

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 bronchioalveolar 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 hema-

topoietic 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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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. 2B; 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. 2C). 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. 2D 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. 3A). 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. 3B; $P = 0.75$).

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. 3B 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. 3C; $P = 0.02$). 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. 3D; $P = 0.7$ 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. 3E 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. S5A), yet colonies from *Bmi1^{-/-}* cells were significantly smaller than colonies from either WT or *Bmi1^{+/+}* populations (Fig. 4A; $P = 0.0003$ 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. S5B; $P = 0.0001$).

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. 4B and S5C). 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. S5D). 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. S5D; 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. 4C; $P < 0.02$), 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. 4D). To determine what role misregulation of the *Ink4a/Arf* locus

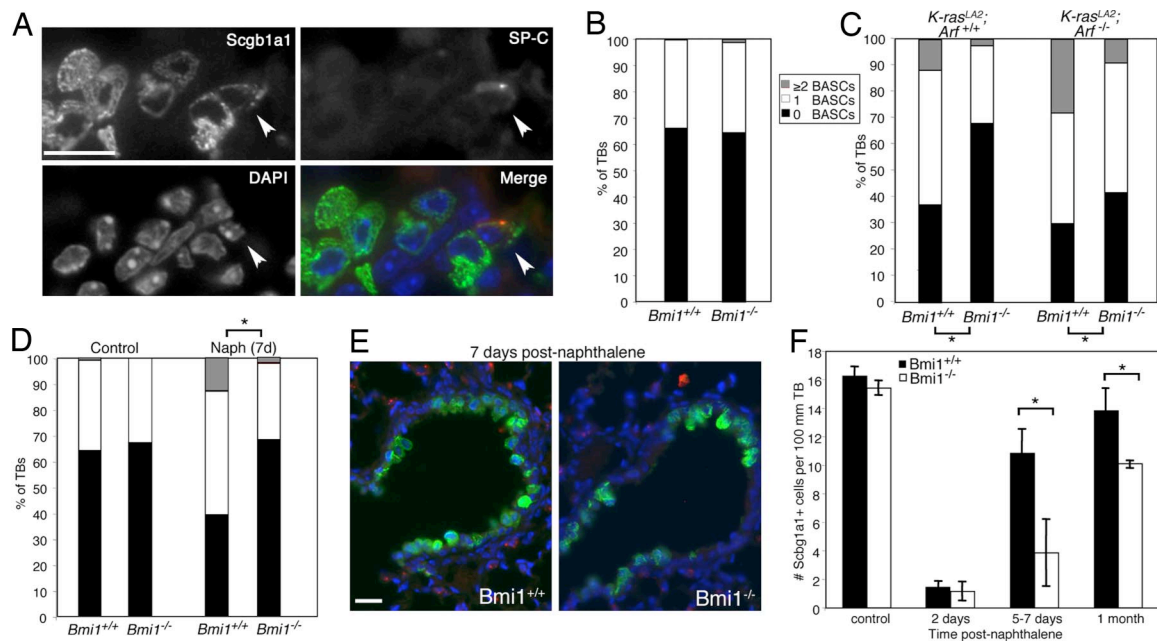


Fig. 3. *Bmi1*^{-/-} BASCs have an impaired response to oncogenic *K-ras* signaling or injury *in vivo*. (A) IF staining of TBs for Scgb1a1 (Upper Left, green in merge), SP-C (Upper Right, red in merge), and DAPI (Lower Left, blue in merge). White arrowhead marks double positive BASC (B) The percentage 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 μ m.) *, $P < 0.01$.

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 $\approx 85\%$ and $\approx 70\%$, respectively, in the *Bmi1*-null BASCs infected with the hairpin compared with the control vector (Fig. 4D; $P = 0.01$ and $P = 0.02$). As a result, p16 and p19^{ARF} mRNAs were present at comparable levels in *Bmi1*^{-/-};*shp16/p19ARF* BASCs and *Bmi1*^{+/+};*shempty* BASCs (Fig. 4D; $P = 0.9$ and $P = 0.2$). In the *Bmi1*^{+/+};*shp16/p19ARF* BASCs, p16 and p19^{ARF} mRNAs were reduced to subphysiological levels relative to the *Bmi1*^{+/+};*shempty* controls (Fig. 4D; $P = 0.02$ and $P = 0.03$). Primary colonies from *Bmi1*^{-/-};*shp16/p19ARF* BASCs were significantly larger than *Bmi1*^{-/-};*shempty* colonies (Fig. 4E; $P = 0.003$), yet the *Bmi1*^{-/-};*shp16/p19ARF* colonies did not reach the same size as the *Bmi1*^{+/+};*shempty* colonies ($P = 0.002$). This partial rescue also applied to the clonal frequency: 1 of 245 plated cells formed colonies in the *Bmi1*^{-/-};*shp16/p19ARF* cultures compared with 1 of 367 in the *Bmi1*^{-/-};*shempty* BASC cultures (Fig. S5E; $P = 0.05$). An examination of secondary colony formation showed that, whereas *Bmi1*^{-/-};*shempty* BASCs did not form any secondary colonies, *Bmi1*^{-/-};*shp16/p19ARF* BASCs were capable of producing secondary colonies in 25% of the wells plated with 1,000 primary colony cells (Figs. 4F and S5F; $P < 0.02$). Notably, the frequency of the *Bmi1*^{-/-};*shp16/p19ARF* BASC secondary colonies was significantly lower than those arising from the *Bmi1*^{+/+};*shempty* 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

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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