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

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

Division of Cancer Biology and Department of Medicine, ENH Research Institute, and Division of Molecular Oncology and Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Evanston, Illinois; and 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 leiomyomatous, 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 p16INK4A-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 p16INK4A and p19ARF (p14ARF in human; refs. 18, 19). By down-regulating p16INK4A 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 p16INK4A (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 p16INK4A, 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 p16INK4A, Bmi-1 regulates AKT activity in MCF10A and MCF7 cells (25). It is thought that the precursor cells for breast cancer are p16INK4A-negative due to promoter methylation and silencing (26), suggesting that overexpression of Bmi-1 in p16INK4A-negative tumors may contribute to oncogenesis via 16INK4A-independent mechanisms. Here, we examined the oncogenic potential of Bmi-1 in an immortal but untransformed HMEC line MCF10A, which does not express p16INK4A, p14ARF, and p15INK4B (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
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 H6B10G7 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, 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 auxiliary mammary fat pad with 1 × 106 cells from each cell line. Tumor growth was measured weekly by caliper, and mice were euthanized by CO2 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 noncancerous but immortal HMEC cell line (Fig. 1A). 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 p16INK4A−negative HMECs. Similar results were obtained using Bmi-1–immortalized 76N HMECs (Supplementary Fig. S1), which also do not express p16INK4A (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).
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. 1B). 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. 1C 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. 1C). 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. 1D).

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 EGFr-starved conditions (Fig. 2A). 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. 2A). 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. 2B). We also determined the expression of pRb and p53 tumor suppressors in control and MCF10A–derived cells. Because MCF10A cells are p16INK4A negative and contained high hyperphosphorylated pRb, no significant differences were found between different forms of pRb in control and MCF10A–derived cells (Fig. 2B). 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. 2B). 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. 3A). 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 passing 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. 3B).
**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. 3D). 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. 3C). 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.

![Figure 3](https://example.com/figure3.png)

**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. 4A). On the other hand, Bmi-1-H-Ras (LP) cells expressed a high level of Ras (Fig. 4A and B). Bmi-1+H-Ras (EP) cells and H-Ras (EP) cells expressed similar levels of Ras (Fig. 4A 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. 4C and D). On the other hand, H-Ras (LP) culture mostly contained cells with low Ras, which stained positive with Ki-67 (Fig. 4C 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. 4C 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. 4C 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. 5A); 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–H-Ras (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–H-Ras-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 immunostaining was done to determine proliferation in MCF10A–H-Ras (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–H-Ras (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.
irregular, whitish-tan on cut surface with well-differentiated vasculature. In contrast, tumors formed by MCF10A–H-Ras (LP) 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).
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. 5C). 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. 5C). 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. 5C). Giemsa staining confirmed the absence of mast cells in these tumors except for a rare mature 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. 5C). 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. 5D).

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 p16INK4A 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. S4A). Because MCF10A cells are p16INK4A-negative, the partial OIS in these cells may depend on p53 and its target genes. Consistent with partial OIS, early-passage MCF10A-LP cells also showed slower growth compared with vector control MCF10A and MCF10A cells co-overexpressing H-Ras and Bmi-1 (Supplementary Fig. S2B).

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. 2B), p53 was down-regulated in MCF10A–H-Ras (LP) cells (Fig. 6A). 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. 6B). 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
MCF10A–Bmi-1 cells, p53 remained inducible by camptothecin in all four sets 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. 6B; 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. 6B; 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. 6B). Interestingly, PRAK expression was up-regulated in H-Ras (LP) cells (Fig. 6B). 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. 6B; 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. 6B; 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. 6B). Interestingly, compared with control MCF10A cells, MCF10A-derived (LP) cells showed significant down-regulation of PIG3 (Fig. 6B).

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 oncprotein in immortalized HMECs. Although immortalized HMECs that we studied lack p16INK4A, 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 p16INK4A, p14ARF, and p15INK4B (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 p16INK4A 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 EGFR 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 p16INK4A 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 anticongenic 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 containing 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 p16INK4A-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. 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 mammmary 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 o-soma immunohistochemistry was negative in these tumors, Masson’s trichrome stain along with positive immunohistochemistry for vimentin would suggest that these cells may represent a myoepithelial phenotype consistent with EMT.

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

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Sonal Datta, Mark J. Hoenerhoff, Prashant Bommi, et al.