcinomas. On the other hand, transgenic mouse models using ErbB2/neu, Ras, Myc, SV40 T or polyomavirus T develop adenocarcinomas, raising the possibility that the parental normal cell subtype may determine the pathological type of breast tumors. Availability of three-dimensional and mammosphere models has led to the identification of putative stem cells, but more studies are needed to define their biologic role and potential as precursor cells for distinct breast cancers. The combined use of transformation strategies in cell culture and mouse models together with molecular definition of human breast cancer subtypes should help to elucidate the nature of breast cancer diversity and to develop individualized therapies.

Introduction

More than 80% of adult human cancers are carcinomas, tumors originating from malignant transformation of epithelial cells. However, much of our understanding of oncogenic transformation comes from fibroblast transformation systems. Breast cancer is the second leading cause of cancer-related deaths among women in the USA [1]. The vast majority of breast cancers are carcinomas that originate from cells lining the milk-forming ducts of the mammary gland (for review [2]). Deliberate transformation of these cells provides a practical window into human epithelial oncogenesis. Malignant transformation represents a complex multistep process in which genetic, environmental, and dietary factors together are thought to alter critical cell growth regulatory pathways resulting in uncontrolled proliferation, which is a hallmark of tumorigenesis [3,4]. Understanding the nature of these cellular pathways is of central importance in cancer biology.

The growth of normal human mammary epithelial cells (HMECs), which include luminal, myoepithelial and/or basal cells (described below), is tightly controlled. These cells grow for a finite life span and eventually senesce (for review [5-7]). Both cell culture and mouse models have provided evidence that essential initial steps in tumorigenesis involve the loss of senescence checkpoints and immortalization, which allow a cell to grow indefinitely and to go through further oncogenic steps, resulting in fully malignant behavior. In addition, cell culture model systems have identified a number of genes whose alterations are involved in HMEC immortalization and thereby have provided significant insights into the biology of early breast cancer [5,7,8]. Use of oncogene combinations has allowed researchers to create cell culture models of full HMEC transformation, thereby illuminating the process of

ASMA = α-smooth muscle actin; CDK = cyclin-dependent kinase; COX = cyclo-oxygenase; ER = estrogen receptor; ESA = epithelial-specific antigen; HMEC = human mammary epithelial cell; HPV = human papillomavirus; hTERT = catalytic subunit of human telomerase; PD = population doubling; Ral-GEF = Ral guanine nucleotide exchange factor; TDLU = terminal ductal–lobular unit.

breast cancer progression [9-11]. Additional insights have come from mouse models, using transgenic overexpression of oncogenesis-promoting genes and deletion of tumor suppressor genes, which often produce breast adenocarcinomas that closely resemble human breast cancers.

Studies using cell culture transformation models have pointed to the existence of HMEC subtypes with distinct susceptibilities to oncogenesis by different oncogenes [5,8]. Remarkably, direct cDNA microarray profiling of human breast cancers has led to similar insights, identifying multiple subtypes of human breast cancer with distinct outcomes; phenotypic and genotypic characteristics of these breast cancer subtypes point to their possible origin from specific subtypes of HMECs, such as basal or luminal cells [12]. Finally, cell culture and mouse model systems have begun to identify mammary stem cells that may provide progenitors for oncogenic transformation [13] and have led to an appreciation of the microenvironment for oncogenesis [14,15].

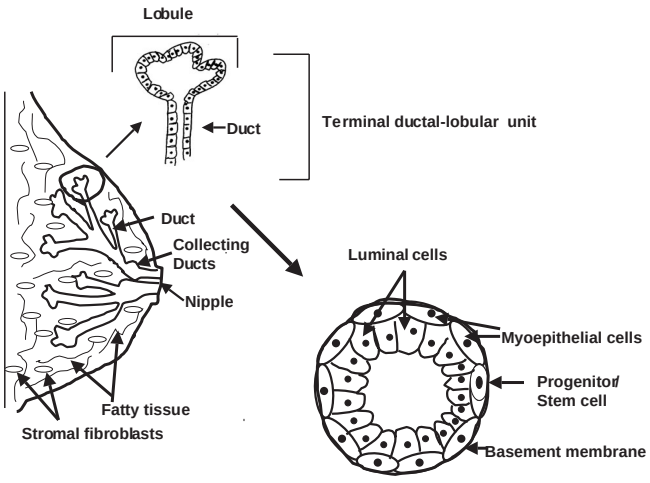
Thus, studies conducted over the past several years have established the importance of HMEC transformation models to our understanding of the pathways that control normal mammary cell growth, development, and oncogenesis. However, many challenges remain, including the identification of mammary cell subtypes or oncogenic strategies that result in cancers that resemble naturally occurring human breast cancers, and translation of new research to devise more specific diagnostic and treatment strategies for different subtypes of breast cancer.

Mammary gland and various epithelial cell subtypes

The mammary gland consists of a branching ductal system that ends in terminal ducts with their associated acinar structures, termed the terminal ductal–lobular units (TDLUs), together with interlobular fat and fibrous tissue [16,17]. Most breast cancers arise in the TDLU (Fig. 1). Unlike other epithelial cancers, such as that of colon, different stages of breast cancer are not clearly defined. However, it is clear that benign stages (such as typical and atypical hyperplasia), noninvasive cancers (such as carcinoma *in situ* – ductal or lobular), and invasive cancers (such as invasive ductal or lobular carcinomas) do exist. Additionally, multiple types of *in situ* carcinomas, such as solid, cribiform, papillary and comedo types, have been reported and it is possible that these represent tumors originating from different epithelial subtype [16,17].

Histological examination of TDLU reveals two major types of cells: inner secretory luminal cells and outer contractile myoepithelial cells (Fig. 1). In addition to luminal and myoepithelial cells, there is emerging evidence that basal cells (presumed to be the progenitor for myoepithelial cells) and stem cells exist in the TDLU [17,18]. Until recently it was believed that the vast majority of breast carcinomas arise from luminal epithelial cells [2]. This was based on the keratin expression and other phenotypic markers of cultured tumor cell lines, mostly derived from metastatic lesions [2]. Unfortunately, the great majority of primary breast tumors have proved difficult to establish in cultures, either on plastic or as three-dimensional cultures [5-7,19-21]. However, recent molecular profiling studies clearly show the existence of multiple subtypes of breast cancers probably originating from luminal, basal, and possibly stem cell compartments [12] (described below in detail).

Figure 1



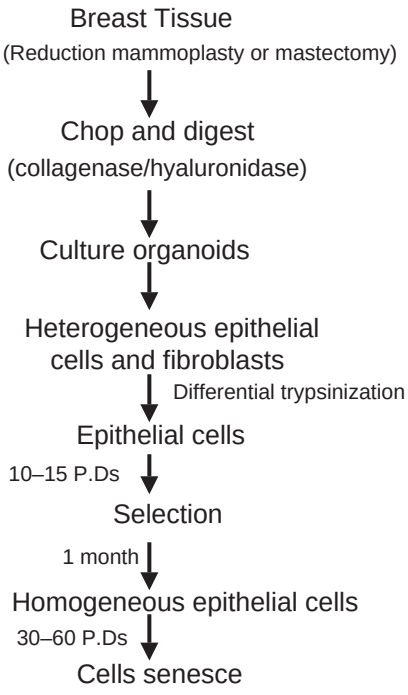
Structure of the mammary gland. Terminal ductal–lobular unit (TDLU), composed of ductal cells, is the unit thought to be the origin of most breast cancer. The stroma is composed of fatty tissue (adipocytes) and fibroblasts. Also shown are the two primary types of cells in normal ducts: outer contractile myoepithelial and inner columnar luminal cells. A putative progenitor/stem cell is also indicated.

Culturing of various epithelial cell subtypes

For more than two decades, various investigators have attempted to develop cell culture models that lead to isolation of breast cancer cells resembling those found in human breast cancers. In order to establish such models, it was essential to culture normal HMECs. In 1980s, work from several laboratories showed that normal HMECs could be cultured in cell culture [22,23] (for review [2,5,7]).

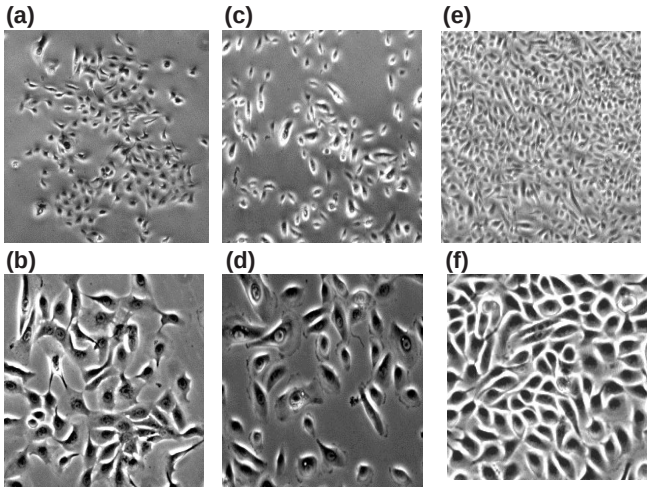
In our laboratory we defined a medium, termed DFCI-1, that helped us to establish and culture normal and some primary breast cancers under identical conditions [20]. However, in general the difficulty in establishing primary tumor cells in cell culture has persisted. Notably, early cultures derived from reduction mammoplasty or mastectomy specimens exhibit considerable heterogeneity (with multiple cell types – luminal, stem cells, basal and myoepithelial cells) and grow for three to four passages or about 15–20 population doublings (PDs), and then senesce (Figs 2 and 3) [5-7]. The senescence in these cells is also termed as M0 stage [24].

Figure 2



Establishment of mammary epithelial cells from reduction mammoplasty/mastectomy specimens. The tissue is chopped, digested with collagenase and hyaluronidase, and plated in medium as organoids. Over a week or so, multiple types of epithelial cells and fibroblasts emerge; fibroblasts are removed by differential trypsinization (fibroblasts are loosely attached), remaining epithelial cells grow for 10–15 population doublings (PDs) followed by senescence of the majority of cells. Occasionally, an homogenous population of cells emerges that continue to proliferate for an additional 30–60 PDs, and eventually these cells also senesce (this step is referred to as agonescence).

Figure 3



Morphological heterogeneity of cells before and after selection. **(a–d)** Two views of mammary epithelial preselection cells (original magnifications: panels a and c, 40×; panels b and d, 100×). Cells shown in panel a grow as compact clusters and are relatively uniform, whereas cells in panel b grow more dispersed and exhibit different types of cells (small and large). **(e,f)** Views of postselection human mammary epithelial cells with relatively uniform morphology (original magnifications: panel e, 40×; panel f, 100×).

However, in some cases (not always) an occasional homogenous cell population emerges that continue to grow further for 30-60 PDs (Figs 2 and 3) [5-7] before senescence occurs (also called agonescence, described below) [25]. This process of emergence of cells that are able to proliferate for extended periods is also known as self-selection; before selection the cells are termed preselection cells, whereas those that emerge after selection are called postselection cells. The keratin profile of preselection cells (K-5, K-6, K-7, K-14, K-17, K-18 and K-19 positive) [8,19,26] suggests the existence of both luminal and basal (myoepithelial) cells. However, postselection cells generally exhibit a loss of expression of K-19 but retain the expression of all other keratins [8,18,25]. These cells also express α-smooth muscle actin (ASMA), suggesting that these may be of myoepithelial origin. Further development of cell sorting techniques and chemically defined media have helped in culturing of luminal and progenitor epithelial cells [14,27] (described below in detail).

It has also been reported that postselection cells lose the expression of $p16^{INK4a}$, a cyclin-dependent kinase (CDK) inhibitor [24,25], and gain expression of cyclo-oxygenase (COX)-2, a gene that is thought to be involved in tumorigenesis [28]. As both of these genes are implicated in oncogenesis, it is conceivable that loss of p16 or gain of COX-2 expression may make these cells more susceptible to transformation, although it is unclear whether the loss of p16 and gain of COX-2 occur *de novo* during self-selection or represent selection of a minor population of cells with pre-existing high COX-2 and low p16 expression. Notably, p16-negative and COX-2-positive cells could be detected using immunohistochemistry in normal mammary tissue [28,29].

Immortalization of various HMEC subtypes in culture

As alluded to above, normal mammoplasty-derived HMECs exhibit a limited life span, which is followed by replicative senescence. Replicative senescence acts as a strong tumor suppressor mechanism and prevents spontaneous immortalization of human cells [30-33]. A major determinant of replicative senescence is the enzyme telomerase, which maintains the length of telomere ends [30,31]. Most somatic cells express little or no telomerase, resulting in telomere shortening with successive cell divisions, which eventually elicits a senescence checkpoint [30-32]. A senescence-like phenotype can also be induced by a variety of nontelomeric signals such as DNA-damaging agents, adverse cell culture conditions, and overexpression of certain oncogenes [30,32]. The tumor suppressor protein p53 and its target gene product p21, and $p16^{INK4a}$ play a crucial role in senescence

induced by telomeric as well as nontelomeric signals [30-33]. Much of our knowledge about senescence comes from studies conducted in human fibroblasts [30-34]. Only recently have we begun to elucidate the mechanisms of senescence in epithelial cells, in particular in HMECs [25].

The senescence associated with the 'selection' phase in HMEC cultures is accompanied by classic features of senescence, such as flat morphology, presence of vacuoles, and positive staining for senescence-associated β-galactosidase (SA-β-gal), a marker of senescence [34]. The block in cell proliferation at this stage is dependent on the pRb/p16 pathway [24,35], because the human papillomavirus (HPV) oncogene E7, which binds and inactivates pRb, can overcome the M0/selection stage [36]. Similarly, a constitutively active p16-insensitive CDK4 mutant can overcome the M0 stage [37]. Thus, senescence of preselection cells appears to be telomere independent. At the end of their replicative life span, postselection HMECs exhibit senescence as well as cell death with a high level of genomic instability. This phenomenon is termed as agonescence, as opposed to replicative senescence [25]. Most importantly, unlike rodent cells, human HMECs derived from reduction mammoplasties or from milk do not exhibit spontaneous immortalization and thus provide suitable models of human cell transformation. Immortalization of HMECs in culture is characterized by their continuous growth beyond the agonescence checkpoint. It is thought that immortalization is an early step in human cancer, and continued proliferation of immortal cells allows the accumulation of additional genetic changes that promote malignant and metastatic behavior.

Stampfer and Bartley [38] presented initial evidence that HMECs could be immortalized in cell culture using benzo(a)-pyrene; however, the immortalization was a rare event in this case. Similar to carcinogen-induced immortalization, we found that γ-radiation induced the transformation of HMECs relatively infrequently [5,8,39]. In general, most viral oncogenes (including SV40 T antigen, adenovirus E1A and E1B, polyoma T antigen) have not proven very efficient as immortalizing genes for human cells [40]. While the introduction of the SV40 T antigen into breast tumor tissue-derived epithelial cells gave rise to immortal cell lines, SV40-transfected cells go through a long crisis period, and emergence of immortal cells is rare [19]. Over the past several years, our studies have defined a system to immortalize human HMECs efficiently and reproducibly, using the urogenital carcinoma-associated HPV oncogenes E6 and E7 [5,8,36].

Comparison of early (preselection) and late-passage (post-selection) cultures revealed that different HMEC subtypes exhibit a remarkably distinct susceptibility to E6 or E7, or their combination [8]. One HMEC subtype was exclusively immortalized by E6 but not by E7; such cells predominated the late-passage cultures but were rare at early passages. Surprisingly, a second cell type, present only in early passages of tissue-derived cultures, showed extension of life span and infrequent immortalization by E7 alone. Finally, E6 and E7 together were required to immortalize fully a large proportion of preselection HMECs [8].

Human milk is an easily available source of relatively pure HMECs that are thought to be differentiated luminal cells [2,19]. However, these cells can be cultured for only a limited number of passages (typically two to three passages, or five to nine PDs), which has precluded their detailed biochemical study [2,18]. Most of the work on milk cells has been carried out in Taylor-Papadimitriou's laboratory and has demonstrated that these cells can be immortalized by SV40 T antigen [41]. Interestingly, neither E6 nor E7 alone could induce the immortalization of milk-derived HMECs, whereas a combination of E6 and E7 was effective [8].

The reproducibility and relatively high efficiency with which E6 (in postselection HMECs) or E6 and E7 combined can induce immortalization of human HMECs have therefore yielded a practical approach to elucidate the biochemical mechanisms of HMEC immortalization. In recent years, using Yeast Two-hybrid analysis, we identified several novel targets of the E6 oncogene in HMECs. These targets represent novel mediator of HMEC immortalization [5]. These include ADA3 (alteration/deficiency in activation 3), a novel coactivator of p53 and steroid receptors (estrogen receptor [ER] and retinoic acid receptor) [42-44]; E6 targeted protein 1 (E6TP1), a novel GTPase activating Rap small G protein; and protein kinase N (PKN), an effector for Rho small G protein [5]. We recently found that MamL1, a human homolog of the *Drosophila* mastermind gene and a known coactivator for Notch [45], also interacts with E6 (I Bhat, V Band, unpublished data). These studies have implicated the p53, Notch, ER, Rho, and Rap signaling pathways in early transformation of human HMECs. Consistent with these analyses, we have shown that expression of mutant p53 [46] or activated Rho (X Zhao, V Band, unpublished data) induces immortalization of HMECs. Furthermore, several studies support a role for p53 mutations as an early event in breast cancer [47]. Taken together, these studies demonstrate that E6 is the most efficient immortalizing gene for postselection HMECs and that E6 immortalizes the HMECs by concurrently altering multiple biochemical pathways. Future studies will need to address the precise role played by these novel oncogene targets in early breast cancer.

In addition to viral oncogenes, alterations in the expression of cellular genes can also help to overcome senescence and promote HMEC immortalization. Among the cellular genes, we recently reported that Bmi-1, a member of the polycomb group of transcriptional repressors, could immortalize postselection HMECs [48]. Although the detailed mechanism of immortalization induced by Bmi-1 remains to be explored, Bmi-1 does not appear to immortalize these cells by down-regulating the INK4a/ARF locus. Interestingly, recent studies have implicated Bmi-1 in stem cell function and renewal

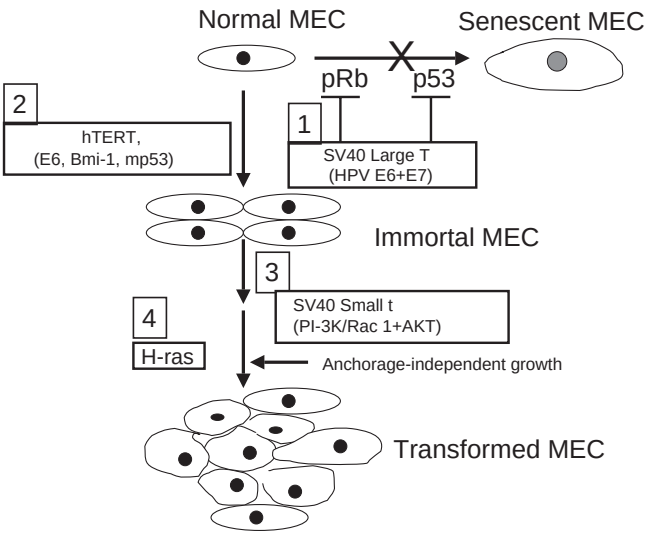
[49,50], suggesting that Bmi-1 could function as a potential breast cancer stem cell marker [50]. Another study showed that ZNF217, a zinc finger protein that is overexpressed in breast cancers, can promote immortalization of postselection HMECs [51]. Furthermore, introduction of hTERT also induces immortalization of postselection cells [5]. Interestingly, induction of telomerase has been documented early after E6 was introduced into HMECs [52], although the cause and effect relationship between telomerase induction and E6-induced immortalization continues to be debated. Recently, the E6 and E6–AP binding protein NFX-91 was implicated in E6-mediated induction of telomerase [53].

Cell culture models of full transformation of HMECs

The ability of researchers to establish normal HMECs and to induce their reproducible immortalization has provided momentum for further efforts to define the nature of biochemical alterations that can lead to full oncogenic transformation. As we and others have demonstrated, HMECs immortalized by most currently known procedures (such as E6 or E6 plus E7, mutant p53, Bmi-1 and hTERT) are preneoplastic and do not grow in an anchorage-independent manner or produce tumors when implanted in immune-deficient mice [5,8]. Weinberg and colleagues [9] recently established a multistep model of full HMEC transformation in cell culture by serial introduction of SV40 large T and small t, hTERT, and activated Ras (Fig. 4). It was shown that introduction of the SV40 large T, which binds and inactivates p53 and pRb, abolished senescence, whereas hTERT was needed to promote immortalization [9]. Notably, these studies showed an essential role for the SV40 small t, which inhibits protein phosphate 2A [54]. HMECs transformed by this method exhibited anchorage independence and produced poorly differentiated carcinoma (but not adenocarcinoma) when implanted in nude mice [9]. Further dissection of the role of small t revealed the importance of the downstream targets of phosphatidylinositol 3-kinase, Akt1 and Rac1, and direct activation of these pathways could fully substitute for small t in the transformation assays [10]. A recent refinement of the transformation in cell culture scheme suggests that perturbation of p53, pRb, protein phosphate 2A, telomerase, Raf, and Ral guanine nucleotide exchange factor (Ral-GEF) pathways are required for the full tumorigenic conversion of normal human cells [11]. The requirement in terms of modulating Raf and Ral-GEF pathways is cell type specific; HMECs require activation of Raf, phosphatidylinositol 3-kinase and Ral-GEFs, whereas human fibroblasts require the activation of Raf and Ral-GEFs [11]. Thus, serial use of viral and/or cellular genes is beginning to unravel the various combinations of genetic lesions that can convert a completely normal mammary epithelial cell into a fully tumorigenic one.

Although these studies have thus far relied on the use of known oncogenes, future studies using the cell culture transformation models with gene libraries should help identify novel cellular genes that participate at various steps of breast cancer progression. Vast majority of human breast cancers are adenocarcinomas, and only a small portion of breast cancers are poorly differentiated carcinomas. Hence, it appears that HMEC transformation in culture system is not optimal because the tumors produced by these transformed HMECs have usually been poorly differentiated carcinomas rather than adenocarcinomas. Breast cancer is associated with overexpression of various cellular proto-oncogenes such as ErbB2, epidermal growth factor receptor, Src family kinases, Bmi-1, cyclin D_1, cyclin E, CDK4, and other potential growth regulators. Use of these oncogenes in the multistep model described above and the use of other HMEC subtypes (such as luminal cells, potential stem cells, or those derived from milk) as a starting population may help to achieve full transformation of HMECs that develop into adenocarcinomas

Figure 4



Current consensus: normal HMECs can be fully transformed in definable serial steps. The first step, bypass of senescence, is achieved by inactivation of p53 and pRb by SV40 large T, human papillomavirus (HPV) E6 and E7, or by inhibition of p53 and pRb expression by the RNAi approach (or expression of dominant-negative mutants in the case of p53). The second step, immortalization, is achieved through the expression of hTERT. Alternatively, expression of HPV E6 or overexpression of Bmi-1, mutant p53, or ZNF217 can be used to induce immortalization of HMECs. The third step, anchorage-independent growth, can be achieved by SV40 small t mediated modulation of PI3K and/or other signaling pathways or by overexpression of activated Rac1 and AKT. The fourth step, full transformation, requires the introduction of activated H-ras, which can be substituted by Raf and Ral-GEFs. Although the current model systems have utilized the serial schemes depicted, other combinations and/or schemes of oncogene introduction are likely also to be effective. Adapted from Elenbaas [9], Zhao [10], and Rangarajan [11] and coworkers. HMEC, human mammary epithelial cell; HPV, human papillomavirus; hTERT, catalytic subunit of human telomerase; PI3K, phosphatidylinositol 3-kinase; Ral-GEF, Ral guanine nucleotide exchange factor; RNAi, RNA interference.

in a nude mouse model. Thus, future studies must focus on developing models that will lead to breast tumors that faithfully reproduce the pathological characteristics of human breast cancers.

Transgenic mouse models of breast cancers

Mouse models of breast cancers have provided a wealth of knowledge about the molecular pathways involved in breast cancers. Initial studies in these models used carcinogens to induce breast carcinomas [55]. Later studies targeted a wide variety of genes expressed under either the MMTV (mouse mammary tumor virus) or the WAP (whey acidic protein) promoter to target genes to the mammary gland. Importantly, such studies invariably produced breast adenocarcinomas in mice that resembled human breast cancers. These include viral proteins, such as SV40 large T, polyoma virus T antigen [56-58], or cellular proteins such as c-Myc, ErbB2/neu, cyclin D_1, cyclin E, ERs, mutant p53, c-Ha-ras, and Wnt-1 [59-63]. Recent studies have focused on mouse models with either a global or a mammary-specific knockout of specific genes to examine the function of obvious players, such as cell cycle related proteins and tumor suppressors, either by themselves or after these deficiencies were combined with transgenic neu or other oncogenes. For example, cyclin D_1-deficient mice are resistant to mammary carcinomas induced by c-neu/ErbB2 and Ha-ras but not to those induced by c-Myc or Wnt-1 [63]. These findings define a pivotal role for cyclin D_1 in selective mammary cancers in a mouse model and imply a functional role for cyclin D_1 overexpression in a subset of human breast cancers. In another study, Cre-mediated deletion of exons 3 and 4 of the mouse *Brca2* gene in mice with a loxP-modified and null *Brca2* allele resulted in high incidence of breast adenocarcinomas [64]. Similarly, the telomere attrition in aging telomerase-deficient and p53-mutant mice promoted the development of breast adenocarcinomas [65]. Another study showed that loss of Stat5a delays mammary cancer progression in a WAP-TAg transgenic mouse model [66].

Collectively, these models have defined a role for p53, pRb, BRCA1/2, cyclins, CDKs, ErbB2, c-Myc, Wnt-1, ER, and progesterone receptor in mammary cell growth and development of breast cancers. Finally, these different oncogenes and the pathways in which they work seem to target different progenitors or cell types in mammary gland to develop mammary tumors [67]. For example, the Wnt signaling pathway targets both luminal and myoepithelial cells, whereas Neu, H-Ras, and polyoma T antigen target only luminal epithelial cells [67]. The take-home lesson here is that the majority of these mouse models result in tumors that resemble human breast adenocarcinomas pathologically. The lack of development of adenocarcinomas from cells transformed in culture models may thus reflect the cell type that was used as the starting normal cell, rather than any peculiarity associated with the use of mouse as a host.

Molecular classification of breast cancers: cues from cell culture studies

A vast body of clinical literature indicates that breast tumors exhibit diverse phenotypes as judged by their distinct clinical course, pathological features, and responsiveness to various therapies. However, it has not been clear whether this diversity reflects cancers arising from distinct subtypes of HMECs. Consistent with such a possibility, several years ago we reported the presence of different subtypes of cells in reduction mammoplasty specimens and in milk that exhibited differential susceptibility to viral oncogenes [5,8]. Direct evidence for the conclusions derived from these cell culture studies was provided by recent work utilizing gene expression patterns in primary human breast cancers, using cDNA microarrays. These studies identified distinct gene expression profiles or molecular portraits based on which breast tumors could be subclassified into groups that appear to reflect the original cellular subtypes found in the mammary gland [12]. Five categories of breast cancers were described [12]: a basal epithelial-like group, an ErbB2-overexpressing group, a normal breast epithelial-like group, luminal epithelial cell type A, and luminal epithelial cell type B. A slightly different classification was proposed by Sotiriou and coworkers [68]. The breast tumors were first divided into ER-positive and ER-negative categories. The ER-negative tumors were further subgrouped into basal-like 1, basal-like 2, and ErbB2/neu tumors, whereas ER-positive tumors were subdivided into luminal-like 1, luminal-like 2, and luminal-like 3 subtypes. Sotiriou and coworkers also re-examined data from the study by Sorlie and coworkers [12] and suggested that luminal-like breast cancer could be classified as luminal A, B, and C subtypes corresponding to luminal-like 1, luminal-like 2, and luminal-like 3 subtypes.

Interestingly, survival analyses conducted in a subcohort of patients with locally advanced breast cancer uniformly treated in a prospective study showed significantly different outcomes for the patients belonging to the various groups, with the basal-like subtype correlating with worst outcome, followed by ErbB2 overexpressing, normal cell type and luminal cell type groups [12,68]. Interestingly, a significant difference in outcome for the two ER-positive groups was also noticed [68]. These studies strongly support the idea that many of the breast tumor subtypes may represent malignancies of biologically distinct cell types producing distinct disease entities that may require different treatment strategies. Importantly, these analyses provide a strong rationale for further definition of various mammary epithelial subtypes and expansion of immortalization and full transformation strategies to derive models that may faithfully reproduce the histological and molecular diversity encountered in human breast cancers.

Do breast cancers arise from stem cells?

Stem cells have enormous replicative potential and capacity for self-renewal, and give rise to different lineages of cells.

Although still a controversial notion, many cancers are thought to originate from cancer stem cells [69]. This idea has also attracted a great interest in the field of breast cancer research, and investigators have begun to examine whether there are mammary stem cells [13,17,27,70-73]. The cellular milieu of the mammary gland undergoes significant changes during pregnancy, lactation, and involution. These include bursts of proliferation of existing cells during pregnancy, continued differentiation during lactation, and apoptosis during involution at the end of the cycle. This cyclical behavior predicts the presence of a stem cell-like population in the mammary gland, which would meet the demand of a pregnancy cycle. The existence of adult mammary epithelial stem cells has therefore been proposed. Direct evidence for the existence of such cells has come from clear fat-pad transplantation, retroviral tagging, and X-chromosome inactivation studies in rodent model [13,16,17,70-73].

Recently, using various putative stem cell and cell surface markers, such as sialomucin (Muc), epithelial-specific antigen (ESA), various cytokeratins, ASMA, and CALLA or CD10, attempts have been made to identify the mouse and human mammary epithelial stem cells [13,27,70-73]. Using immunomagnetic cell sorting based on surface antigen markers (Muc and ESA) and subsequent immortalization with E6 and E7, Gudjonsson and coworkers [27] separated $Muc^-/ESA^+/K\text{-}19^+$ cells that were able both to self-renew and to give rise to Muc^-/ESA^+ epithelial cells and $ASMA^+$ myoepithelial cells, thus exhibiting characteristic of breast stem cells. Dontu and coworkers [13] isolated undifferentiated mammospheres from single cell suspensions of HMECs obtained by mechanical and enzymatic dissociations. Primary mammospheres can be further passaged to generate secondary mammospheres. Primary as well as secondary mammospheres were highly enriched in early progenitor or stem cells capable of differentiating along multiple lineages and of self-renewal. Immunostaining of these mammospheres showed the presence of CD10, α_6 integrin and K-5 on early progenitors, and ESA and K-14 on late progenitor cells [13]. However, MUC1, K-18, and ASMA were not expressed in cells present in mammospheres [13]. Detailed expression profiling of mammospheres suggests the presence of additional markers that are upregulated in mammospheres such as stem cell growth factor, hepatocyte growth factor antagonist, stem cell growth factor B and apolipoprotein E. Some markers are exclusively expressed in mammospheres such as FZD2 (frizzled homolog 2), glypican 4, interleukin-6, CXCR4 (CXC chemokine receptor), and FGFR1 (fibroblast growth factor receptor 1). Several genes that are expressed in mammospheres are also expressed in similar structures derived from other cell types (such as neurospheres formed by neural stem cells) [13].

Thus, culture of human HMECs in mammospheres may provide a tool with which to isolate and study mammary epithelial stem cells and their oncogenic susceptibilities. Based on the above and other related studies [13,17,27], the candidate mammary stem cells appear to be ESA^+, $MUC1^-$, α_6 $integrin^+$, and $CD10^+$, and the mammary stem cell niche appears to be at the suprabasal location within the luminal cell layer. Further work by other laboratories and adoption of the schemes employed by Gudjonsson [27] and Dontu [13] and their groups should help in determining the general feasibility of these novel approaches.

Apart from normal mammary stem cells, the possible existence of a breast cancer stem cell has been reported in the literature [74,75]. In a NOD/SCID xenotransplants model, Al-Hajj and coworkers [75] used four cell surface markers, CD44, CD24, ESA and B38.1 (a Breast/ovarian cancer specific marker), and lineage markers to sort different populations of breast cells from breast tumor tissues. All mice injected with $Lin^-/CD44^+/B38.1^+/CD24^{-/low}$ generated tumors, whereas none of the mice injected with $CD44^-/B38.1^-$ cells developed tumors. $Lin^-/CD44^+/B38.1^+$ fractions were further subdivided based on ESA expression. When used in numbers as low as 200, $Lin^-/ESA^+/CD44^+/CD24^{-/low}$ cells in xenotransplants generated tumors that were similar to initial tumors in term of phenotypic heterogeneity [75]. The presence of such a population in breast tumor tissue, which is able to self-renew and differentiate, supports the stem-cell model of breast tumorigenesis.

Conclusion

Our ability to culture and immortalize normal HMECs has provided a wealth of knowledge about the behavior of mammary cells and the genes involved in normal cell growth and oncogenesis. Characterization of these cells has provided novel markers that may permit early diagnosis and prognosis of breast cancers, and has yielded knowledge about potential precursor cells for breast cancers. Transformation analyses in cell culture models have also proven important to our understanding of the multistep nature of breast cancer. Transgenic mouse models have identified the roles played by various tumor suppressors, cell cycle proteins, and other proto-oncogenes in breast cancers. Recent studies using three-dimensional models have proven useful to our understanding of the normal and tumor mammary stem cells and the relationship of microenvironment to epithelial cell growth. Finally, using gene profiling, we have begun to appreciate that breast cancers do not originate only from luminal cells but also from basal and myoepithelial cells, and that there are subtypes of breast cancers that possibly originate from distinct normal precursors that have distinct clinical outcomes and may require different treatment strategies.

However, a number of critical questions remain. What are breast stem cells and what is their role in breast cancer? Are myoepithelial cells and basal cells similar or distinct? Why can we not culture most of the primary breast cancers? How can we develop transformed breast cells in culture that would give rise to breast tumors that resemble human breast cancer –

adenocarcinomas as opposed to poorly differentiated carcinomas? How do different subtypes of breast cancer originate?

In conclusion, experimental immortalization and transformation models have led to substantial progress in our understanding of the biology of breast cancer. Future studies in these model systems should go a long way toward elucidating the nature of breast cancer heterogeneity and thus facilitate the development of more individualized therapies for breast cancer patients.

Competing interests

The author(s) declare that they have no competing interests.

Acknowledgments

We apologize to many of our colleagues whose original work could not be cited due to space constrains.

We thank past and present members of our respective laboratories for their contribution to work published from our laboratories. Work in our laboratories was supported by the NIH Grants CA94143, CA96844, CA81076, and DAMD BC010093 (VB); CA 87986, CA 76118, CA 99900, CA99163, and DAMD17-02-1-0303 (HB); and CA 094150 and DAMD17-02-1-0509 (GD). VB and HB gratefully acknowledge the support of the Duckworth Family Chair in Breast Cancer Research and Jean Ruggles-Romoser Chair for Cancer Research, respectively.

References

1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ: **Cancer statistics, 2005.** *CA Cancer J Clin* 2005, **55:**10-30.
2. Taylor-Papadimitriou J, Berdichevsky F, D'Souza B, Burchell J: **Human models of breast cancer.** *Cancer Surv* 1993, **16:**59-78.
3. Farber E: **The multistep nature of cancer development.** *Cancer Res* 1984, **44:**4217-4223.
4. Hanahan D, Weinberg RA: **The hallmarks of cancer.** *Cell* 2000, **100:**57-70.
5. Ratsch SB, Gao Q, Srinivasan S, Wazer DE, Band V: **Multiple genetic changes are required for efficient immortalization of different subtypes of normal human mammary epithelial cells.** *Radiat Res* 2001, **155:**143-150.
6. Ethier SP, Summerfelt RM, Cundiff KC, Asch BB: **The influence of growth factors on the proliferative potential of normal and primary breast cancer-derived human breast epithelial cells.** *Breast Cancer Res Treat* 1991, **17:**221-230.
7. Yaswen P, Stampfer MR: **Molecular changes accompanying senescence and immortalization of cultured human mammary epithelial cells.** *Int J Biochem Cell Biol* 2002, **34:** 1382-1394.
8. Wazer DE, Liu XL, Chu Q, Gao Q, Band V: **Immortalization of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7.** *Proc Natl Acad Sci USA* 1995, **92:** 3687-3691.
9. Elenbaas B, Spirio L, Koerner F, Fleming MD, Zimonjic DB, Donaher JL, Popescu NC, Hahn WC, Weinberg RA: **Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells.** *Genes Dev* 2001, **15:**50-65.
10. Zhao JJ, Gjoerup OV, Subramanian RR, Cheng Y, Chen W, Roberts TM, Hahn WC: **Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase.** *Cancer Cell* 2003, **3:**483-495.
11. Rangarajan A, Hong SJ, Gifford A, Weinberg RA: **Species- and cell type-specific requirements for cellular transformation.** *Cancer Cell* 2004, **6:**171-183.
12. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, *et al.*: **Repeated observation of breast tumor subtypes in independent gene expression data sets.** *Proc Natl Acad Sci USA* 2003, **100:**8418-8423.
13. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS: **In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells.** *Genes Dev* 2003, **17:**1253-1270.
14. Schmeichel KL, Bissell MJ: **Modeling tissue-specific signaling and organ function in three dimensions.** *J Cell Sci* 2003, **116:** 2377-2388.
15. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A, *et al.*: **Molecular characterization of the tumor microenvironment in breast cancer.** *Cancer Cell* 2004, **6:**17-32.
16. Cardiff RD, Wellings SR: **The comparative pathology of human and mouse mammary glands.** *J Mammary Gland Biol Neoplasia* 1999, **4:**105-122.
17. Smalley M, Ashworth A: **Stem cells and breast cancer: a field in transit.** *Nat Rev Cancer* 2003, **3:**832-844.
18. Lakhani SR, O'Hare MJ: **The mammary myoepithelial cell – Cinderella or ugly sister?** *Breast Cancer Res* 2001, **3:**1-4.
19. Taylor-Papadimitriou J, Stampfer M, Bartek J, Lewis A, Boshell M, Lane EB, Leigh IM: **Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to in vivo phenotypes and influence of medium.** *J Cell Sci* 1989, **94:**403-413.
20. Band V, Sager R: **Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types.** *Proc Natl Acad Sci USA* 1989, **86:**1249-1253.
21. Petersen OW, Ronnov-Jessen L, Howlett AR, Bissell MJ: **Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells.** *Proc Natl Acad Sci USA* 1992, **89:**9064-9068.
22. Janss DH, Hillman EA, Malan-Shibley LB, Ben TL: **Methods for the isolation and culture of normal human breast epithelial cells.** *Methods Cell Biol* 1980, **21B:**107-135.
23. Easty GC, Easty DM, Monaghan P, Ormerod MG, Neville AM: **Preparation and identification of human breast epithelial cells in culture.** *Int J Cancer* 1980, **26:**577-584.
24. Wong DJ, Foster SA, Galloway DA, Reid BJ: **Progressive region-specific de novo methylation of the p16 CpG island in primary human mammary epithelial cell strains during escape from M(0) growth arrest.** *Mol Cell Biol* 1999, **8:**5642-5651.
25. Romanov SR, Kozakiewicz BK, Holst CR, Stampfer MR, Haupt LM, Tlsty TD: **Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes.** *Nature* 2001, **409:**633-637.
26. Trask DK, Band V, Zajchowski DA, Yaswen P, Suh T, Sager R: **Keratins as markers that distinguish normal and tumor-derived mammary epithelial cells.** *Proc Natl Acad Sci USA* 1990, **87:**2319-2323.
27. Gudjonsson T, Villadsen R, Nielsen HL, Ronnov-Jessen L, Bissell MJ, Petersen OW: **Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties.** *Genes Dev* 2002, **16:**693-706.
28. Crawford YG, Gauthier ML, Joubel A, Mantei K, Kozakiewicz K, Afshari CA, Tlsty TD: **Histologically normal human mammary epithelia with silenced p16(INK4a) overexpress COX-2, promoting a premalignant program.** *Cancer Cell* 2004, **5:**263-273.
29. Holst CR, Nuovo GJ, Esteller M, Chew K, Baylin SB, Herman JG, Tlsty TD: **Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia.** *Cancer Res* 2003, **63:**1596-1601.
30. Itahana K, Campisi J, Dimri GP: **Mechanisms of cellular senescence in human and mouse cells.** *Biogerontology* 2004, **5:**1-10.
31. Shay JW, Wright WE: **Senescence and immortalization: role of telomeres and telomerase.** *Carcinogenesis* 2005, **26:**867-874.
32. Ben-Porath I, Weinberg RA: **When cells get stressed: an integrative view of cellular senescence.** *J Clin Invest* 2004, **113:**8-13.
33. Itahana K, Dimri G, Campisi J: **Regulation of cellular senescence by p53.** *Eur J Biochem* 2001, **268:**2784-2791.
34. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, *et al.*: **A biomarker that identifies senescent human cells in culture and in aging skin in vivo.** *Proc Natl Acad Sci USA* 1995, **92:**9363-9367.
35. Brenner AJ, Stampfer MR, Aldaz CM: **Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation.** *Oncogene* 1998, **17:**199-205.

36. Boyer SN, Wazer DE, Band V: **E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway.** *Cancer Res* 1996, **56:**4620-4624.
37. Ramirez RD, Herbert BS, Vaughan MB, Zou Y, Gandia K, Morales CP, Wright WE, Shay JW: **Bypass of telomere-dependent replicative senescence (M1) upon overexpression of Cdk4 in normal human epithelial cells.** *Oncogene* 2003, **22:**433-444.
38. Stampfer MR, Bartley JC: **Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo [A]pyrene**. *Proc Natl Acad Sci USA* 1985, **82:**2394-2398.
39. Wazer DE, Chu Q, Liu XL, Gao Q, Safaii H, Band V: **Loss of p53 protein during radiation transformation of primary human mammary epithelial cells.** *Mol Cell Biol* 1994, **14:**2468-2478.
40. Toouli CD, Huschtscha LI, Neumann AA, Noble JR, Colgin LM, Hukku B, Reddel RR: **Comparison of human mammary epithelial cells immortalized by simian virus 40 T-antigen or by the telomerase catalytic subunit.** *Oncogene* 2002, **21:**128-139.
41. Bartek J, Bartkova J, Kyprianou N, Lalani EN, Staskova Z, Shearer M, Chang S, Taylor-Papadimitriou J: **Efficient immortalization of luminal epithelial cells from human mammary gland by introduction of simian virus 40 large tumor antigen with a recombinant retrovirus.** *Proc Natl Acad Sci USA* 1991, **88:** 3520-3524.
42. Kumar A, Zhao Y, Meng G, Zeng M, Srinivasan S, Delmolino LM, Gao Q, Dimri G, Weber GF, Wazer DE, *et al.*: **Human papillomavirus oncoprotein E6 inactivates the transcriptional coactivator human ADA3.** *Mol Cell Biol* 2002, **22:**5801-5812.
43. Zeng M, Kumar A, Meng G, Gao Q, Dimri G, Wazer D, Band H, Band V: **Human papilloma virus 16 E6 oncoprotein inhibits retinoic X receptor-mediated transactivation by targeting human ADA3 coactivator.** *J Biol Chem* 2002, **277:**45611-45618.
44. Meng G, Zhao Y, Nag A, Zeng M, Dimri G, Gao Q, Wazer DE, Kumar R, Band H, Band V: **Human ADA3 binds to estrogen receptor (ER) and functions as a coactivator for ER-mediated transactivation.** *J Biol Chem* 2004, **279:**54230-54240.
45. Wu L, Aster JC, Blacklow SC, Lake R, Artavanis-Tsakonas S, Griffin JD: **MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors.** *Nat Genet.* 2000, **26:**484-489.
46. Gao Q, Hauser SH, Liu XL, Wazer DE, Madoc-Jones H, Band V: **Mutant p53-induced immortalization of primary human mammary epithelial cells.** *Cancer Res* 1996, **56:**3129-3133.
47. Lukas J, Niu N, Press MF: **p53 mutations and expression in breast carcinoma *in situ*.** *Am J Pathol* 2000, **156:**183-191.
48. Dimri GP, Martinez JL, Jacobs JL, Keblusek P, Itahana K, van Lohuizen M, Campisi J, Wazer DE, Band V: **Bmi-1 oncogene induces telomerase and immortalizes human mammary epithelial cells.** *Cancer Res* 2002, **62:**4736-4745.
49. Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, Morrison SJ, Clarke MF: **Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells.** *Nature* 2003, **423:** 302-305.
50. Lessard J, Sauvageau G: **Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells.** *Nature* 2003, **423:**255-260.
51. Nonet GH, Stampfer MR, Chin K, Gray JW, Collins CC, Yaswen P: **The ZNF217 gene amplified in breast cancers promotes immortalization of human mammary epithelial cells.** *Cancer Res* 2001, **61:**1250-1254.
52. Klingelhutz AJ, Foster SA, McDougall JK: **Telomerase activation by the E6 gene product of human papillomavirus type 16.** *Nature* 1996, **380:**79-82.
53. Gewin L, Myers H, Kiyono T, Galloway DA: **Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex.** *Genes Dev* 2004, **18:**2269-2282.
54. Hahn WC, Dessain SK, Brooks MW, King JE, Elenbaas B, Sabatini DM, DeCaprio JA, Weinberg RA: **Enumeration of the simian virus 40 early region elements necessary for human cell transformation.** *Mol Cell Biol* 2002, **22:**2111-2123.
55. Sukumar S, McKenzie K, Chen Y: **Animal models for breast cancer.** *Mutat Res* 1995, **333:**37-44.
56. Guy CT, Cardiff RD, Muller WJ: **Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease.** *Mol Cell Biol* 1992, **12:**954-961.
57. Li M, Lewis B, Capuco AV, Laucirica R, Furth PA: **WAP-TAg transgenic mice and the study of dysregulated cell survival, proliferation, and mutation during breast carcinogenesis.** *Oncogene* 2000, **19:**1010-1019.
58. Husler MR, Kotopoulis KA, Sundberg JP, Tennent BJ, Kunig SV, Knowles BB: **Lactation-induced WAP-SV40 Tag transgene expression in C57BL/6J mice leads to mammary carcinoma.** *Transgenic Res* 1998, **7:**253-263.
59. Blackshear PE: **Genetically engineered rodent models of mammary gland carcinogenesis: an overview.** *Toxicol Pathol* 2001, **29:**105-116.
60. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P: **Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene.** *Cell* 1988, **54:** 105-115.
61. Li Y, Hively WP, Varmus HE: **Use of MMTV-Wnt-1 transgenic mice for studying the genetic basis of breast cancer.** *Oncogene* 2000, **19:**1002-1009.
62. D'Cruz CM, Gunther EJ, Boxer RB, Hartman JL, Sintasath L, Moody SE, Cox JD, Ha SI, Belka GK, Golant A, *et al.*: **c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations.** *Nat Med* 2001, **7:**235-239.
63. Yu Q, Geng Y, Sicinski P: **Specific protection against breast cancers by cyclin D1 ablation.** *Nature* 2001, **411:**1017-1021.
64. Ludwig T, Fisher P, Murty V, Efstratiadis A: **Development of mammary adenocarcinomas by tissue-specific knockout of Brca2 in mice.** *Oncogene* 2001, **20:**3937-3948.
65. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, DePinho RA: **Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice.** *Nature* 2000, **406:**641-645.
66. Ren S, Cai HR, Li M, Furth PA: **Loss of Stat5a delays mammary cancer progression in a mouse model.** *Oncogene* 2002, **21:** 4335-4339.
67. Li Y, Welm B, Podsypanina K, Huang S, Chamorro M, Zhang X, Rowlands T, Egeblad M, Cowin P, Werb Z, *et al.*: **Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells.** *Proc Natl Acad Sci USA* 2003, **100:**15853-15858.
68. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET: **Breast cancer classification and prognosis based on gene expression profiles from a population-based study.** *Proc Natl Acad Sci USA* 2003, **100:** 10393-10398.
69. Marx J: **Cancer research. Mutant stem cells may seed cancer.** *Science* 2003, **301:**1308-1310.
70. Kordon EC, Smith GH: **An entire functional mammary gland may comprise the progeny from a single cell.** *Development* 1998, **125:**1921-1930.
71. Smith GH, Chepko G: **Mammary epithelial stem cells.** *Microsc Res Tech* 2001, **52:**190-203.
72. Stingl J, Eaves CJ, Zandieh I, Emerman JT: **Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue.** *Breast Cancer Res Treat* 2001, **67:**93-109.
73. Stingl J, Eaves CJ, Kuusk U, Emerman JT: **Phenotypic and functional characterization in vitro of a multipotent epithelial cell present in the normal adult human breast.** *Differentiation* 1998, **63:**201-213.
74. Dick JE: **Breast cancer stem cells revealed.** *Proc Natl Acad Sci USA* 2003, **100:**3547-3549.
75. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF: **Prospective identification of tumorigenic breast cancer cells.** *Proc Natl Acad Sci USA* 2003, **100:**3983-3988.

Molecular Biology of the Cell
Vol. 18, 536–546, February 2007

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

Wei-Jian Guo,* Sonal Datta,* Vimla Band,*†‡ and Goberdhan P. Dimri*†

*Division of Cancer Biology and Department of Medicine, Evanston Northwestern Healthcare Research Institute, Evanston, IL 60201; and †Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, and ‡Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60201

Submitted May 22, 2006; Revised November 14, 2006; Accepted November 27, 2006
Monitoring Editor: John Cleveland

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).

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–05–0447) on December 6, 2006.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

Address correspondence to: Goberdhan P. Dimri (g-dimri@northwestern.edu).

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-

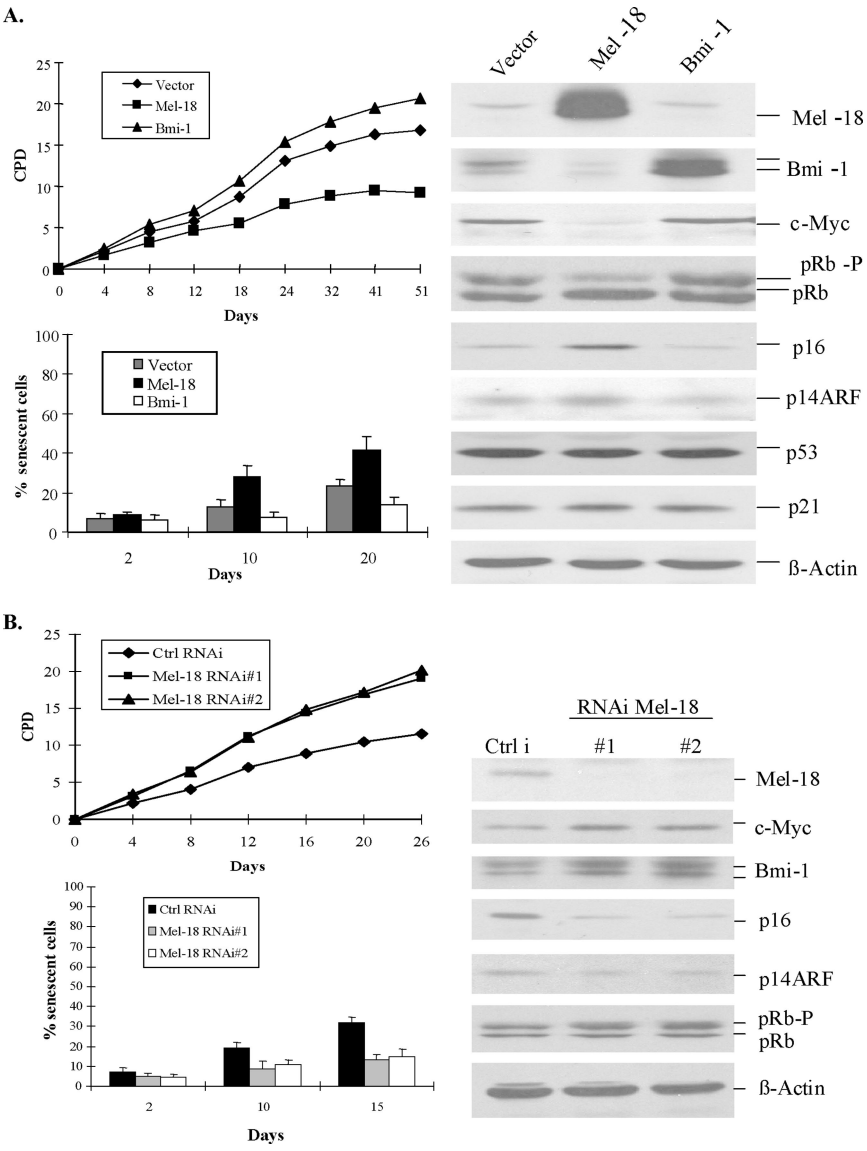


Figure 1. Mel-18 regulates cellular senescence in human fibroblasts. (A) Overexpression of Mel-18 induces premature senescence in proliferating fibroblasts. MRC-5 fibroblasts overexpressing Mel-18 or Bmi-1 and vector-infected control cells were serially passaged in culture to determine the replicative 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

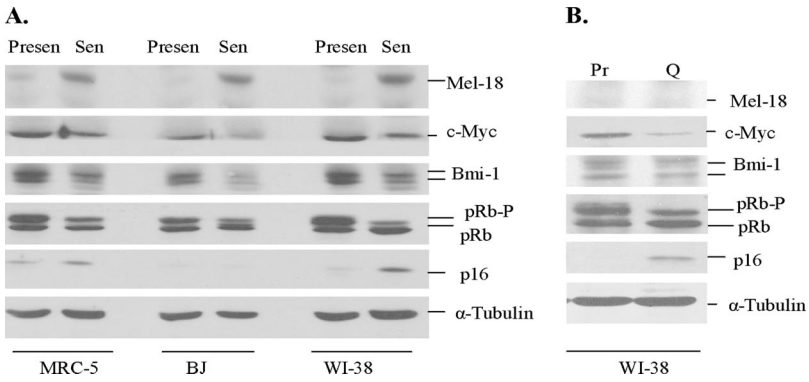


Figure 2. Mel-18 is overexpressed in senescent human fibroblasts. (A) MRC-5, BJ, and WI-38 strains of human fibroblast were serially passaged in culture until senescence as determined by measuring the SA-β-gal index. Mel-18, Bmi-1, pRb, p16, and α-tubulin in total cell lysates from proliferating presenescent (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

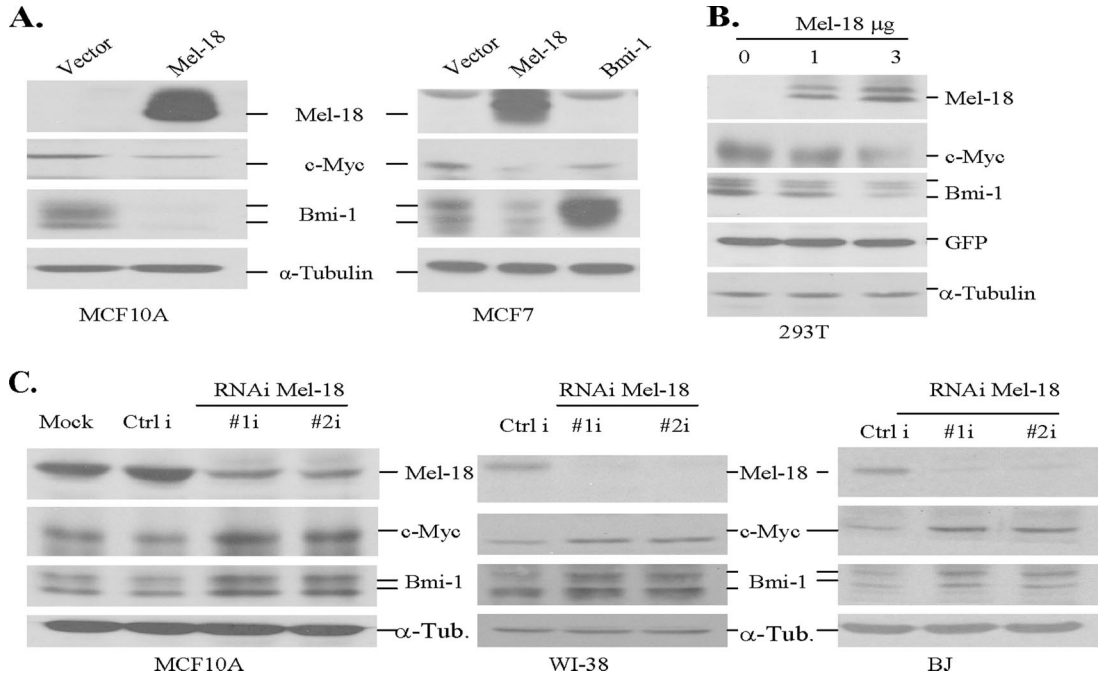


Figure 3. Mel-18 regulates Bmi-1 in human cells. (A) Stable expression of Mel-18 in MCF10A and MCF7 cells leads to down-regulation of Bmi-1 and c-Myc oncoprotein. Total cell lysate from indicated cells was analyzed by Western blot analysis using antibody against Bmi-1, Mel-18, c-Myc, and α-tubulin (loading 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

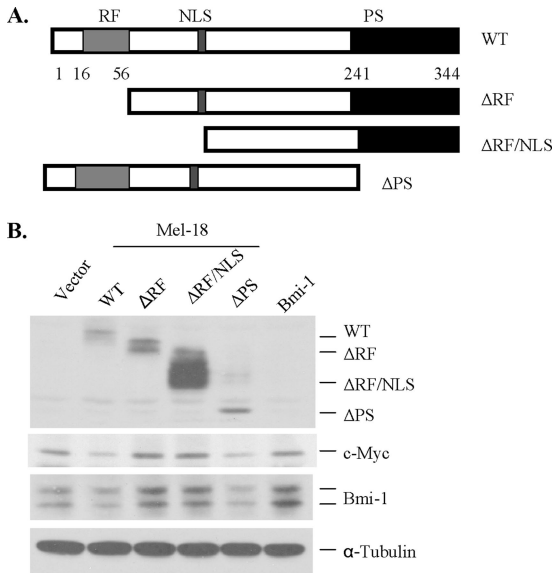


Figure 4. Structural analysis of Mel-18. (A) Schematic representation of mutants of Mel-18 depicting various domains. These mutants were generated by PCR and cloned in the pLPC retroviral vector. (B) Stable overexpression of wild type (WT) and the mutants of Mel-18 in MCF10A cells; WT and the PS mutant 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.*

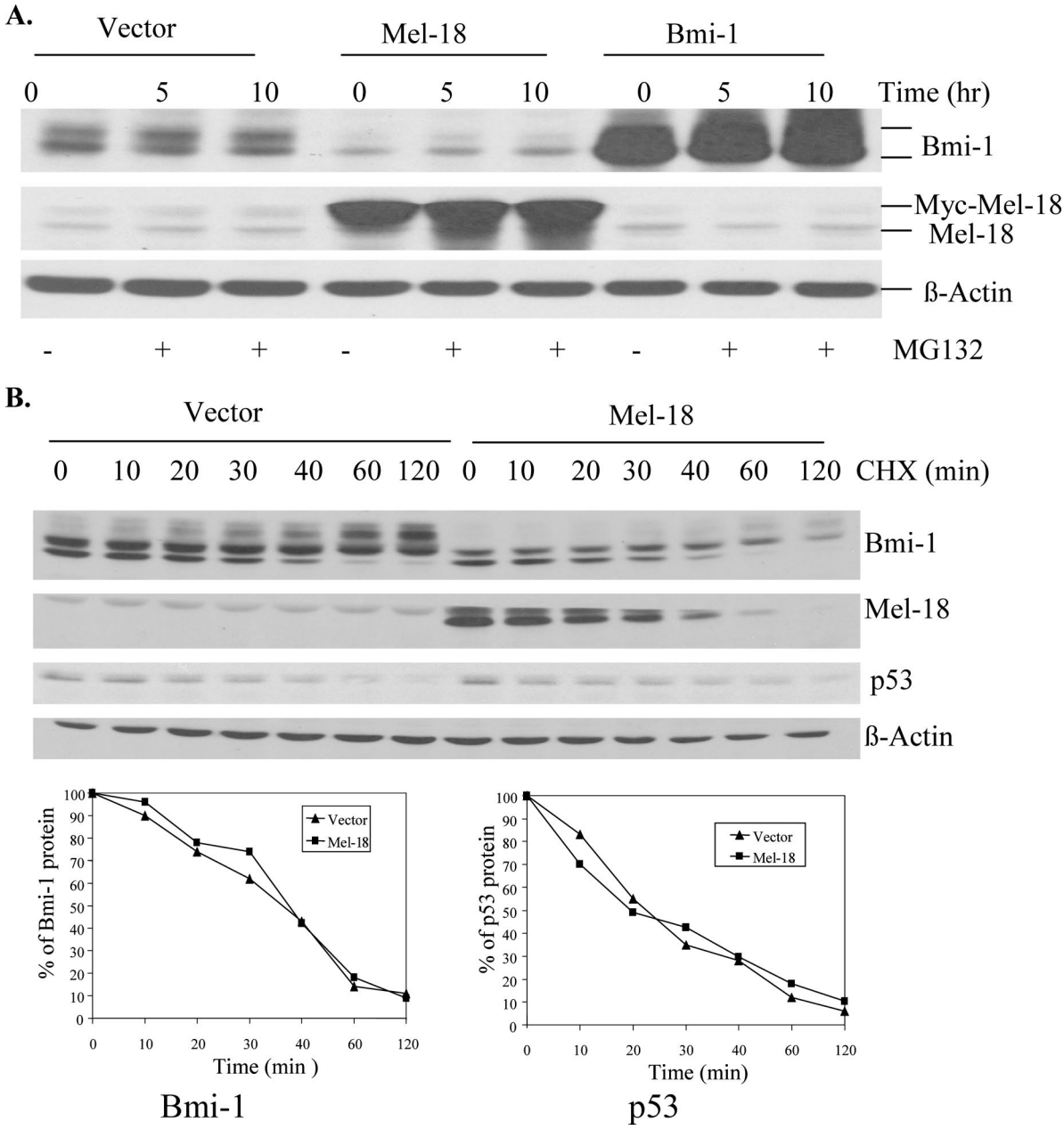


Figure 5. Mel-18 does not regulate Bmi-1 protein level. (A) Treatment with MG132, a proteosome inhibitor does not restore Bmi-1 expression in Mel-18–overexpressing cells. Control, Mel-18– and Bmi-1–overexpressing cells were treated with 10 μM MG132 for the indicted time period and analyzed by Western 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

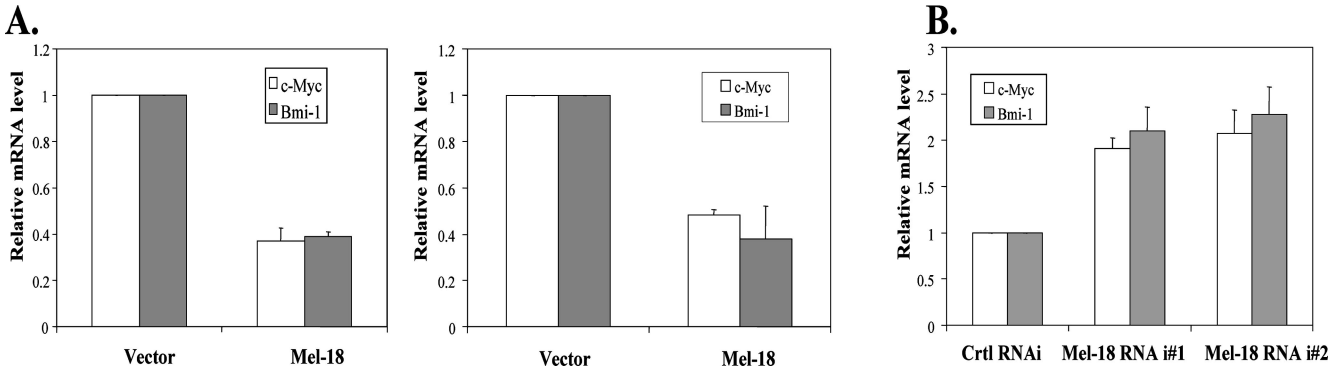


Figure 6. Mel-18 regulates mRNA levels of c-Myc and Bmi-1 as determined by QRT-PCR analysis. (A) The mRNA levels of Bmi-1 and c-Myc in Mel-18–overexpressing and control MCF10A and MCF7 cells were quantified by QRT-PCR and normalized to GAPDH mRNA levels as described in *Materials and Methods.* (B) Using 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

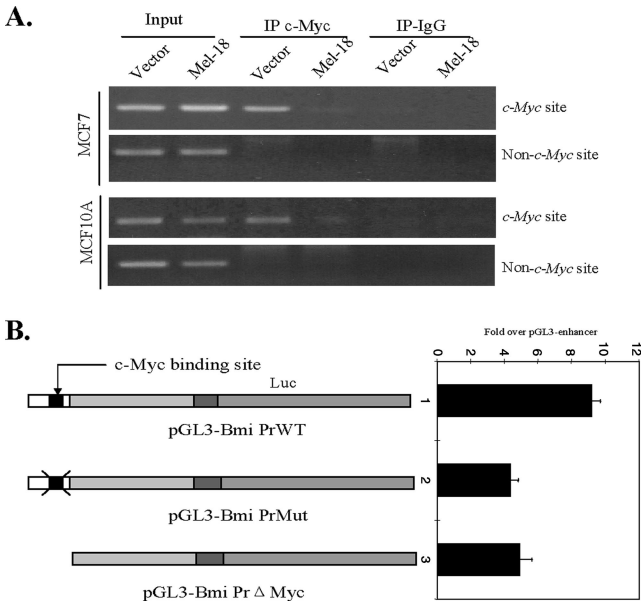


Figure 7. c-Myc binds to the Bmi-1 promoter and regulates its activity. (A) c-Myc binds to the E-box sequences in the Bmi-1 promoter as shown by the ChIP analysis. The ChIP analysis was performed using vector control or Mel-18–overexpressing MCF10A and MCF7 cells as indicated. The cell lysates were IPed 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

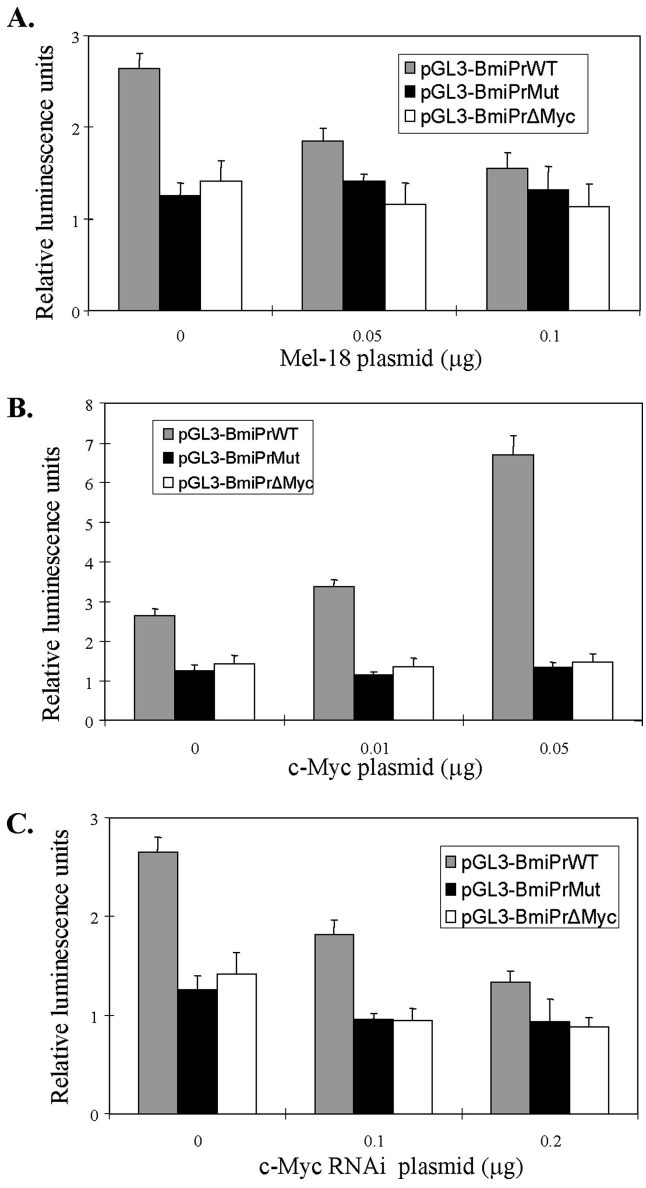


Figure 8. Mel-18 and c-Myc regulate Bmi-1 promoter activity. (A) Overexpression of Mel-18 down-regulates only the wild-type Bmi-1 promoter. pGL3-Bmi-1 PrWT, pGL3-Bmi-1PrMut, and pGL3- Bmi-1PrΔMyc plasmids were transiently transfected into 293T cells together with an increasing amount of Mel-18–overexpressing 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-

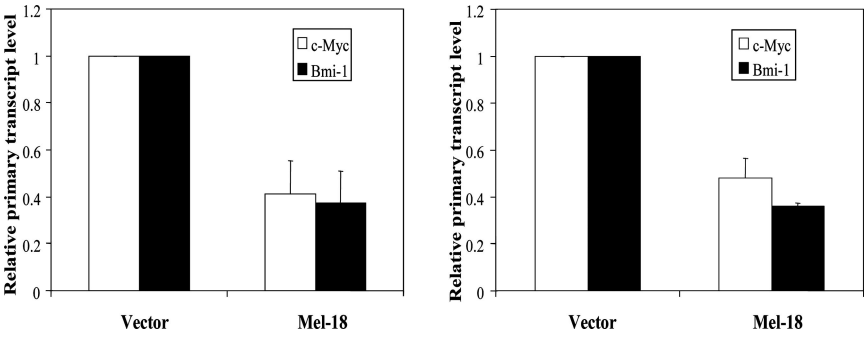


Figure 9. Mel-18 regulates c-Myc and Bmi-1 transcription. Quantitative PT RT-PCR analysis of primary transcripts of c-Myc and Bmi-1 in control and Mel-18–overexpressing MCF7 (left panel) and MCF10A cells (right panel). PT RT-PCR analysis was performed in triplicate as described in *Materials and Methods.*

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.

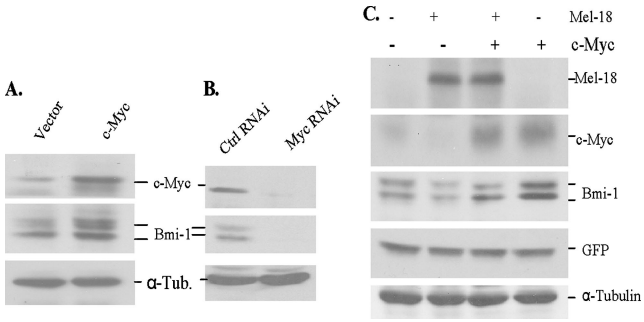


Figure 10. c-Myc regulates endogenous Bmi-1 expression and transient expression of c-Myc in Mel-18–overexpressing cells restores Bmi-1 expression. (A) Stable overexpression of c-Myc leads to Bmi-1 up-regulation. MCF10A cells were infected with a c-Myc expressing retrovirus (pLNCX2-Myc), selected in G418, 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.

ACKNOWLEDGMENTS

We thank Dr. J. Campisi and Dr. M. van Lohuizen for the gift of reagents used in this study. We thank Dr. J. Campisi and the members of G.D.'s laboratory for helpful comments. We also thank Dr. C. Barry for help in performing real-time PCR analysis and Prashant Bommi for providing technical assistance. This work was supported by the grants from the National Cancer Institute (RO1CA 094150) and the US Department of Defense (DAMD17-02-1-0509) to G.D.

REFERENCES

Adhikary, S., and Eilers, M. (2005). Transcriptional regulation and transformation by Myc proteins. Nat. Rev. Mol. Cell Biol. *6*, 635–645.

Akasaka, T., van Lohuizen, M., van der Lugt, N., Mizutani-Koseki, Y., Kanno, M., Taniguchi, M., Vidal, M., Alkema, M., Berns, A., and Koseki, H. (2001). Mice doubly deficient for the Polycomb Group genes *Mel18* and *Bmi1* reveal synergy and requirement for maintenance but not initiation of Hox gene expression. Development *128*, 1587–1597.

Alkema, M. J., Bronk, M., Verhoeven, E., Otte, A., van't Veer, L. J., Berns, A., and van Lohuizen, M. (1997). Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian polycomb complex. Genes Dev. *11*, 226–240.

Boyer, S. N., Wazer, D. E., and Band, V. (1996). E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteosome pathway. Caner Res. *56*, 4620–4624.

Bracken, A. P., Pasini, D., Capra, M., Prosperini, E., Colli, E., and Helin, K. (2003). EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J. *22*, 5323–5335.

Bruggeman, S. W., *et al.* (2005). Ink4a and Arf differentially affect cell proliferation and neural stem cell self-renewal in Bmi1-deficient mice. Genes Dev. *19*, 1438–1443.

Campisi, J. (2005). Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell *120*, 513–522.

Cao, R., Tsukada, Y., and Zhang, Y. (2005). Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol. Cell *20*, 845–854.

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

Dimri, G. P., Itahana, K., Acosta, M., and Campisi, J. (2000). Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14(ARF) tumor suppressor. Mol. Cell. Biol. *20*, 273–285.

Dimri, G. P., *et al.* (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA *92*, 9363–9367.

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

Gil, J., Bernard, D., and Peters, G. (2005). Role of polycomb group proteins in stem cell self-renewal and cancer. DNA Cell Biol. *24*, 117–125.

Glinsky, G. V., Berezovska, O., and Glinskii, A. B. (2005). Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. J. Clin. Invest. *115*, 1503–1521.

Guney, I., Wu, S., and Sedivy, J. M. (2006). Reduced c-Myc signaling triggers telomere-independent senescence by regulating Bmi-1 and p16INK4a. Proc. Natl. Acad. Sci. USA *103*, 3645–3650.

Ho, A. D., Wagner, W., and Mahlknecht, U. (2005). Stem cells and ageing. The potential of stem cells to overcome age-related deteriorations of the body in regenerative medicine. EMBO Rep. Jul; 6 Spec No, S35–S38.

Ishida, A., Asano, H., Hasegawa, M., Koseki, H., Ono, T., Yoshida, M. C., Taniguchi, M., and Kanno, M. (1993). Cloning and chromosome mapping of the human Mel-18 gene which encodes a DNA-binding protein with a new 'RING-finger' motif. Gene *129*, 249–255.

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

Itahana, K., Zou, Y., Itahana, Y., Martinez, J.-L., Beausejour, C., Jacobs, J.J.L., van Lohuizen, M., Band, V., Campisi, J., and Dimri, G. P. (2003). Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1. Mol. Cell. Biol. *23*, 389–401.

Iwama, A., *et al.* (2004). Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. Immunity *21*, 843–851.

Jacobs, J.J.L., Kieboom, K., Marino, S., DePinho, R. A., and van Lohuizen, M. (1999). The oncogene and polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature *397*, 164–168.

Kajiume, T., Ninomiya, Y., Ishihara, H., Kanno, R., and Kanno, M. (2004). Polycomb group gene mel-18 modulates the self-renewal activity and cell cycle status of hematopoietic stem cells. Exp. Hematol. *32*, 571–578.

Kanno, M., Hasegawa, M., Ishida, A., Isono, K., and Taniguchi, M. (1995). mel-18, a Polycomb group-related mammalian gene, encodes a transcriptional negative regulator with tumor suppressive activity. EMBO J. *14*, 5672–5678.

Kim, J. H., Yoon, S. Y., Jeong, S. H., Kim, S. Y., Moon, S. K., Joo, J. H., Lee, Y., Choe, I. S., and Kim, J. W. (2004). Overexpression of Bmi-1 oncoprotein correlates with axillary lymph node metastases in invasive ductal breast cancer. Breast *13*, 383–388.

Kleer, C. G., *et al.* (2003). EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc. Natl. Acad. Sci. USA *100*, 11606–11611.

Lessard, J., and Sauvageau, G. (2003). Bmi-1 determines the proliferative capacity of normal and leukemic stem cells. Nature *423*, 255–260.

Liu, S., Dontu, G., Mantle, I. D., Patel, S., Ahn, N. S., Jackson, K. W., Suri, P., and Wicha, M. S. (2006). Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res. *66*, 6063–6071.

Molofsky, A. V., He, S., Bydon, M., Morrison, S. J., and Pardal, R. (2005). Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. Genes Dev. *19*, 1432–1437.

Molofsky, A. V., Pardal, R., Iwashita, T., Park, I. K., Clarke, M. F., and Morrison, S. J. (2003). Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature *425*, 962–967.

Murray, P. J. (2005). The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. Proc. Natl. Acad. Sci. USA *102*, 8686–8691.

Nowak, K., Kerl, K., Fehr, D., Kramps, C., Gessner, C., Killmer, K., Samans, B., Berwanger, B., Christiansen, H., and Lutz, W. (2006). BMI1 is a target gene of E2F-1 and is strongly expressed in primary neuroblastomas. Nucleic Acids Res. *34*, 1745–1754.

Park, I.-K., Qian, D., Kiel, M., Becker, M., Pihalja, M., Weissman, I. L., Morrison, S. J., and Clarke, M. (2003). Bmi-1 is required for the maintenance of adult self-renewing hematopoietic stem cells. Nature *423*, 302–305.

Pickart, C. M. (2001). Mechanisms underlying ubiquitination. Annu. Rev. Biochem. *70*, 503–533.

Ringrose, L., and Paro, R. (2004). Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu. Rev. Genet. *38*, 413–443.

Rosenthal, N. (2005). Youthful prospects for human stem-cell therapy. In another few decades, revised attitudes toward stem cells could lead to disease prevention and life extension. EMBO Rep. Jul; 6 Spec No, S30–S34.

Sherr, C. J., Bertwistle, D., Den Bestn, W., Kuo, M. L., Sugimoto, M., Tago, K., Williams, R. T., Zindy, F., and Roussel, M. F. (2005). p53-Dependent and -independent functions of the Arf tumor suppressor. Cold Spring Harb. Symp. Quant. Biol. *70*, 129–137.

Sun, L. Q., Lee, D. W., Zhang, Q., Xiao, W., Raabe, E. H., Meeker, A., Miao, D., Huso, D. L., and Arceci, R. J. (2004). Growth retardation and premature aging phenotypes in mice with disruption of the SNF2-like gene, PASG. Genes Dev. *18*, 1035–1046.

Tetsu, O., Ishihara, H., Kanno, R., Kamiyasu, M., Inoue, H., Tokuhisa, T., Taniguchi, M., and Kanno, M. (1998). mel-18 negatively regulates cell cycle progression upon B cell antigen receptor stimulation through a cascade leading to c-myc/cdc25. Immunity *9*, 439–448.

Valk-Lingbeek, M. E., Bruggeman, S. W., and van Lohuizen, M. (2004). Stem cells and cancer: the polycomb connection. Cell *118*, 409–418.

Varambally, S., *et al.* (2002). The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature *419*, 624–629.

Waters, C. M., Littlewood, T. D., Hancock, D. C., Moore, J. P. and Evan, G. I. (1991). c-myc protein expression in untransformed fibroblasts. Oncogene *6*, 797–805.

Zeller, K. I., Jegga, A. G., Aronow, B. J., O'Donnell, K. A., and Dang, C. V. (2003). An integrated database of genes responsive to the Myc oncogenic transcription factor: identification of direct genomic targets. Genome Biol. *4*, R69.

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/).

Requests for reprints: Goberdhan P. Dimri, ENH Research Institute, 1001 University Place, Evanston, IL 60201. Phone: 224-364-7521; Fax: 224-364-7402; E-mail: gdimri@enh.org or g-dimri@northwestern.edu.

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

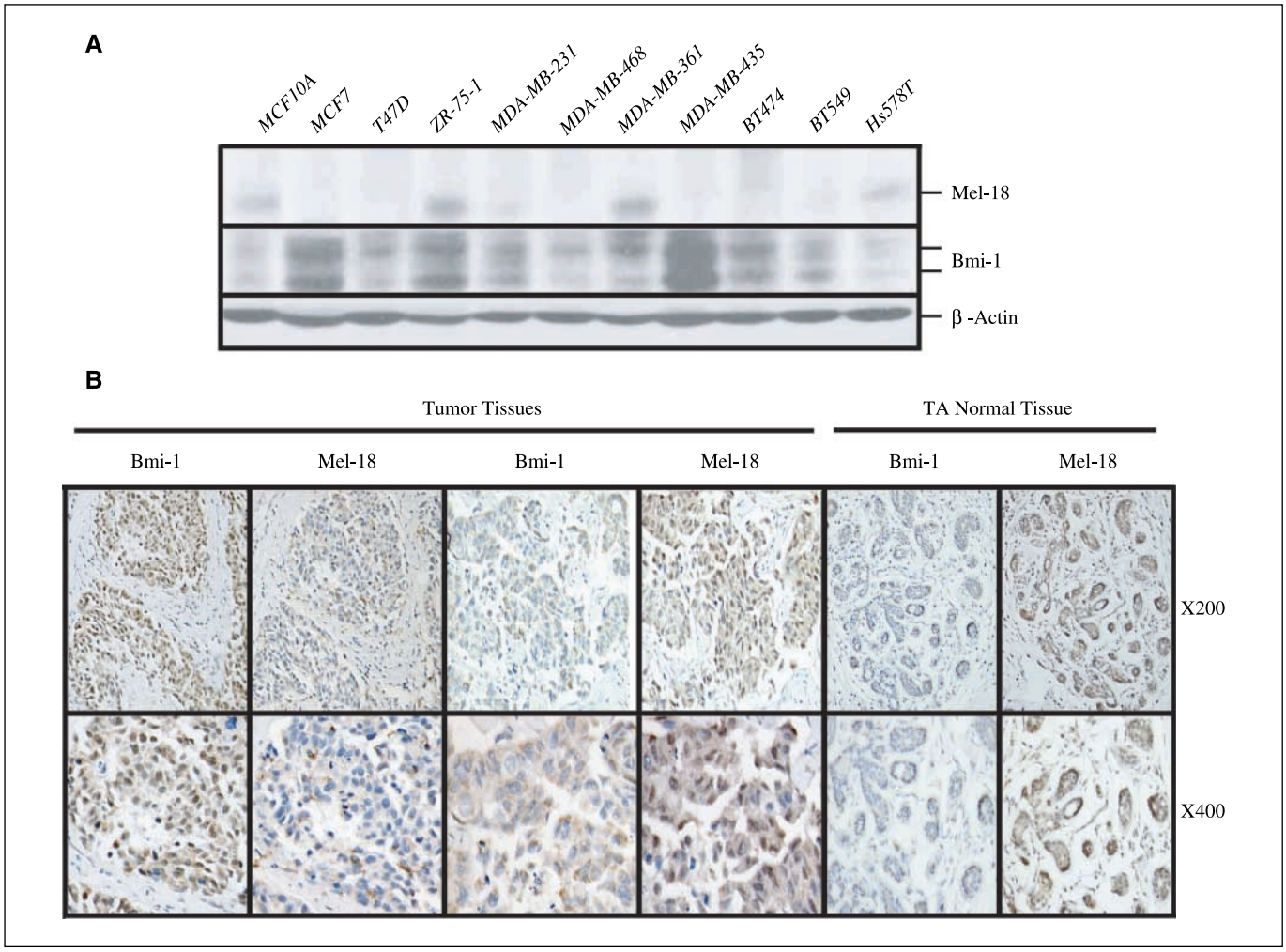


Figure 1. Mel-18 and Bmi-1 expression inversely correlates in breast cancer cell lines and breast tumors. *A,* Bmi-1 and Mel-18 expression in various breast cancer cell lines as detected by Western blot analysis. *B,* representative of two tumor samples: sample 1 expresses high Bmi-1 and low Mel-18, 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

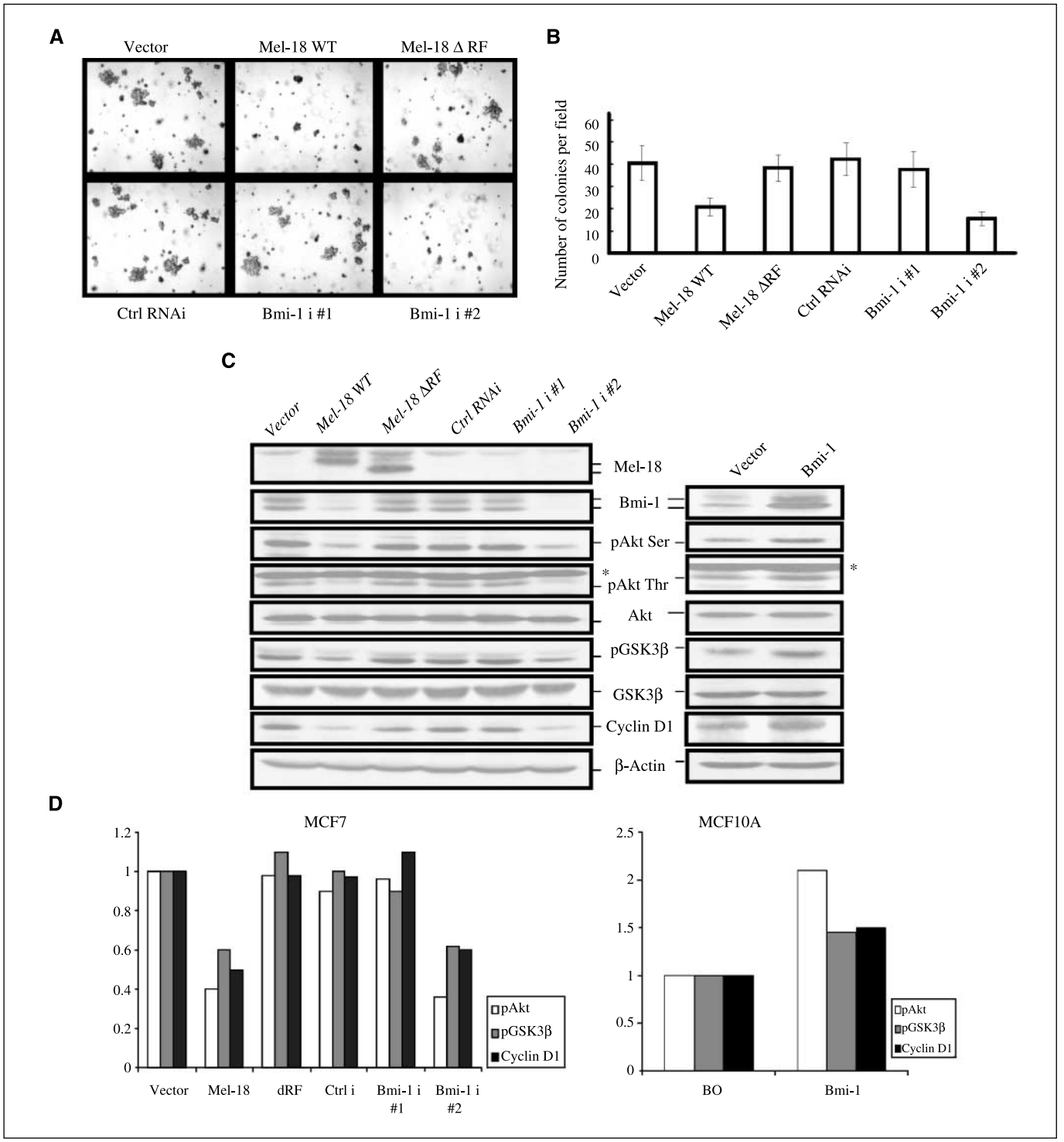


Figure 2. Reduction of transformed phenotype of MCF7 cells by Mel-18 overexpression and knockdown of Bmi-1 expression. *A,* overexpression of Mel-18 and knockdown of Bmi-1 expression in MCF7 decreases colony formation in soft agar. Control or Mel-18–overexpressing MCF7 cells (*top*), and control (*Ctrl 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*).

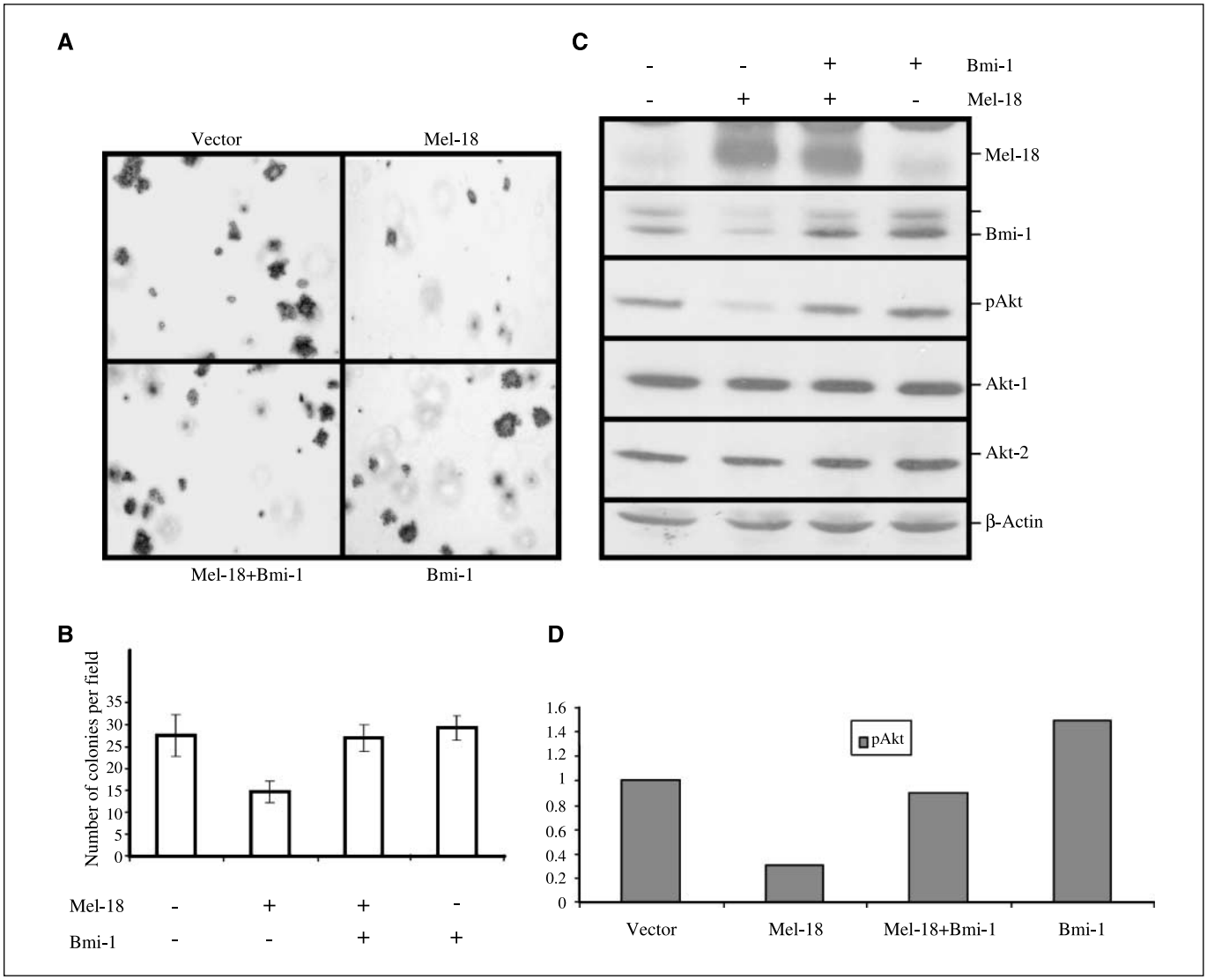


Figure 3. Exogenous Bmi-1 restores Akt activity and anchorage-independent growth potential of Mel-18–overexpressing cells. *A,* MCF7 cells were infected with a control retrovirus or Bmi-1–overexpressing retrovirus. Cells were selected in hygromycin and super-infected with Mel-18–expressing retrovirus. 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*.

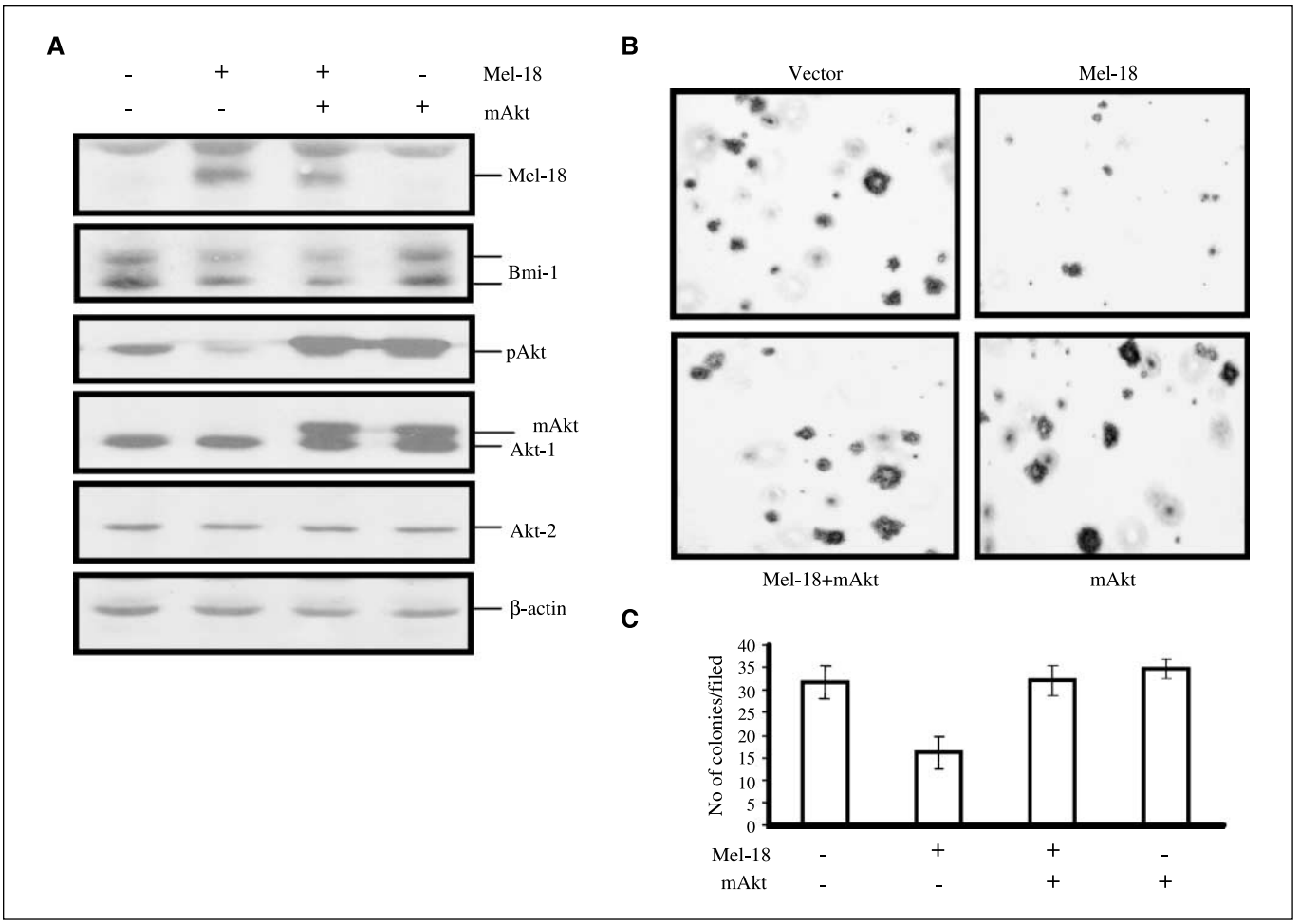


Figure 4. Exogenous overexpression of activated Akt (mAkt) restores anchorage-independent growth potential of Mel-18–overexpressing MCF7 cells. *A,* mAkt was stably expressed in Mel-18–overexpressing cells using a retroviral expression vector as described in Materials and Methods. Cell expressing Mel-18, 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

Received 11/28/2006; revised 3/13/2007; accepted 4/6/2007.

Grant support: National Cancer Institute grant RO1CA 094150 and U.S. Department of Defense grant DAMD17-02-1-0509 (G.P. Dimri).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. N. Hay for providing mAkt expression vector.

References

1. Valk-Lingbeek ME, Bruggeman SW, van Lohuizen M. Stem cells and cancer; the polycomb connection. Cell 2004;118:409–18.
2. Glinsky GV, Berezovska O, Glinskii AB. Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. J Clin Invest 2005;115:1503–21.
3. Kim JH, Yoon SY, Jeong SH, et al. Overexpression of Bmi-1 oncoprotein correlates with axillary lymph node metastases in invasive ductal breast cancer. Breast 2004; 13:383–8.
4. Kleer CG, Cao Q, Varambally S, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci U S A 2003;100:11606–11.
5. Vonlanthen S, Heighway J, Altermatt HJ, et al. The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. Br J Cancer 2001;84:1372–6.
6. Kim JH, Yoon SY, Kim CN, et al. The Bmi-1 oncoprotein is overexpressed in human colorectal cancer and correlates with the reduced p16INK4a/p14ARF proteins. Cancer Lett 2004;203:217–24.
7. Song LB, Zeng MS, Liao WT, et al. Bmi-1 is a novel molecular marker of nasopharyngeal carcinoma progression and immortalizes primary human nasopharyngeal epithelial cells. Cancer Res 2006;66: 6225–32.
8. Kang MK, Kim RH, Kim SJ, et al. 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 2007;96: 126–33.
9. Liu S, Dontu G, Mantle ID, et al. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res 2006;66: 6063–71.
10. Dimri GP. What has senescence got to do with cancer? Cancer Cell 2005;7:505–12.
11. Itahana K, Zou Y, Itahana Y, et al. Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1. Mol Cell Biol 2003;23: 389–401.

12. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature 1999; 397:164–8.
13. Dimri GP, Martinez JL, Jacobs JJ, et al. The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells. Cancer Res 2002;62: 4736–45.
14. Guo WJ, Datta S, Band V, Dimri GP. Mel-18, a polycomb group protein, regulates cell proliferation and senescence via transcriptional repression of Bmi-1 and c-Myc oncoproteins. Mol Biol Cell 2007; 18:536–46.
15. Band V, Zajchowski D, Kulesa V, Sager R. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. Proc Natl Acad Sci U S A 1990;87:463–7.
16. Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle 2003;2:339–45.
17. Silva J, Garcia JM, Pena C, et al. Implication of polycomb members Bmi-1, Mel-18, and Hpc-2 in the regulation of p16INK4a, p14ARF, h-TERT, and c-Myc expression in primary breast carcinomas. Clin Cancer Res 2006;12:6929–36.
18. Hay N. The Akt-mTOR tango and its relevance to cancer. Cancer Cell 2005;8:179–83.
19. Bose S, Chandran S, Mirocha JM, Bose N. The Akt pathway in human breast cancer: a tissue-array-based analysis. Mod Pathol 2006;19:238–45.
20. Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. Genes Dev 1999; 13:2678–90.

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

Sonal Datta,[1] Mark J. Hoenerhoff,[4] Prashant Bommi,[1] Rachana Sainger,[1] Wei-Jian Guo,[1] Manjari Dimri,[2] Hamid Band,[2,3] Vimla Band,[1,3] Jeffrey E. Green,[4] and Goberdhan P. Dimri[1,3]

[1]Division of Cancer Biology and Department of Medicine, ENH Research Institute, and [2]Division of Molecular Oncology and [3]Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Evanston, Illinois; and
[4]Laboratory of Cancer Biology and Genetics, National Cancer Institute, Bethesda, Maryland

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):10286–95]

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 HMECs. However, the combined

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

S. Datta and M.J. Hoenerhoff contributed equally to this work.

G.P. Dimri and J.E. Green are cosenior authors.

Requests for reprints: Goberdhan P. Dimri, ENH Research Institute, 1001 University Place, Evanston, IL 60201. Phone: 224-364-7521; Fax: 224-364-7402; E-mail: g-dimri@northwestern.edu or Jeffrey E. Green, Laboratory of Cancer Biology and Genetics, National Cancer Institute, Bethesda, MD 20892. Phone: 301-435-5193; Fax: 301-496-8709; E-mail: jegreen@nih.gov.

doi:10.1158/0008-5472.CAN-07-1636

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).

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.

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; ref. 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).

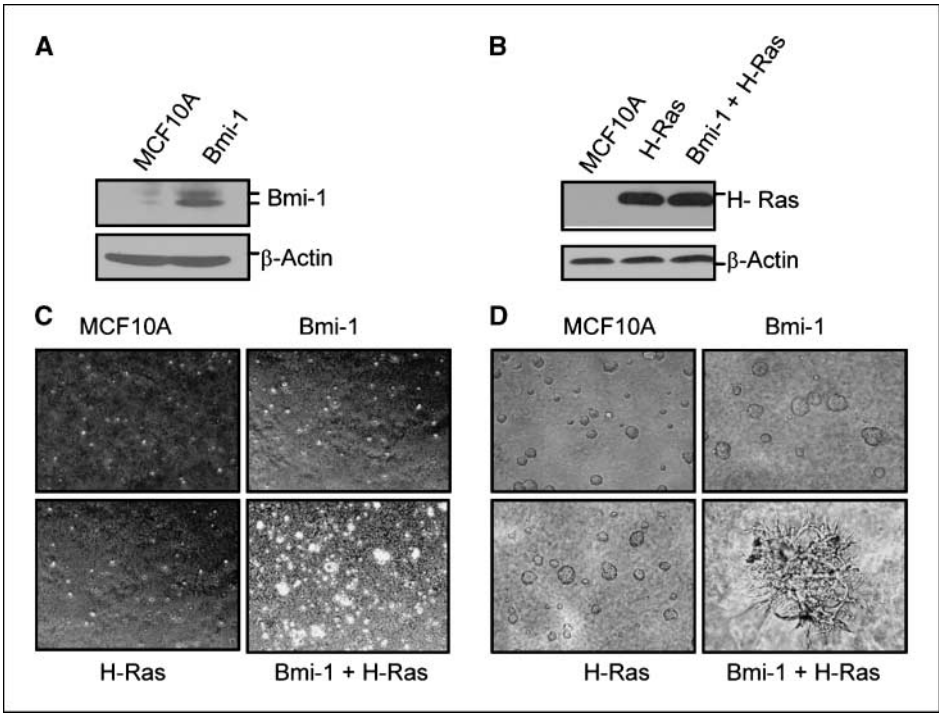


Figure 1. Bmi-1 and H-Ras co-overexpression transforms HMECs. *A,* Bmi-1–overexpressing MCF10A cells were generated by stable overexpression of Bmi-1, and cells (as indicated) were analyzed for Bmi-1 overexpression by Western blot analysis. *B,* H-Ras was introduced in control MCF10A and MCF10A–Bmi-1 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).

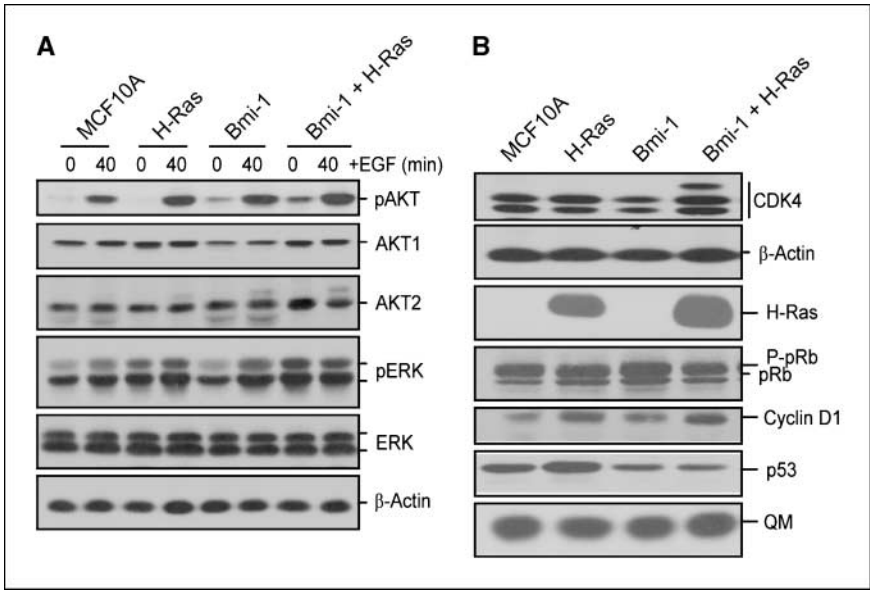


Figure 2. Various growth-regulatory pathways are dysregulated in cells co-overexpressing Bmi-1 and H-Ras. All cells were analyzed at passage 2 after Ras selection and/or mock infection. *A,* Western blot analysis of phosphorylated AKT, total AKT (AKT1 and AKT2), phosphorylated ERK, and total ERK in control 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.

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 HMECs 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*).

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

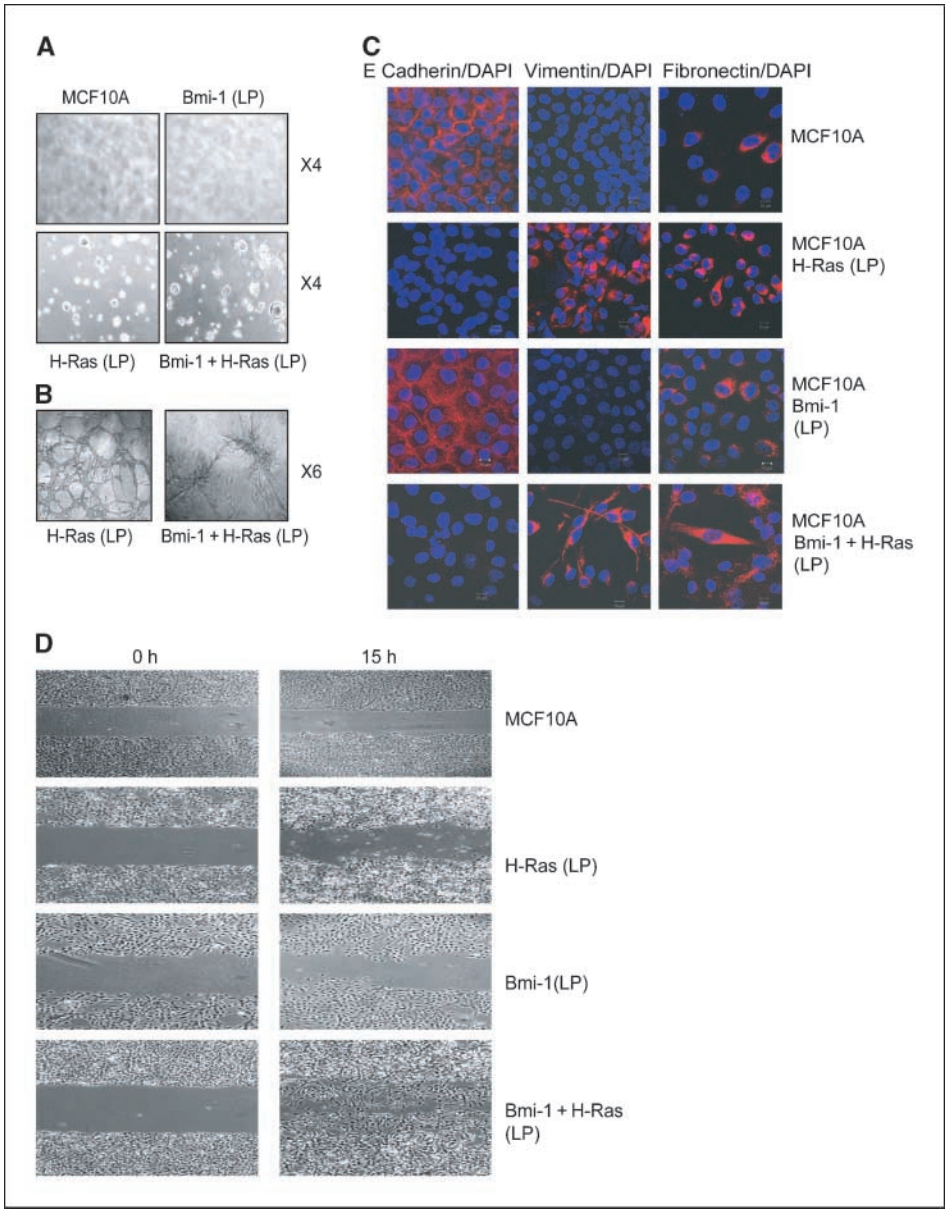


Figure 3. Late-passage H-Ras–expressing MCF10A cells exhibit transformed features. All MCF10A-derived cells were analyzed at passage 8. *A,* control MCF10A and MCF10A-derived late-passage cells (as indicated) were grown in soft agar to determine anchorage-independent growth potential of these cells. 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).

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*); MCF10A–Bmi-1+H-Ras (LP) cells formed tumors that were solid, firm, and

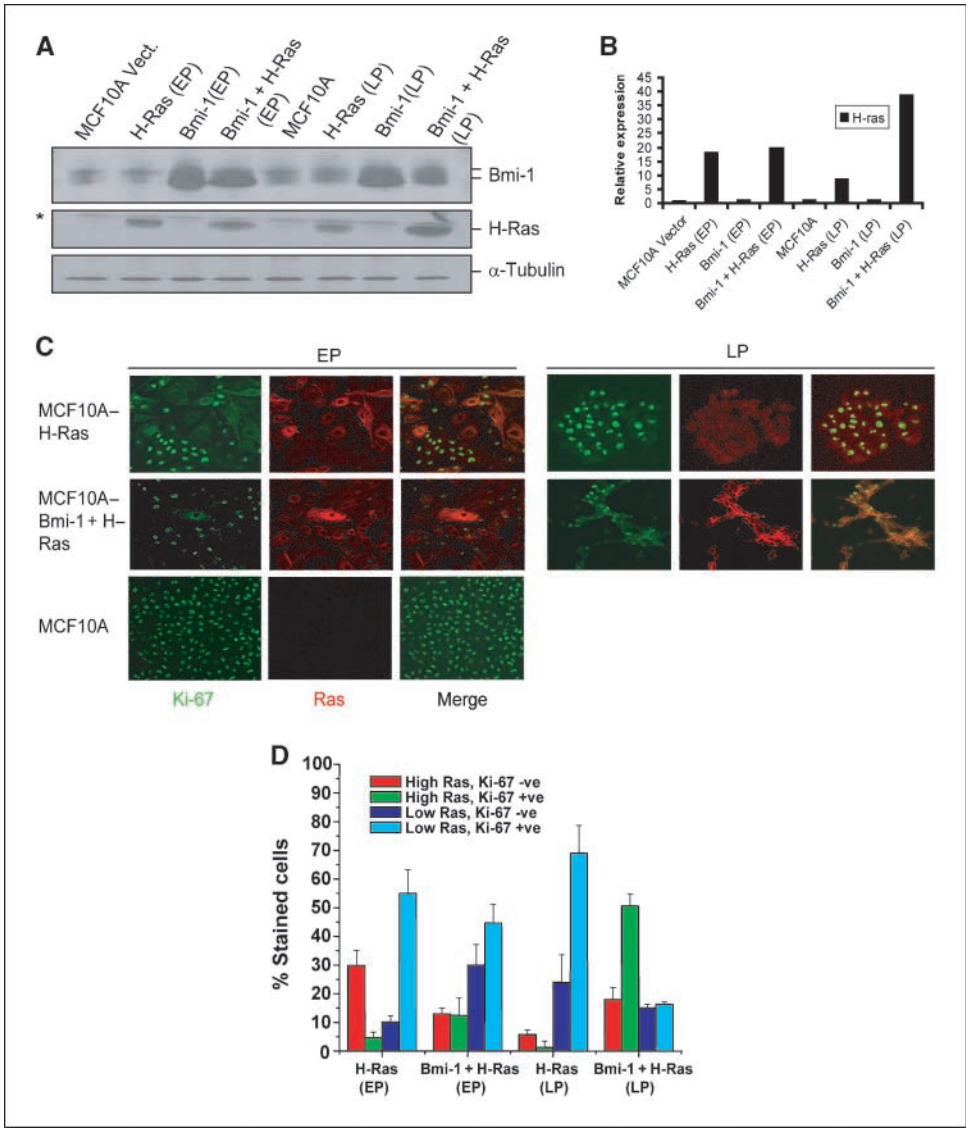


Figure 4. Expression level of H-Ras determines proliferation in MCF10A cells overexpressing H-Ras. *A,* H-Ras expression in MCF10A control and MCF10A-derived early-passage (passage 2 after Ras selection) and late-passage cells (passage 8) was determined by Western blot analysis as described in Materials 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.

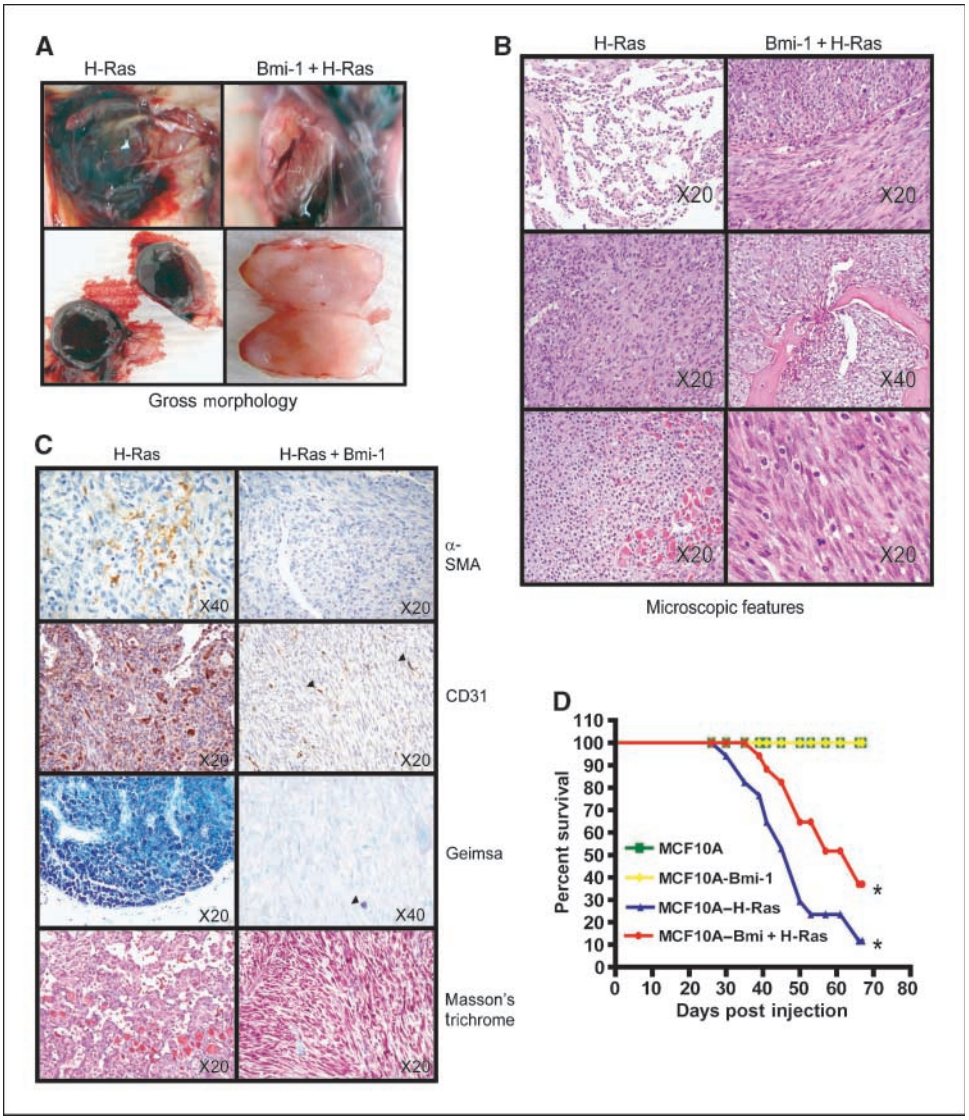


Figure 5. Gross morphology, histopathology, and immunohistochemistry of tumors originating from xenografts. *A,* gross morphology of tumors resulting from injection of MCF10A–H-Ras (LP) and MCF10A–Bmi-1+H-Ras cells (LP; as indicated). *Left,* tumors induced by MCF10A–H-Ras. *Right,* tumors induced by 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 ($P < 0.002$).

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*).

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*).

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.

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

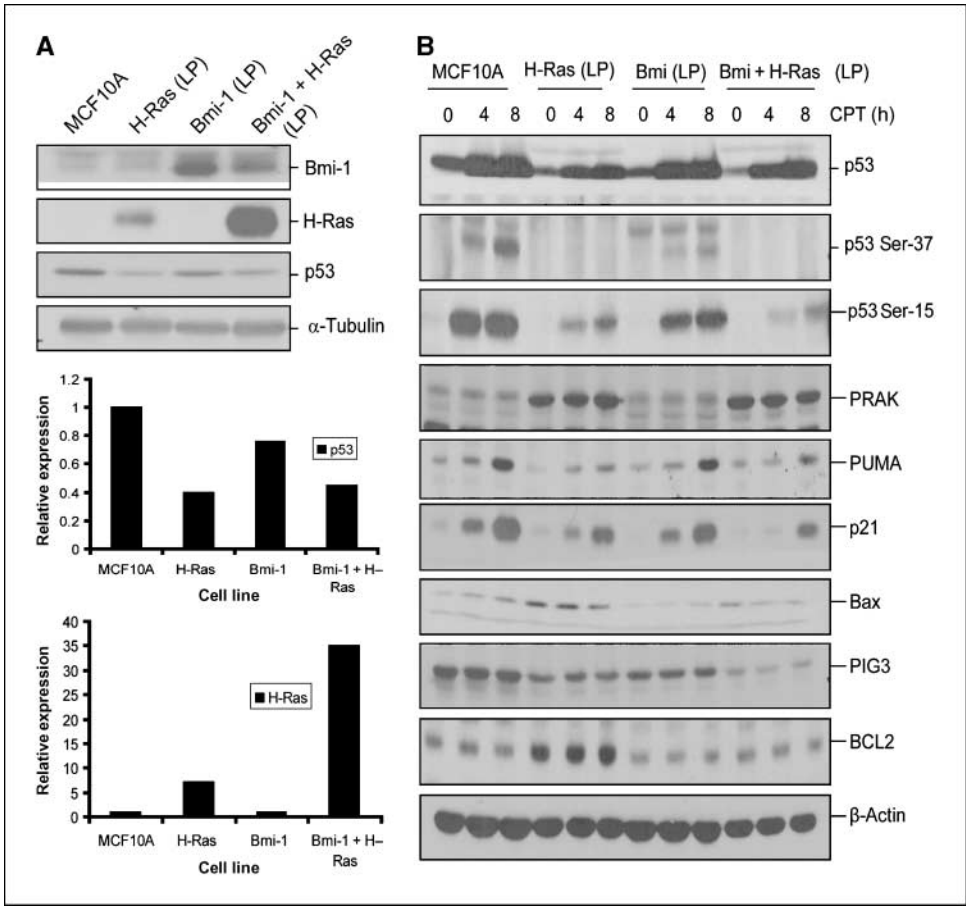


Figure 6. Analysis of p53 pathway in control MCF10A and MCF10A-derived late-passage cells. All cells except parental MCF10A cells, passage 9 cells, were used for the analysis. *A, top,* Western blot analysis of Bmi-1, H-Ras, and p53 in control MCF10A and MCF10A-derived (LP) cells (as indicated) was done 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).

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 phosphoryated 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*.

Acknowledgments

Received 5/3/2007; revised 8/2/2007; accepted 8/31/2007.

Grant support: National Cancer Institute grant RO1CA 094150 and Department of Defense grant DAMD17-02-1-0509 (G.P. Dimri).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Ringrose L, Paro R. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu Rev Genet 2004;38:413–43.
2. Raaphorst FM. Deregulated expression of Polycomb-group oncogenes in human malignant lymphomas and epithelial tumors. Hum Mol Genet 2005;14:R93–100.
3. Bea S, Tort F, Pinyol M, et al. BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. Cancer Res 2001;61:2409–12.
4. van Kemenade FJ, Raaphorst FM, Blokzijl T, et al. Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. Blood 2001;97:3896–901.
5. Sawa M, Yamamoto K, Yokozawa T, et al. BMI-1 is highly expressed in M_0-subtype acute myeloid leukemia. Int J Hematol 2005;82:42–7.
6. Vonlanthen S, Heighway J, Altermatt HJ, et al. The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. Br J Cancer 2001;84:1372–6.
7. Kim JH, Yoon SY, Kim CN, et al. The Bmi-1 oncoprotein is overexpressed in human colorectal cancer and correlates with the reduced p16INK4a/p14ARF proteins. Cancer Lett 2004;203:217–24.
8. Kim JH, Yoon SY, Jeong SH, et al. Overexpression of Bmi-1 oncoprotein correlates with axillary lymph node metastases in invasive ductal breast cancer. Breast 2004;13:383–8.
9. Glinsky GV, Berezovska O, Glinskii AB. Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. J Clin Invest 2005;115:1503–21.
10. Kang MK, Kim RH, Kim SJ, et al. 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 2007;96:126–33.
11. Song LB, Zeng MS, Liao WT, et al. Bmi-1 is a novel molecular marker of nasopharyngeal carcinoma progression and immortalizes primary human nasopharyngeal epithelial cells. Cancer Res 2006;66:6225–32.
12. Iwama A, Oguro H, Negishi M, et al. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. Immunity 2004;21:843–51.
13. Lessard J, Sauvageau G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature 2003;423:255–60.
14. Molofsky AV, He S, Bydon M, Morrison SJ, Pardal R. Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. Genes Dev 2005;19:1432–7.
15. Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ. Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature 2003;425:962–7.
16. Liu S, Dontu G, Mantle ID, et al. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer Res 2006;66:6063–71.

17. Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res 2005;7:86–95.

18. Itahana K, Zou Y, Itahana Y, et al. Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1. Mol Cell Biol 2003;23:389–401.

19. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature 1999;397:164–8.

20. Dimri GP. What has senescence got to do with cancer? Cancer Cell 2005;7:505–12.

21. Dimri GP, Martinez JL, Jacobs JJ, et al. The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells. Cancer Res 2002;62: 4736–45.

22. Kim RH, Kang MK, Shin KH, et al. Bmi-1 cooperates with human papillomavirus type 16 E6 to immortalize normal human oral keratinocytes. Exp Cell Res 2007;313: 462–72.

23. Guo WJ, Datta S, Band V, Dimri GP. Mel-18, a polycomb group protein, regulates cell proliferation and senescence via transcriptional repression of Bmi-1 and c-Myc oncoproteins. Mol Biol Cell 2007;18:536–46.

24. Lee K, Adhikary G, Balasubramanian S, et al. Expression of Bmi-1 in epidermis enhances cell survival by altering cell cycle regulatory protein expression and inhibiting apoptosis. J Invest Dermatol. Epub 2007 July 12.

25. Guo WJ, Zeng MS, Yadav A, et al. Mel-18 acts as a tumor suppressor by repressing Bmi-1 expression and down-regulating Akt activity in breast cancer cells. Cancer Res 2007;67:5083–9.

26. Berman H, Zhang J, Crawford YG, et al. Genetic and epigenetic changes in mammary epithelial cells identify a subpopulation of cells involved in early carcinogenesis. Cold Spring Harb Symp Quant Biol 2005;70:317–27.

27. Cowell JK, LaDuca J, Rossi MR, Burkhardt T, Nowak NJ, Matsui S. Molecular characterization of the t(3;9) associated with immortalization in the MCF10A cell line. Cancer Genet Cytogenet 2005;163:23–9.

28. Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 2003;30:256–68.

29. Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. Proc Natl Acad Sci U S A 1995;92: 9363–7.

30. Band V, Zajchowski D, Kulesa V, Sager R. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. Proc Natl Acad Sci U S A 1990;87: 463–7.

31. Dimri M, Naramura M, Duan L, 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;67:4164–72.

32. Elenbaas B, Spirio L, Koerner F, et al. Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev 2001;15: 50–65.

33. Seeburg PH, Colby WW, Capon DJ, Goeddel DV, Levinson AD. Biological properties of human c-Ha-ras1 genes mutated at codon 12. Nature 1984;312:71–5.

34. Landis MW, Pawlyk BS, Li T, Sicinski P, Hinds PW. Cyclin D1-dependent kinase activity in murine development and mammary tumorigenesis. Cancer Cell 2006;9: 13–22.

35. Yu Q, Sicinska E, Geng Y, et al. Requirement for CDK4 kinase function in breast cancer. Cancer Cell 2006;9:23–32.

36. Dawson PJ, Wolman SR, Tait L, Heppner GH, Miller FR. MCF10AT: a model for the evolution of cancer from proliferative breast disease. Am J Pathol 1996;148:313–9.

37. Giunciuglio D, Culty M, Fassina G, et al. Invasive phenotype of MCF10A cells overexpressing c-Ha-ras and c-erbB-2 oncogenes. Int J Cancer 1995;63:815–22.

38. Moon A, Kim MS, Kim TG, et al. H-ras, but not N-ras, induces an invasive phenotype in human breast epithelial cells: a role for MMP-2 in the H-ras-induced invasive phenotype. Int J Cancer 2000;85:176–81.

39. Santner SJ, Dawson PJ, Tait L, et al. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res Treat 2001;65:101–10.

40. Shin I, Kim S, Song H, Kim HR, Moon A. H-Ras-specific activation of Rac-MKK3/6-p38 pathway: its critical role in invasion and migration of breast epithelial cells. J Biol Chem 2005;280:14675–83.

41. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997;88:593–602.

42. Sun P, Yoshizuka N, New L, et al. PRAK is essential for ras-induced senescence and tumor suppression. Cell 2007;128:295–308.

43. Wang W, Chen JX, Liao R, et al. Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced premature senescence. Mol Cell Biol 2002;22:3389–403.

44. Chen G, Hitomi M, Han J, Stacey DW. The p38 pathway provides negative feedback for Ras proliferative signaling. J Biol Chem 2000;275:38973–80.

45. el-Deiry WS. Regulation of p53 downstream genes. Sem Cancer Biol 1998;8:345–57.

46. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 2001;7:683–94.

47. Flatt PM, Polyak K, Tang LJ, et al. p53-dependent expression of PIG3 during proliferation, genotoxic stress, and reversible growth arrest. Cancer Lett 2000; 156:63–72.

48. Boehm JS, Hahn WC. Understanding transformation: progress and gaps. Cur Opin Genet Dev 2005;15:13–7.

49. Clark GJ, Der CJ. Aberrant function of the Ras signal transduction pathway in human breast cancer. Breast Cancer Res Treat 1995;35:133–44.

50. von Lintig FC, Dreilinger AD, Varki NM, Wallace AM, Casteel DE, Boss GR. Ras activation in human breast cancer. Breast Cancer Res Treat 2000;62:51–62.

51. Sarkisian CJ, Keister BA, Stairs DB, Boxer RB, Moody SE, Chodosh LA. Dose-dependent oncogene-induced senescence *in vivo* and its evasion during mammary tumorigenesis. Nature Cell Biol 2007;9:493–505.

In Search of Breast Cancer Culprits: Suspecting the Suspected and the Unsuspected

Goberdhan P. Dimri

Department of Medicine, ENH Research Institute, Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Evanston, IL 60201.

Abstract: I would like to welcome breast cancer research community to the first editorial of our newest journal "Breast Cancer: Basic and Clinical Research". In pursuit of breast cancer culprits, we have come a long way since the early 90's when the first breast cancer susceptibility gene *BRCA1* was mapped and cloned. In the past few years, several new loci associated with the various degree of breast cancer risk have been identified using "Candidate Gene Association Study (CGAS) and Genome-Wide Association Study (GWAS)" approaches. This editorial is meant to quickly glance over recent findings of these population-based association studies.

Introduction

Breast cancer is one of the most terrifying diseases that human civilization has ever known. Legend has it that powerful queen Atossa of the ancient Babylon had breast cancer, and the specific mention of breast cancer can be found in "Hippocratic Corpus" written by Hippocrates and his peers dating back to fourth and fifth centuries B.C. (Karpozilos and Pavlidis, 2004). Throughout our past and present civilization, we are reminded that several notable figures and ordinary citizens have suffered or are suffering from this dreadful disease. Although the life-time risk of developing breast cancer may vary in different geographic regions of the world, nobody is immune to developing breast cancer. In the United States of America and most western countries, the life-time risk of developing breast cancer in women is close to 1 in 8. The most intriguing question is- what determines this risk?

It is well known that early onset breast cancer tends to cluster in families and usually first degree relatives of affected individuals have twofold higher risk of developing breast cancer (2001). This increased risk is independent of lifestyle and environmental factors, and thought to be due to genetic susceptibility of individuals to develop breast cancer (Lichtenstein et al. 2000). The early onset breast cancer, which tends to cluster in families, is also known as familial breast cancer. Overall, 20%–25% of familial breast cancer is attributed to high penetrance genes *BRCA1, BRCA2*, *TP53*, and *PTEN* (Easton, 1999).

The high penetrance genes were identified using family-based linkage studies. These studies also identified additional breast cancer susceptibility genes. In all, these family-based linkage studies were instrumental in identifying ten important genes- *BRCA1, BRCA2, TP53, PTEN, CHEK2, ATM, NBS1, RAD50, BRIP1,* and *PALB2* for inherited breast cancer (Walsh and King, 2007). These ten genes, which are critical for genome integrity account for roughly 50% of familial breast cancer (Walsh and King, 2007). Despite intense efforts, linkage studies have failed to identify additional breast cancer susceptibility genes for familial breast cancer.

The late onset breast cancer, which is primarily sporadic in nature, is by far the most prevalent. In sporadic breast cancer, "the 10 genes for inherited breast cancer" have very minimal role. Hence, the 50% of the familial or early onset cases and majority of late onset cases of breast cancer must involve low to medium penetrance genes. The linkage studies lack the power to detect alleles responsible for low to moderate risk of developing breast cancer. Such alleles are now being identified using population-based gene-association studies. These studies take advantage of thousands of known single-nucleotide polymorphisms (SNPs) present in the human genome. The earlier studies focused on candidate gene approach and looked for SNPs in limited number of genes and their possible

Correspondence: Goberdhan P. Dimri, ENH Research Institute, 1001 University Place, Evanston, IL 60201, U.S.A. Tel: (224) 364-7521; Fax: (224) 364-7402; Email: gdimri@enh.org or g-dimri@northwestern.edu

association with breast cancer. More recent studies known as Genome-Wide Association Studies (GWASs) are taking advantage of unbiased scan of the whole genome for SNPs associated with breast cancer risk.

The Usual Suspects: Candidate Gene Association Studies

Several breast cancer research groups have studied the association of breast cancer risk with common variants (SNPs) of candidate genes. More comprehensive CGASs have been carried out by SEARCH breast cancer study group and the "Breast Cancer Association Consortium" (BCAC). Results of CGASs have been mixed and very confusing. Some of the association studies may not be directly comparable because they used different population groups, while in other studies, the sample size may not be sufficiently large enough.

Starting with *HER-2*, in some studies a common variant *HER-2* V655I was reported to be associated with breast cancer (Xie et al. 2000), however other studies found no such association (Benusiglio et al. 2006; Einarsdottir et al. 2006). Human *CYP19* gene, which encodes aromatase cytochrome P450 is another plausible candidate gene where some studies have suggested association of common variants with significant breast cancer risk (Haiman et al. 2003; Ralph et al. 2007), while other studies suggested no association of any SNPs in *CYP19* with breast cancer risk (Healey et al. 2000). Recently, Ralph et al. suggested possible age-specific association of certain SNPs present in genes encoding steroid hormone pathway (Ralph et al. 2007). Specifically, it was reported that cytosine/cytosine homozygous genotype of cytochrome P450 XIB2 (*CYP11B2*) was associated with reduced breast cancer risk at younger age, but increased risk at older age (Ralph et al. 2007), and homozygous cytosine-guanine (CG/CG) genotype of uridine phosphorylase glycosyltransferase 1A7 (*UGT1A7*) was associated with increased breast cancer risk at younger ages but decreased risk at older ages (Ralph et al. 2007).

By analyzing 4,474 breast cancer cases and 4,560 controls from SEARCH collection (United Kingdom), Baynes et al. reported that in contrast to rare variants, the common variants in the *ATM*, *BRCA1*, *BRCA2*, *CHEK2* and *TP53* are unlikely to increase the breast cancer risk (Baynes et al. 2007). A study be Onay et al. also concluded that 19 individual commonly occurring SNPs associated with 18 key cancer genes *XPD, PTEN, GADD45, p27, ESR1, CYP17, GSTM3, MTHFR, IL1a, IL10, IL13, TNFa, G-CSF, CCND1, COMT, BARD1, GSTP1* and *MMP1* did not contribute to breast cancer risk (Onay et al. 2006). A very recent BCAC study also did not find an association of *MDM2* SNP309 and *TP53* R72P SNP with breast cancer risk (Schmidt et al. 2007).

On the other hand, common variants of few genes, which were suspected to play a role in breast cancer did turned out to have weak association with breast cancer risk. For example, BCAC reported that common coding variants *CASP8* D302H in the gene encoding Caspase 8, and *TGFB1* L10P in the gene encoding transforming growth factor-β (TGFβ), in one allele (heterozygote) were associated with significant risk to invasive breast cancer (Cox et al. 2007). In an earlier study, *CASP8* D302H variant was reported to be associated with reduced breast cancer risk in a dose-dependent manner; it provided better protection against breast cancer in the homozygous condition (MacPherson et al. 2004).

BCAC also analyzed data from 12 studies for 16 SNPs in various candidate genes and concluded that only 5 SNPS (*CASP*8 D302H, *IGFBP3*-202 *c* > *a*, *PGR*V660L, *SOD2* V16A, and *TGFB1* L10P) were associated with breast cancer, but the statistical significance of the association was only borderline (Breast Cancer Association, 2006). The remaining 11 SNPs in other candidate genes showed no significant association with breast cancer risk (Breast Cancer Association, 2006). Another recent study (SEARCH investigators), which analyzed association between common variants found in 120 candidate genes and breast cancer concluded that a proportion of SNPs in candidate genes in the cell-cycle control pathway, genes involved in steroid hormone metabolism and signaling were weakly associated with breast cancer risk but large sample-sizes from multicentre collaboration is needed to identify SNPs that are associated with definitive breast cancer risk (Pharoah et al. 2007). Some borderline significance of SNPs in few selected antioxidant defense genes (for example *CAT g*27168*a*, *TXN t*2715*c*, *TXNRD2* A66S and *TXNRD2 g*23524*a*) and epigenetic genes (for example *DNMT3b-c*31721*t*) with breast cancer risk has been reported but these observations need to be confirmed in larger

epidemiological studies (Cebrian et al. 2006a; Cebrian et al. 2006b).

Not so Usual Suspects: Genome-Wide Association Studies

As discussed above, the candidate gene approach studies to identify breast cancer risk has not been very successful. With the advent of rapid SNP screening technologies and completion of "Hap Map", it is now possible to rapidly scan the genome of several thousand individuals to find association of SNPs with a particular disease. Recently, four such GWASs have been conducted to identify novel breast cancer susceptibility loci (Easton et al. 2007; Hunter et al. 2007; Murabito et al. 2007; Stacey et al. 2007).

Stacey et al. genotyped 4,554 breast cancer patients and 17,577 controls using the Illumina Hap300 platform and reported that individuals of European descent with homozygous allele A of rs13387042 SNP on chromosome 2q35 have an estimated 1.44 fold higher risk of estrogen receptor-positive (ER-positive) breast cancer compared to noncarriers, while homozygous allele T of rs3803662 on chromosome 16q12 was associated with 1.64 fold risk of ER-positive breast cancer (Stacey et al. 2007). Among other ethnicities, both variants were only marginally significant; in fact T-rs3803662 allele was protective in African Americans (Stacey et al. 2007). Functional significance of both these SNPs is not clear, although rs3803662 is near the 5' end of *TNRC9*, a gene implicated in bone metastasis of breast cancer cells. Remarkably, significant breast cancer association of rs3803662 SNP near the 5' end of *TNRC9* was also reported in an independent study (Easton et al. 2007).

The discovery of association of SNPs in intron 2 of *FGFR2*, which encode fibroblast growth factor receptor 2, with breast cancer risk was also reported in two independent GWASs (Easton et al. 2007; Hunter et al. 2007). In the first study, which also tagged rs3803662, GWAS was carried out using a two-stage analysis of 4,398 breast cancer cases and 4,316 controls. At second stage, authors found significant association of 1,792 SNPs with breast cancer risk, but chose to study 30 SNPs with highest level of significance for subsequent confirmation in 21,860 cases and 22,578 controls chosen from 22 studies (Easton et al. 2007). The following SNPs showed the most significant and consistent evidence of association- rs2981582 (*FGFR2*), and rs12443621, rs8051542 and rs3803662 (*TNRC9*), rs889312 (*MAP3K1*), rs13281615 (8q) and rs3817198 (*LSP1*) (Easton et al. 2007). Although *FGFR, TNRC9, MAP3K1 and LSP1* are plausible breast cancer culprits, the functional significance of SNPs in these genes remain unclear at this point (Easton et al. 2007).

In the second GWAS, Hunter et al. genotyped 528,173 SNPs in 1,145 postmenopausal women of European descent with invasive breast cancer and 1,142 controls (Hunter et al. 2007). The GWAS identified four SNPs (rs1219648, rs2420946, rs11200014 and rs2981579) in intron 2 of *FGFR2*, which showed significant association with breast cancer (Hunter et al. 2007). The association was confirmed using three additional studies using 1,776 cases and 2,072 controls (Hunter et al. 2007). Again, although *FGFR2* is a plausible breast cancer gene, the functional significance of these common variants in *FGFR2* loci is not clear.

Another GWAS was conducted by Murabito et al. using study subjects from NHLBI's Framingham Heart Study (Murabito et al. 2007). The study involved 1,335 participants, including 58 women with breast cancer and 59 men with prostate cancer (Murabito et al. 2007). Possibly, because of limited size of the population, authors did not find significant association of any SNP with breast or prostate cancer risk. Although in the same study, using candidate gene approach, authors reported significant association of two SNPs (rs9325782 and rs2410373) in *MSRI* gene with prostate cancer, and three SNPs (rs905883, rs7564590 and rs7558615) in *ERBB4* with breast cancer (Murabito et al. 2007).

Conclusion: Devil is Hiding in the Genome

With the rapid advent of genotyping technologies, we have entered an exciting era of genome-based discoveries for human diseases. CGAS and GWAS clearly have the power to identify common variants that are associated with low susceptibility loci for a particular disease. At present, due to continued drop in genotyping costs, GWAS appears to be a better approach than CGAS. However, a great degree of caution is needed in the correct interpretation of such studies. There are several issues which need to be addressed in each GWAS; the caveats range from sample size to genotyping

quality controls to successful replication of results. Several of these points are discussed in NCI-NHGRI (National Cancer Institute-National Human Genome Research Institute) working group recommendations on replicating GWAS results (Chanock et al. 2007).

The next legitimate question is- what is the overall risk of a particular disease associated with these so called common variants? Although statistically significant, the effect of individual SNP is generally very small in terms of increasing breast cancer risk. Such risk is usually close to 1.2 to 1.5. However, the overall effect of combinations of SNPs on breast cancer risk may be substantial. This overall effect, which is also termed as polygenic effect (of SNPs) may be additive or synergistic and may account for most of the genetic risk associated with developing breast cancer. Although, only a handful of these common variants have been identified so far, the presumption is that there are many more of these, and they may genetically interact.

For now, the breast cancer community will wait for the identification of all of the common variants in the genome, which would be associated with breast cancer, and then all of us- clinical and basic scientists, and other stake holders will debate what is in the best interest of naïve general public. Should we prepare for the genetic counseling of would be breast cancer patients even though the overall risk factor may still be below 1.5 to 2.0? In summary, each GWAS starts with an assumption that the evildoers are in the genome and that they most probably conspire together to increase the risk of developing a particular disease such as breast cancer. On an optimistic note, the gene hunters or SNP hunters to be more accurate, are busy hunting these evildoers, wherever they may be- in the introns, exons or the regulatory regions in the genome.

References

2001. Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58,209 women with breast cancer and 101,986 women without the disease. *Lancet*, 358:1389–99.

Baynes, C., Healey, C.S., Pooley, K.A., Scollen, S., Luben, R.N., Thompson, D.J., Pharoah, P.D., Easton, D.F., Ponder, B.A. and Dunning, A.M. 2007. Common variants in the ATM, BRCA1, BRCA2, CHEK2 and TP53 cancer susceptibility genes are unlikely to increase breast cancer risk. *Breast Cancer Res.*, 9:R27.

Benusiglio, P.R., Pharoah, P.D., Smith, P.L., Lesueur, F., Conroy, D., Luben, R.N., Dew, G., Jordan, C., Dunning, A., Easton, D.F. and Ponder, B.A. 2006. HapMap-based study of the 17q21 ERBB.2 amplicon in susceptibility to breast cancer. *Br. J. Cancer*, 95:1689–95.

Breast Cancer Association, C. 2006. Commonly studied single-nucleotide polymorphisms and breast cancer: results from the Breast Cancer Association Consortium. *J. Natl. Cancer Inst.*, 98:1382–96.

Cebrian, A., Pharoah, P.D., Ahmed, S., Ropero, S., Fraga, M.F., Smith, P.L., Conroy, D., Luben, R., Perkins, B., Easton, D.F., Dunning, A.M., Esteller, M. and Ponder, B.A. 2006a. Genetic variants in epigenetic genes and breast cancer risk. *Carcinogenesis.*, 27:1661–9.

Cebrian, A., Pharoah, P.D., Ahmed, S., Smith, P.L., Luccarini, C., Luben, R., Redman, K., Munday, H., Easton, D.F., Dunning, A.M. and Ponder, B.A. 2006b. Tagging single-nucleotide polymorphisms in antioxidant defense enzymes and susceptibility to breast cancer. *Cancer Res.*, 66:1225–33.

Chanock, S.J., Manolio, T., Boehnke, M., Boerwinkle, E., Hunter, D.J., Thomas, G. et al. 2007. Replicating genotype-phenotype associations. *Nature*, 447:655–60.

Cox, A., Dunning, A.M., Garcia-Closas, M., Balasubramanian, S. et al. 2007. A common coding variant in CASP8 is associated with breast cancer risk. *Nat. Genet.*, 39:352–8.

Easton, D.F. 1999. How many more breast cancer predisposition genes are there. *Breast Cancer Res.*, 1:14–7.

Easton, D.F., Pooley, K.A., Dunning, A.M., Pharoah, P.D. et al. 2007. Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature*, 447:1087–93.

Einarsdottir, K., Rosenberg, L.U., Humphreys, K., Bonnard, C., Palmgren, J., Li, Y., Li, Y., Chia, K.S., Liu, E.T., Hall, P., Liu, J. and Wedren, S. 2006. Comprehensive analysis of the ATM, CHEK2 and ERBB2 genes in relation to breast tumour characteristics and survival: a population-based case-control and follow-up study. *Breast Cancer Res.*, 8:R67.

Haiman, C.A., Stram, D.O., Pike, M.C., Kolonel, L.N., Burtt, N.P., Altshuler, D., Hirschhorn, J. and Henderson, B.E. 2003. A comprehensive haplotype analysis of CYP19 and breast cancer risk: the Multiethnic Cohort. *Hum. Mol. Genet.*, 12:2679–92.

Healey, C.S., Dunning, A.M., Durocher, F., Teare, D., Pharoah, P.D., Luben, R.N., Easton, D.F. and Ponder, B.A. 2000. Polymorphisms in the human aromatase cytochrome P450 gene (CYP19) and breast cancer risk. *Carcinogenesis*, 21:189–93.

Hunter, D.J., Kraft, P., Jacobs, K.B., Cox, D.G., Yeager, M., Hankinson, S.E. et al. 2007. A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. *Nat Genet.*, 39:870–4.

Karpozilos, A. and Pavlidis, N. 2004. The treatment of cancer in Greek antiquity. *Eur. J. Cancer*, 40:2033–40.

Lichtenstein, P., Holm, N.V., Verkasalo, P.K., Iliadou, A., Kaprio, J., Koskenvuo, M., Pukkala, E., Skytthe, A. and Hemminki, K. 2000. Environmental and heritable factors in the causation of cancer—analyses of cohorts of twins from Sweden, Denmark, and Finland. *N. Engl. J. Med.*, 343:78–85.

MacPherson, G., Healey, C.S., Teare, M.D., Balasubramanian, S.P., Reed, M.W., Pharoah, P.D., Ponder, B.A., Meuth, M., Bhattacharyya, N.P. and Cox, A. 2004. Association of a common variant of the CASP8 gene with reduced risk of breast cancer. *J. Natl. Cancer Inst.*, 96:1866–9.

Murabito, J.M., Rosenberg, C.L., Finger, D., Kreger, B.E., Levy, D., Splansky, G.L., Antman, K. and Hwang, S.J. 2007. A genome-wide association study of breast and prostate cancer in the NHLBI's Framingham Heart Study. *BMC Med. Genet.*, 8(Suppl 1):S6.

Onay, V.U., Briollais, L., Knight, J.A., Shi, E., Wang, Y., Wells, S., Li, H., Rajendram, I., Andrulis, I.L. and Ozcelik, H. 2006. SNP-SNP interactions in breast cancer susceptibility. *BMC Cancer*, 6:114.

Pharoah, P.D., Tyrer, J., Dunning, A.M., Easton, D.F. and Ponder, B.A. 2007. Association between common variation in 120 candidate genes and breast cancer risk. *PLoS Genet.*, 3:e42.

Ralph, D.A., Zhao, L.P., Aston, C.E., Manjeshwar, S., Pugh, T.W., DeFreese, D.C., Gramling, B.A., Shimasaki, C.D. and Jupe, E.R. 2007. Age-specific association of steroid hormone pathway gene polymorphisms with breast cancer risk. *Cancer*, 109:1940–8.

Schmidt, M.K., Reincke, S., Broeks, A., Braaf, L.M., Hogervorst, F.B., Tollenaar, R.A. et al. 2007. Do MDM2 SNP309 and TP53 R.72P interact in breast cancer susceptibility? A large pooled series from the breast cancer association consortium. *Cancer Res.*, 67:9584–90.

Stacey, S.N., Manolescu, A., Sulem, P., Rafnar, T., Gudmundsson, J. et al. 2007. Common variants on chromosomes 2q35 and 16q12 confer susceptibility to estrogen receptor-positive breast cancer. *Nat. Genet.*, 39:865–9.

Walsh, T. and King, M.C. 2007. Ten genes for inherited breast cancer. *Cancer Cell.*, 11:103–5.

Xie, D., Shu, X.O., Deng, Z., Wen, W.Q., Creek, K.E., Dai, Q., Gao, Y.T., Jin, F. and Zheng, W. 2000. Population-based, case-control study of HER2 genetic polymorphism and breast cancer risk. *J. Natl. Cancer Inst.*, 92:412–7.