Bmi1 is critical for lung tumorigenesis and bronchioalveolar stem cell expansion

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Understanding the pathways that control epithelial carcinogenesis is vital to the development of effective treatments. The Polycomb group family member Bmi1 is overexpressed in numerous epithelial tumors, but its role in their development has not been established. We now show a key role for *Bmi1* in lung adenocarcinoma. Whereas lung development occurs normally in *Bmi1*-deficient mice, loss of *Bmi1* decreases the number and progression of lung tumors at a very early point in an oncogenic *K-ras*-initiated mouse model of lung cancer. This correlates with a defect in the ability of *Bmi1*-deficient putative bronchiolalveolar stem cells (BASCs) to proliferate in response to the oncogenic stimulus. Notably, in the absence of oncogenic *K-ras*, *Bmi1*-deficient BASCs show impaired proliferation and self-renewal capacity in culture and after lung injury *in vivo*. Abrogated lung cancer development and BASC self-renewal occur partially in a $p19^{ARF}$-dependent manner. Our data suggest that *Bmi1* deficiency suppresses tumor development by limiting the expansion potential of BASCs, the apparent lung cancer cells of origin. Because Bmi1 is elevated in additional tumor types, this suggests that Bmi1 plays a key role in regulating proliferation of both stem cells and tumor cells in diverse adult epithelial tissues.

Arf | Ink4a | non-small-cell lung cancer | p16

Cancers of epithelial origin are the most prevalent tumors in adults (1), yet little is known about the requirements for initiation and maintenance of these solid tumors. Lung cancer is the most common epithelial tumor and the leading cause of cancer death worldwide. Analysis of human tumors has identified a number of dysregulated genes that are associated with lung cancer including the Polycomb group member, Bmi1, which is overexpressed in non-small-cell lung cancer (NSCLC) and other epithelial malignancies, including colorectal carcinoma and liver carcinoma (reviewed in ref. 2). In particular, Bmi1 overexpression is correlated with poor prognosis for lung cancer patients (3). Bmi1 is an epigenetic chromatin modifier that acts as a key component of the PRC1 complex to mediate transcriptional repression. It was initially identified as an oncogene that cooperates with *c-myc* in generation of B-cell lymphomas (4, 5). The oncogenic potential of Bmi1 is, in part, because of negative regulation of the *Ink4a/Arf* locus that encodes two proteins, $p16^{INK4a}$ and $p19^{ARF}$, that suppress proliferation and promote apoptosis (6, 7).

Notably, Bmi1 has been implicated in the control of tissue stem cells and the tumors to which they may give rise. Analysis of *Bmi1*-deficient animals reveals the presence of defects in hematopoiesis and both the central and peripheral nervous systems (8). Characterization of these defects demonstrates that Bmi1 is required for self-renewal of hematopoietic and neural stem cells (9, 10). In addition to its effect on these normal cell compartments, *Bmi1* deficiency suppresses hematopoietic malignancy (11), and leukemic stem cells lacking *Bmi1* fail to propagate disease (12). Similarly, granule cell precursors from *Bmi1*-null animals show decreased proliferation, and medulloblastomas arising from this cell type have increased levels of Bmi1 (13). Loss of *Ink4a/Arf* partially rescues *Bmi1*-null hematopoietic and neurosphere self-renewal capacity and restores transformation potential to *Bmi1*-deficient hematopoietic progenitors (11, 14, 15). Similarly, Bmi1 contributes to glioma development through a combination of *Ink4a/Arf*-dependent and -independent mechanisms (16). Apart from the skeletal, hematopoietic, and neural defects, all other tissues in *Bmi1* null animals, including the lungs, appear normal.

Several populations of putative stem or progenitor cells have been identified in the adult murine lung, providing a means to address the possible connections between maintenance of adult lung tissue and lung tumorigenesis (17, 18). Particularly in the distal lung where lung adenocarcinomas most frequently arise, the nonciliated bronchiolar cells, Clara cells, and the secretory epithelial cells in the alveolar space, alveolar type II (AT2) cells, have been implicated as stem or progenitor cells. Lung injury studies suggest that a population of damage-resistant, or "variant", Clara cells are stem or progenitor cells that repair damaged Clara cells (19, 20). Expanding on these seminal studies, we identified a putative pulmonary stem cell population called bronchioalveolar stem cells (BASCs). BASCs coexpress SP-C, a marker of AT2 cells, and Scgb1a1, a marker of Clara cells (also known as CCSP, CCA, and CC10) (21, 22), and reside in the bronchioalveolar duct junction (BADJ). BASCs proliferate before Clara cells, in response to Clara cell injury, and also respond to alveolar cell injury *in vivo*. BASCs exhibit bona fide stem cell characteristics *in vitro*. BASCs have self-renewal capacity, and they can give rise to both bronchiolar and alveolar cells in culture. Thus, we hypothesize that BASCs are a distal lung stem cell that maintains bronchiolar and alveolar homeostasis. Importantly, BASCs are the initial cells to proliferate in response to activation of oncogenic Ras *in vivo* (22). Thus, BASCs appear to be the cell of origin for lung adenocarcinomas initiated by oncogenic *K-ras*.

We were intrigued by the observations that Bmi1 is overexpressed in NSCLC, and that Bmi1 has been implicated in stem cell maintenance and tumorigenicity in other tumors. Thus, we examined the consequences of *Bmi1* deficiency in a murine lung cancer model and in BASCs to determine whether Bmi1 impacts the tumorigenic capacity of tissues that develop normally in the absence of Bmi1. Our analysis shows that *K-ras*-induced lung tumorigenesis is impaired in *Bmi1*-null animals. Additionally, Bmi1 is necessary for BASC proliferation and self-renewal *in*

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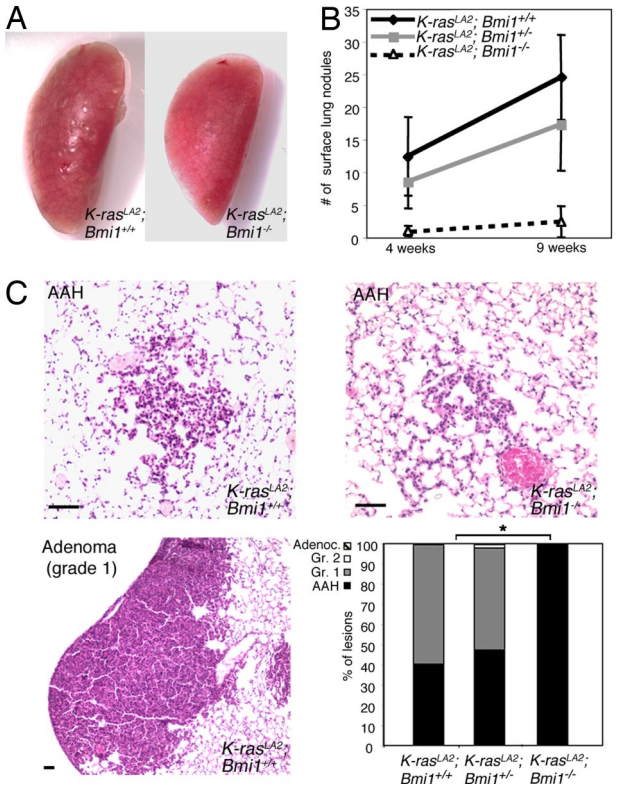

Fig. 1. Loss of *Bmi1* causes a reduction in the number and stage of lung lesions in latent *K-ras*^{G12D} mice. (*A*) Surface lesions visible in the lungs in 9-week-old *K-ras*^{LA2};*Bmi1*$^{+/+}$ (*Left*) and *K-ras*^{LA2};*Bmi1*$^{-/-}$ (*Right*) mice. (*B*) Number of lung surface lesions in 4-week and 9-week-old *K-ras*^{LA2};*Bmi1*$^{+/+}$, *K-ras*^{LA2};*Bmi1*$^{+/-}$ and *K-ras*^{LA2};*Bmi1*$^{-/-}$ mice. (*C*) Lesions present in *K-ras*^{LA2};*Bmi1*$^{+/+}$ mice (AAH, *Upper Left*, and grade 1 adenoma, *Lower Left*) and *K-ras*^{LA2};*Bmi1*$^{-/-}$ mice (AAH, *Upper Right*). (*Lower Right*) Percentage of lesions that represent each tumor grade in 9-week-old *K-ras*^{LA2};*Bmi1*$^{+/+}$, *K-ras*^{LA2};*Bmi1*$^{+/-}$, and *K-ras*^{LA2};*Bmi1*$^{-/-}$ mice. (Scale bars, 50 μm.) *, $P < 0.0001$. Error bars indicate standard deviation.

vivo and in culture. Our data suggest that Bmi1 serves an unrecognized role in epithelial tumorigenesis and provide a molecular link between putative lung stem cells and lung tumorigenesis.

Results

Loss of Bmi1 Abrogates Lung Tumorigenesis in Oncogenic K-ras Mice. Given the observed overexpression of Bmi1 in human lung cancers and the requirement for Bmi1 in lymphomagenesis, we hypothesized that Bmi1 would also play a role in lung tumorigenesis. To investigate this possibility, we crossed mice carrying an inactivated *Bmi1* gene (8) and a latent oncogenic *K-ras*^{G12D} allele (*K-ras*^{LA2}) that is activated by spontaneous *in vivo* recombination (23). *K-ras*^{LA2} mice developed lung tumors with complete penetrance and lesions are first detectable at 1 week of age. Importantly, the age of onset of this tumor phenotype occurred well within the 3 month lifespan of the *Bmi1*-null animals.

At both 4 and 9 weeks of age, *K-ras*^{LA2};*Bmi1*$^{+/+}$, *K-ras*^{LA2};*Bmi1*$^{+/-}$, and *K-ras*^{LA2};*Bmi1*$^{-/-}$ mice were killed and examined for visible lung tumors (Fig. 1*A*). There were no detectable differences in lung morphology or the ratio of lung size to body weight between *Bmi1*$^{-/-}$ and WT mice, demonstrating that loss of Bmi1 has no noticeable effect on lung development (data not shown). However, there was a significant reduction in the number of visible tumors at both 4 and 9 weeks in K-*ras*^{LA2};*Bmi1*$^{-/-}$ animals compared with either *K-ras*^{LA2};*Bmi1*$^{+/+}$ or *K-ras*^{LA2};*Bmi1*$^{+/-}$ animals (Fig. 1*B*; $P <$ 0.00001 and $P = 0.003$, 4 and 9 weeks, respectively). In addition, at 9 weeks, the difference in lesions in *K-ras*^{LA2};*Bmi1*$^{+/+}$ and *K-ras*^{LA2};*Bmi1*$^{+/-}$ mice was also highly significant ($P = 0.0004$). Screening for the earliest detectable lesions by histopathological examination also detected a marked decrease in the number of early lesions in the *K-ras*^{LA2};*Bmi1*$^{-/-}$ animals compared with *K-ras*^{LA2};*Bmi1*$^{+/-}$ or *K-ras*^{LA2};*Bmi1*$^{+/+}$ animals [supporting information (SI) Fig. S1]. Taken together, these data demonstrate a dose-dependent requirement for Bmi1 in early lung tumor development.

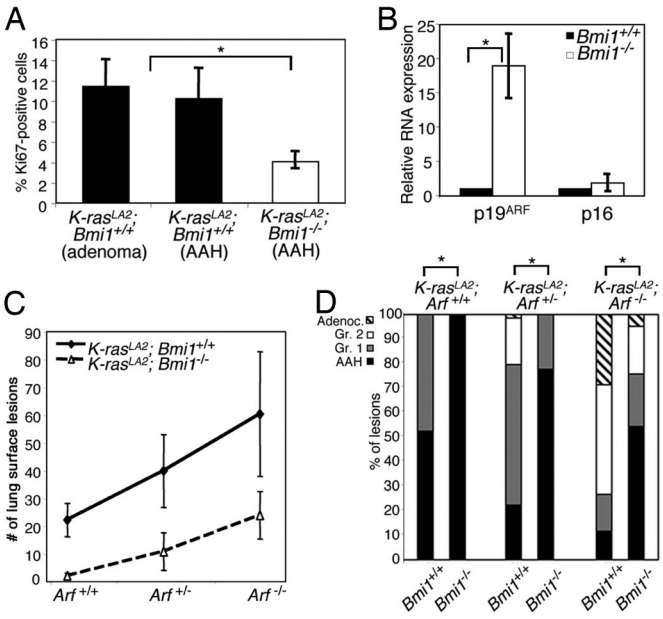

Fig. 2. *Bmi1*-null lung lesions have decreased proliferation and are partially rescued by loss of p19^{ARF} (*A*) Quantification of Ki67 staining in AAH from *K-ras*^{LA2};*Bmi1*$^{+/+}$ or *K-ras*^{LA2};*Bmi1*$^{-/-}$ mice and adenomas from *K-ras*^{LA2};*Bmi1*$^{+/+}$ mice. (*B*) Quantification of mRNA levels of p16 and p19 ^{ARF} from total lung cells of 7-week-old *Bmi1*$^{+/+}$ or *Bmi1*$^{-/-}$ animals. WT levels are normalized to one. (*C*) Number of lung surface lesions in 9-week-old *K-ras*^{LA2};*Bmi1*$^{+/+}$ and *K-ras*^{LA2};*Bmi1*$^{-/-}$ mice that are either *Arf*$^{+/+}$, *Arf*$^{+/-}$, or *Arf*$^{-/-}$. (*D*) Percentage of lesions that represent each tumor grade in 9-week-old *K-ras*^{LA2};*Bmi1*$^{+/+}$ and *K-ras*^{LA2};*Bmi1*$^{-/-}$ mice that are either *Arf*$^{+/+}$, *Arf*$^{+/-}$ or *Arf*$^{-/-}$. *, $P < 0.0001$. Error bars indicate standard deviation.

In addition to determining that loss of *Bmi1* affects the number of lung tumors, we also examined the effect of *Bmi1* deficiency on tumor progression. We observed the early stage lesions of atypical adenomatous hyperplasia (AAH) and grade 1 alveolar adenoma at approximately equal levels in both the *K-ras*^{LA2};*Bmi1*$^{+/+}$ and *K-ras*^{LA2};*Bmi1*$^{+/-}$ animals (Fig. 1*C*). In contrast, there was a dramatic shift in tumor spectrum in the *K-ras*^{LA2};*Bmi1*$^{-/-}$ animals: the infrequently observed lesions were entirely AAH (Fig. 1*C*). These data suggest that loss of *Bmi1* also impairs tumor progression. Taken together, these observations indicate that *Bmi1* deficiency does not prevent transformation, *per se*, but rather blocks tumor development at a very early point during the earliest hyperplastic stage.

Bmi1-Null Lung Lesions Have Decreased Proliferation and Are Partially Rescued by Loss of p19^{ARF}. We wished to establish how *Bmi1* deficiency blocks progression of lung lesions in this model. Because we found no significant apoptosis in WT or *Bmi1*-null lung lesions (Fig. S2), we examined the amount of cellular proliferation given *Bmi1*'s role in its regulation (6). Immunohistochemical analysis of Ki67 expression indicated that there was a reduction in the percentage of cycling cells in *K-ras*^{LA2};*Bmi1*$^{-/-}$ tumors compared with the *K-ras*^{LA2};*Bmi1*$^{+/+}$ tumors (Figs. 2*A*

and Fig. S2). The Ki67-positive status of AAH in *K-ras*^{LA2};*Bmi1*$^{-/-}$ mice was 2.5-fold lower than AAH or grade 1 adenomas in *K-ras*^{LA2};*Bmi1*$^{+/+}$ mice ($P < 0.0001$). These data suggest that the decreased number and grade of lung lesions in *Bmi1*$^{-/-}$ versus *Bmi1*$^{+/+}$ animals correlate with a loss of proliferative capacity rather than a change in apoptosis.

A potential cause for the decreased cycling status of the lung tumors is the misregulation of the *Ink4a/Arf* locus. Bmi1 has been shown to be an important regulator of *Ink4a/Arf*, and expression of both p16 and p19^{ARF} is increased in cells deficient for *Bmi1* (6). Because derepression of these two genes can impair cell cycle progression and impact tumorigenesis, we examined the level of p16 and p19^{ARF} mRNA in total lung from both *Bmi1*$^{+/+}$ and *Bmi1*$^{-/-}$ animals at ≈7 weeks of age. Levels of p19^{ARF} were 19 ± 5-fold greater in *Bmi1*$^{-/-}$ lungs compared with WT lungs ($P < 0.001$). Levels of p16 were not significantly greater ($P = 0.2$) nor were levels of p15 transcript from the neighboring INK4b locus (Fig. 2*B*; data not shown). This indicates that Bmi1 is required for appropriate repression of *Arf* in the lung.

To determine the extent to which misregulation of p19^{ARF} is responsible for the *Bmi1*-null tumor phenotypes, we interbred the *K-ras*^{LA2};*Bmi1* compound mutant animals with *Arf* mutant animals (24) and quantified tumor number at 9 weeks of age. Whereas loss of *Arf* increased the number of tumors in *K-ras*^{LA2};*Bmi1*$^{-/-}$;Arf$^{-/-}$ animals to the same level as in *K-ras*^{LA2};*Bmi1*$^{+/+}$ animals, the *K-ras*^{LA2};*Bmi1*$^{+/+}$;Arf$^{-/-}$ animals exhibited an even greater number of tumors (Fig. 2*C*). A similar effect was also seen on tumor stage: loss of either one or both copies of *Arf* increased the percentage of advanced stage tumors in both *K-ras*^{LA2};*Bmi1*$^{+/+}$ and *K-ras*^{LA2};*Bmi1*$^{-/-}$ animals (Figs. 2*D* and S3). Although the loss of *Arf* in *Bmi1*-null lung increased the stage of lung lesions, such that we detected adenomas (40%) and occasional adenocarcinomas (6%), this did not approach the much higher frequency of adenomas (60%) and adenocarcinomas (28%) seen in the *Bmi1*,WT; *Arf*-null animals. This suggests that, though *Arf* plays a role in the suppression of tumors caused by loss of *Bmi1*, there are additional downstream targets that are also necessary for tumor formation.

Bmi1-Deficient BASCs Fail to Expand *In Vivo*. The presence of BASCs in mouse lung adenocarcinomas and the documented expansion of BASCs in response to oncogenic *K-ras* supports the hypothesis that BASCs are the tumor-initiating cells in *K-ras*^{G12D}-induced lung tumors (21, 22). Given that *Bmi1* affects very early tumorigenesis, we were interested in exploring the role of *Bmi1* in BASCs. We identified BASCs in tissue sections at the BADJ by using dual immunofluorescence (IF) for SP-C and Scgb1a1 (Fig. 3*A*). Analysis of terminal bronchioles (TBs) from single sections determined that there was no difference in the number of BASCs in WT and *Bmi1*$^{-/-}$ mice at 9 weeks of age (Fig. 3*B*; $P = 0.75$).

Having established that *Bmi1* loss is not affecting early tumorigenesis by simply reducing the starting numbers of BASCs, we next asked whether the *Bmi*-null BASCs were capable of expanding in response to oncogenic *K-ras*. Our analysis showed a significant increase in the number of BASCs per TB when the *K-ras*^{LA2} allele was expressed in the *Bmi1*$^{+/+}$ animals (Fig. 3 *B* and *C*; $P < 0.0001$). In contrast, BASCs in *K-ras*^{LA2};*Bmi1*$^{-/-}$ mice exhibited a significantly reduced expansion ($P < 0.0001$). As with the tumor analyses, the loss of p19 ^{ARF} in the *Bmi1*-null animals allowed BASC numbers to expand to levels indistinguishable from *K-ras*^{LA2} WTs ($P = 0.5$), and the loss of p19^{ARF} in the presence of Bmi1 further increased BASC expansion in response to oncogenic *K-ras* (Fig. 3*C*; $P = 0.02$). These data indicate that *Bmi1* is required for the *K-ras*-induced expansion of BASCs *in vivo* in a partially p19^{ARF}-dependent manner, linking a defect in BASCs to reduced lung tumorigenesis in *Bmi1*-deficient mice.

We wished to determine whether the impaired expansion of BASCs observed in the *K-ras*^{LA2};*Bmi1*$^{-/-}$ lungs reflects a specific defect in the response to oncogenic *K-ras* or a more general failure to respond to proliferative signals. In the absence of lung injury, the distal pulmonary epithelial cells are rarely proliferative. Thus, we do not detect BASC proliferation unless the cells are subjected to oncogenic stimuli or lung injury, or they are removed from the niche and placed into culture (22). Thus, we administered naphthalene to ≈8-week-old WT and mutant mice to ablate Clara cells and stimulate BASC expansion (20, 22, 25). In contrast to WT mice, naphthalene-treated *Bmi1*$^{-/-}$ mice did not demonstrate a significant expansion of BASCs at 1 week after naphthalene treatment (Fig. 3*D*; $P = 0.7$ compared with *Bmi1*$^{-/-}$ corn oil controls; $P < 0.001$ compared with naphthalene-treated *Bmi1*$^{+/+}$ animals). Additionally, the extent of repair of Clara cells was impaired in *Bmi1*-null animals at both 1 week and 1 month after injury (Fig. 3 *E* and *F*; $P \leq 0.01$). This repair defect was accompanied by a failure of *Bmi1*$^{-/-}$ BASCs to proliferate at early time points after injury, as measured by BrdU incorporation (data not shown). These data indicate that *Bmi1* is required for BASC expansion and the ability to replenish Clara cells after lung injury *in vivo*.

Bmi1-Deficient BASCs Demonstrate Defective Proliferation and Self-Renewal in Culture. We next studied the performance of *Bmi1*-deficient BASCs, in culture conditions that support self-renewal or differentiation, to further explore the role of Bmi1 in BASCs. We isolated BASCs from ≈7-week-old WT, *Bmi1*$^{+/-}$, and *Bmi1*$^{-/-}$ animals (Fig. S4; data not shown) and plated the cells on feeders for limiting dilution analysis. Consistent with our results showing the lack of expansion of *Bmi1*-null BASCs *in vivo* in response to oncogenic signaling or injury (Fig. 3), *Bmi1*-deficient BASCs in culture exhibited a defect in proliferation. Primary epithelial colonies formed from all genotypes (Fig. S5*A*), yet colonies from *Bmi1*$^{-/-}$ cells were significantly smaller than colonies from either WT or *Bmi1*$^{+/-}$ populations (Fig. 4*A*; $P = 0.0003$ and $P = 0.01$, respectively). This indicates that *Bmi1* influences BASC proliferation in culture in a dose-dependent manner. Additionally, the limiting dilution analysis of the primary colonies demonstrated that, whereas 1 of 110 WT BASCs gave rise to a primary colony, the clonal frequency from *Bmi1*$^{-/-}$ populations was 1 of 288 (Fig. S5*B*; $P = 0.0001$).

When primary colonies were dissociated and replated for secondary colony formation, a profound self-renewal defect in BASCs lacking Bmi1 was revealed. Cells from *Bmi1*$^{-/-}$ primary colonies did not form any secondary colonies, whereas *Bmi1*$^{+/+}$ and *Bmi1*$^{+/-}$ formed secondary colonies equivalently (Figs. 4*B* and S5*C*). These data demonstrate that Bmi1 is required for proliferation and self-renewal of BASCs in culture, suggest that the BASC defects we observed *in vivo* were cell intrinsic.

Importantly, *Bmi1*-deficiency did not impair the differentiation potential of BASCs. *Bmi1*$^{-/-}$ BASCs formed three-dimensional structures similar to those seen in WT cultures when plated in differentiation conditions (Fig. S5*D*). Both WT and mutant BASCs were multipotent, giving rise to bronchiolar and alveolar cells, and there was no statistically significant difference in differentiation frequency between the genotypes (Fig. S5*D*; data not shown). These results suggest that the primary defects in *Bmi1*-mutant BASCs are a result of self-renewal abrogation rather than altered differentiation.

We reasoned that the limitation on self-renewal capacity in BASCs from *Bmi1*-null animals could in part be explained by misregulation of the *Ink4a/Arf* locus. When we analyzed BASCs directly after sorting, we found that the levels of p19^{ARF} mRNA are increased 19-fold ± 8 in the *Bmi1*-null population (Fig. 4*C*; $P < 0.02$), but the increase in p16 levels was not significant ($P = 0.1$). However, once the BASCs were cultured, we observed an increase in the levels of both p16 and p19^{ARF} transcripts (Fig. 4*D*). To determine what role misregulation of the *Ink4a/Arf* locus

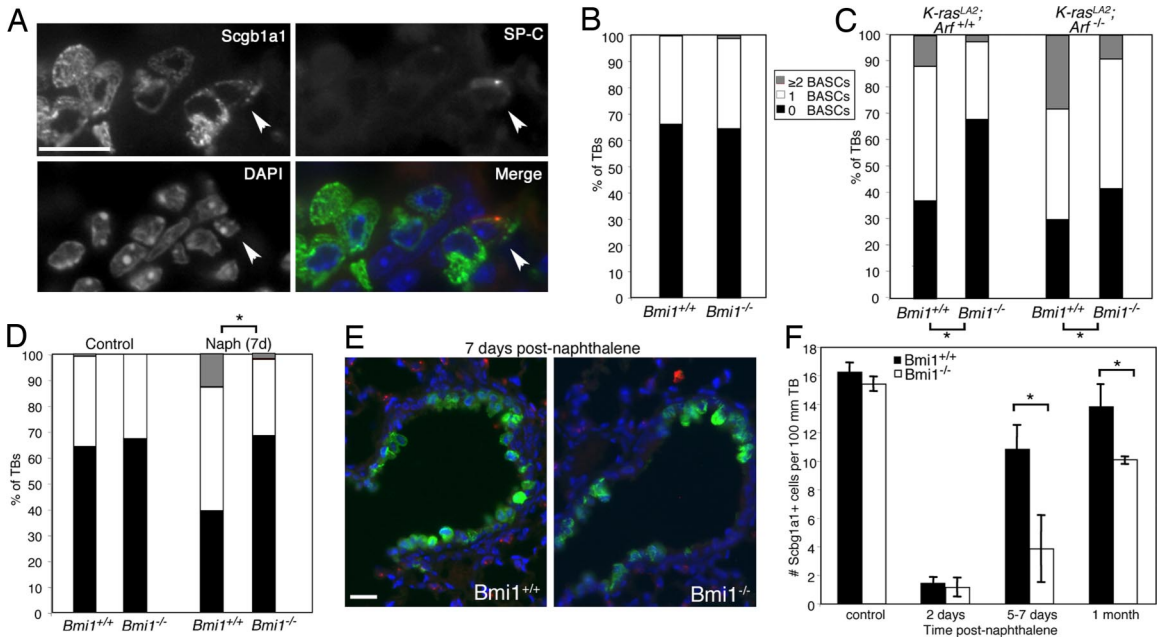

Fig. 3. *Bmi1*$^{-/-}$ BASCs have an impaired response to oncogenic *K-ras* signaling or injury *in vivo*. (*A*) IF staining of TBs for Scgb1a1 (*Upper Left*, green in merge), SP-C (*Upper Right*, red in merge), and DAPI (*Lower Left*, blue in merge). White arrowhead marks double positive BASC (*B*) The percentage of TBs that have 0, 1, or ≥2 BASCs from *Bmi1*$^{+/+}$ or *Bmi1*$^{-/-}$ lungs. (*C*) The percentage of TBs that have 0, 1, or ≥2 BASCs from *K-ras*^{LA2};Bmi1$^{+/+}$, *K-ras*^{LA2};Bmi1$^{+/+}$;*Arf*$^{-/-}$, *K-ras*^{LA2};*Bmi1*$^{-/-}$, and *K-ras*^{LA2};Bmi1$^{-/-}$;*Arf*$^{-/-}$ lungs. (*D*) The percentage of TBs that have 0, 1 or ≥2 BASCs from 8-week-old *Bmi1*$^{+/+}$ or *Bmi1*$^{-/-}$ mice treated with either corn oil or naphthalene 1 week prior. (*E*) Extent of Clara cell repair demonstrated by IF analysis of TBs (Scgb1a1, green; SP-C, red; and DAPI, blue) from *Bmi1*$^{+/+}$ or *Bmi1*$^{-/-}$ mice treated with naphthalene 1 week prior. (*F*) Quantification of postnaphthalene injury repair in TBs from *Bmi1*$^{+/+}$ or *Bmi1*$^{-/-}$ mice. (Scale bars, 20 μm.) ⋆, $P < 0.01$.

plays in the impairment of the *Bmi1*-deficient BASCs, we used a retroviral shRNA-GFP vector with a hairpin that targets both p16 and p19^{ARF} to knockdown expression of these two transcripts. Quantitative PCR showed that levels of p16 and p19^{ARF} mRNA were reduced ≈85% and ≈70%, respectively, in the *Bmi1*-null BASCs infected with the hairpin compared with the control vector (Fig. 4*D*; $P = 0.01$ and $P = 0.02$). As a result, p16 and p19^{ARF} mRNAs were present at comparable levels in *Bmi1*$^{-/-}$;*sh*$^{p16/p19ARF}$ BASCs and *Bmi1*$^{+/+}$;*sh*^{empty} BASCs (Fig. 4*D*; $P = 0.9$ and $P = 0.2$). In the *Bmi1*$^{+/+}$;*sh*$^{p16/p19ARF}$ BASCs, p16 and p19^{ARF} mRNAs were reduced to subphysiological levels relative to the *Bmi1*$^{+/+}$;*sh*^{empty} controls (Fig. 4*D*; $P = 0.02$ and $P = 0.03$). Primary colonies from *Bmi1*$^{-/-}$;*sh*$^{p16/p19ARF}$ BASCs were significantly larger than *Bmi1*$^{-/-}$;*sh*^{empty} colonies (Fig. 4*E*; $P = 0.003$), yet the *Bmi1*$^{-/-}$;*sh*$^{p16/p19ARF}$ colonies did not reach the same size as the *Bmi1*$^{+/+}$;*sh*^{empty} colonies ($P = 0.002$). This partial rescue also applied to the clonal frequency: 1 of 245 plated cells formed colonies in the *Bmi1*$^{-/-}$;sh$^{p16/p19ARF}$ cultures compared with 1 of 367 in the *Bmi1*$^{-/-}$;*sh*^{empty} BASC cultures (Fig. S5*E*; $P = 0.05$). An examination of secondary colony formation showed that, whereas *Bmi1*$^{-/-}$;*sh*^{empty} BASCs did not form any secondary colonies, *Bmi1*$^{-/-}$;*sh*$^{p16/p19ARF}$ BASCs were capable of producing secondary colonies in 25% of the wells plated with 1,000 primary colony cells (Figs. 4*F* and S5*F*; $P < 0.02$). Notably, the frequency of the *Bmi1*$^{-/-}$;*sh*$^{p16/p19ARF}$ BASC secondary colonies was significantly lower than those arising from the *Bmi1*$^{+/+}$;*sh*^{empty} BASCs, even though these two genotypes have comparable p16 and p19^{ARF} mRNA levels. Taken together, these data show that increased levels of *Ink4a/Arf* inhibit the proliferation and self-renewal potential of *Bmi1*-deficient BASCs *in vitro*. The observed partial rescue suggests that either other downstream targets of Bmi1 are involved in establishing these properties, or that the BASCs have suffered irreversible consequences because of constitutive *Bmi1* deficiency. Importantly, these data indicate that Bmi1 is critical for BASC proliferation and self-renewal, the key stem cell property of BASCs.

Discussion

Although the analysis of human tumors has shown a positive correlation between the overexpression of Bmi1 and the development of various epithelial tumors, a causal relationship had not been established before this study. We clearly establish that Bmi1 plays a key role in the development of lung cancer in the mouse. Thus, further investigation into the effects of Bmi1 expression on human lung cancer development or progression is warranted. Loss of *Bmi1* has a profound and early effect on lung tumor development in a mouse model of lung cancer initiated by oncogenic *K-ras*^{G12D}. There is a dramatic decrease in the number of lung lesions at very early time points in the *Bmi1*-deficient animals. Additionally, the lesions that arise appear to be blocked at an earlier stage of tumor development compared with WT animals and exhibit decreased proliferation. These data indicate that Bmi1 plays a vital role in very early epithelial tumor development. Studies have shown that Bmi1 is required for formation of hematopoietic and neural tumors, but normal development of these tissues was also impaired by *Bmi1* deficiency (8, 12, 13). Our data now reveal a role for Bmi1 in tumors originating from a tissue that normally develops under homeostatic conditions in adult *Bmi1*-deficient mice. Bmi1 is known to be up-regulated in a variety of epithelial tumors; therefore, we propose that Bmi1 may play a broad role in tumorigenesis in tissues that were previously considered to be independent of Bmi1 function, as judged by normal development in the absence of Bmi1.

Our data show that the requirement for Bmi1 in lung tumor development correlates with a requirement for Bmi1 to maintain the function of BASCs, the apparent tumor-initiating cells. BASCs are present at the appropriate levels in the *Bmi1*$^{-/-}$ mice, and the lungs exhibit normal appearance. However, whereas

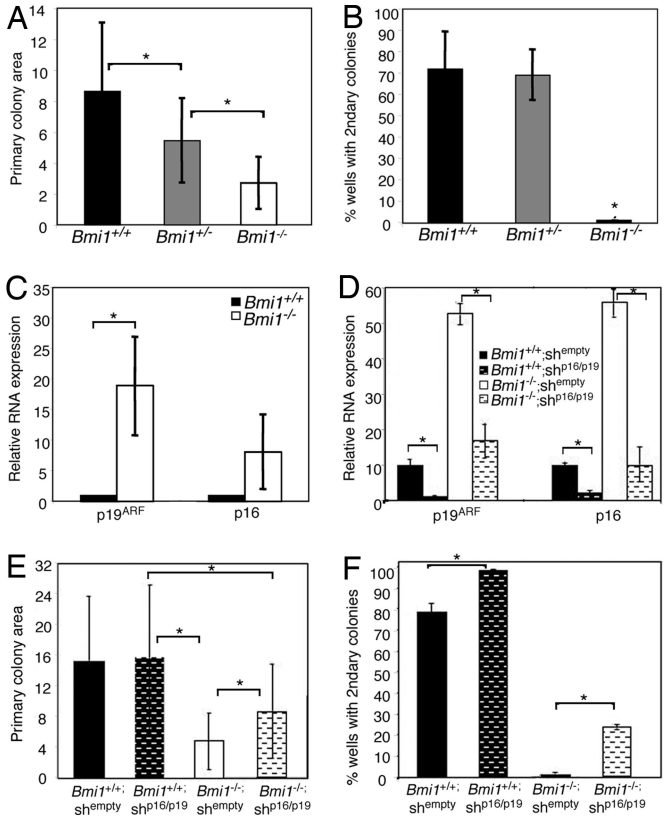

Fig. 4. *Bmi1*$^{-/-}$ BASCs exhibit proliferation and self-renewal defects *in vitro*, that are partially *Ink4a/Arf*-independent. (*A*) Area of primary colonies from *Bmi1*$^{+/+}$, *Bmi1*$^{+/-}$, or *Bmi1*$^{-/-}$ BASCs at 7 days after plating. (*B*) Percentage of wells containing secondary colonies. Each well was plated with 1,000 cells from *Bmi1*$^{+/+}$, *Bmi1*$^{+/-}$, or *Bmi1*$^{-/-}$ primary colonies. (*C*) Quantitation of mRNA levels of p16 and p19 ^{ARF} from BASCs of 7-week-old *Bmi1*$^{+/+}$ or *Bmi1*$^{-/-}$ animals. WT levels are normalized to one. (*D*) Quantitation of p16 and p19^{ARF} mRNA levels from *Bmi1*$^{-/-}$ and *Bmi1*$^{+/+}$ BASC primary colonies that were infected with an empty shRNA vector or a p16/p19^{ARF} shRNA vector. (*E*) Area of primary colonies from *Bmi1*$^{+/+}$ or *Bmi1*$^{-/-}$ BASCs infected with empty shRNA or p16/p19^{ARF} shRNA virus 7 days after plating. (*F*) Percentage of wells containing secondary colonies. Each well was plated with 1,000 cells from *Bmi1*$^{+/+}$ or *Bmi1*$^{-/-}$ primary colonies infected with empty shRNA or p16/p19^{ARF} shRNA. (Scale bars, 100 μm.) All error bars represent standard deviation. *, $P <$ 0.05.

expression of oncogenic *K-ras* or distal lung injury *in vivo* triggered the proliferation of BASCs in WT animals, this response was impaired by the loss of *Bmi1*. Similarly, *Bmi1*-deficient BASCs showed reduced proliferation and an inability to self-renew in culture. Clearly, Bmi1 is not the only determinant of BASCs' proliferative capacity. Recent studies already implicate negative regulators, such as MAPK and p27, in BASC control and lung tumorigenesis (26, 27). Interestingly, we found that the defects in BASCs lacking *Bmi1* were only uncovered when they encountered increased proliferative demand. This raises the possibility that other tissues with infrequent turnover may also rely on Bmi1 function in physiological settings that have not yet been examined.

These data lead to several interpretations about the functions of BASCs and Bmi1. First, the observed normal lung phenotype in *Bmi1*$^{-/-}$ mice, despite defective BASC expansion in *Bmi1*$^{-/-}$ adult lung, may suggest that BASCs are not required for lung development. Alternatively, BASCs may play a role in lung development, and the requirement for Bmi1 function may not occur in these cells until they have undergone a threshold of proliferative rounds (28). Supporting the former, we and others have identified BASCs in adult lung but not during lung development (C. Kim, unpublished data) (29). We favor the hypothesis that BASCs function specifically in response to lung insults in adults. In this model, BASCs elicit the initial proliferative response to injury and give rise to more specialized cells that function as progenitor cells to expand and repair damaged lung epithelia. Cell-type-specific lineage tracing and transplantation studies are needed to test the ability of BASCs and other lung cells to function as stem, progenitor, or differentiated cells *in vivo* and to determine the potential overlap of BASCs, variant Clara cells, and other putative lung stem cells. The clear requirement for Bmi1 in the expansion of BASCs, in response to lung injury and oncogenic stimuli, may help to elucidate lung cell lineages.

Our studies provide evidence, along with previous work, that oncogenic *K-ras*-induced tumors may depend on Bmi1 to suppress the p19^{ARF} locus. Bmi1 is known to repress loci that suppress proliferation, including *Ink4a/Arf*. In both the *Bmi1*-null lungs and BASCs, we see significantly increased levels of p19^{ARF} but not p16 (Figs. 2*B* and 4*C*). This result is opposite to that seen in *Bmi1*-null neural tissue where p16, not p19^{ARF}, is more significantly up-regulated (10). In ES cells, mutant *K-ras* causes up-regulation of p19^{ARF}, and Arf overexpression has been reported in latent *K-ras*^{G12D}-induced lung tumors (30, 31). Bmi1 overexpression contributes to Ras-induced transformation in culture and *in vivo* (6, 32). The incomplete rescue of BASC function and lung tumorigenesis after p19 depletion, along with similar effects seen in both the hematopoietic and neural systems (14–16), supports the notion that Bmi1 acts through both *Arf*-dependent and -independent mechanisms. We have some evidence to suggest that levels of p16 increase in the *Bmi1*-null total lung and BASCs on loss of *Arf*, suggesting a potential compensatory mechanism, which may, in part, explain the partial rescue (Dovey and Lees, unpublished data). However, the failure of *Arf* and p16 knockdown to fully rescue the proliferation of BASCs *in vitro* suggests that Bmi1 has targets beyond the *Ink4a/Arf* locus, although we cannot rule out any irreversible effects of the *Bmi1* deficiency. Additional work is required to explore the relevant downstream targets of Bmi1 that may contribute to Bmi1's role in lung stem cell function and tumorigenesis.

The dependence of diverse tumor types on Bmi1 makes it an attractive target for cancer therapy. However, a dangerous side effect of depleting Bmi1 function in cancer patients would be abrogation of normal tissue stem cell function. Studies to determine whether there are mechanistic differences between the roles of Bmi1 in cancer cells versus stem cells will be crucial to identify more amenable strategies to eliminate cancer cells without affecting tissue homeostasis.

Materials and Methods

The materials and methods used for immunohistochemistry, IF, quantitative RT-PCR, naphthalene treatment, differentiation cultures, and RNA interference can be found in *SI Text*.

Mice and Tissues. *Bmi1* knockout mice (8), *K-ras*^{LA2} mice (23), and WT littermates were maintained on a mixed background (C57BL/6 $\times$ 129/sv), and *Bmi1* and *K-ras*^{LA2} mice were interbred. *Arf* (24) mice were maintained on a 129/sv background and interbred with *K-ras*^{LA2}; *Bmi1* mice. Four- and nine-week-old mice were killed for lung tumor analysis or IF BASC analysis. Tissue preparation was as described in ref. 23.

BASC Sorting and Culture. Single cell suspensions and FACS analysis were performed as described in ref. 22, with the following modifications: lungs were incubated with 2 μg/ml collagenase/dispase (in HBSS-Mg-Ca) for 45 min at 37°C and then incubated with 0.002% DNase for 10 min on ice. BASC cultures and regression analysis were conducted as described in ref. 22.

Statistics. Statistical analysis of the limiting dilution assays was done by using L-calc software (Stem Cell Technologies). For the comparison of the distribution of BASC number per TB with the distribution of lung tumor stages, the Cochran-Armitage test was performed with MStat software. Other analyses were done by using the two-tailed Student's *t test*.

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