Dysregulation of the Bmi-1/p16Ink4a pathway provokes an aging-associated decline of submandibular gland function

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Summary

Bmi-1 prevents stem cell aging, at least partly, by blocking expression of the cyclin-dependent kinase inhibitor p16Ink4a. Therefore, dysregulation of the Bmi-1/p16Ink4a pathway is considered key to the loss of tissue homeostasis and development of associated degenerative diseases during aging. However, because Bmi-1 knockout (KO) mice die within 20 weeks after birth, it is difficult to determine exactly where and when dysregulation of the Bmi-1/p16Ink4a pathway occurs during aging in vivo. Using real-time in vivo imaging of p16Ink4a expression in Bmi-1-KO mice, we uncovered a novel function of the Bmi-1/p16Ink4a pathway in controlling homeostasis of the submandibular glands (SMGs), which secrete saliva into the oral cavity. This pathway is dysregulated during aging in vivo, leading to induction of p16Ink4a expression and subsequent declined SMG function. These findings will advance our understanding of the molecular mechanisms underlying the aging-related decline of SMG function and associated salivary gland hypofunction, which is particularly problematic among the elderly.

Key words: aging; Bmi-1; homeostasis; p16Ink4a; stem/progenitor cells; submandibular gland.

Introduction

In higher eukaryotes, maintenance of adult stem and progenitor cells is indispensable for tissue homeostasis throughout the lifespan of the organism (Cheung & Rando, 2013). However, regulation of these processes declines with age, resulting in an increased incidence of various aging-associated degenerative diseases (Sharpless & DePinho, 2007; Liu & Rando, 2011; Behrens et al., 2014). Bmi-1 belongs to the PRC1, which is recruited to a locus due to the PRC2 which trimethylates H3K27, priming the Bmi-1-containing PRC1 ubiquitin ligase complex to silence a locus (Hernandez-Munoz et al., 2005; Bracken et al., 2007; Kotake et al., 2007) and is essential for self-renewal of several types of adult stem cells and/or proliferation of certain types of differentiated cells, such as pancreatic β cells (Lessard & Sauvageau, 2003; Molofsky et al., 2003, 2005; Park et al., 2003; Iwama et al., 2004; Dhawan et al., 2009; Bihs et al., 2013). For example, although knockout (KO) mice lacking Bmi-1 are born with normal numbers of stem cells, Bmi-1-KO mice exhibit postnatal self-renewal defects that lead to premature depletion of adult stem cells, which resembles accelerated aging (van der Lugt et al., 1994; Lessard & Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003; Robson et al., 2011). Therefore, the aging-associated decline of Bmi-1 function may lead to failure of adult stem cell homeostasis and subsequent aging-associated disruption of tissue repair mechanisms and subsequent onset of degenerative diseases. Thus, better understanding of the downstream mediators of the Bmi-1 pathway will likely facilitate the development of new strategies for prevention or intervention of aging-associated degenerative diseases.

Several downstream targets of the Bmi-1 pathway have been proposed (van der Lugt et al., 1996; Jacobs et al., 1999; Oguro et al., 2010). Among them, p16Ink4a may be the strongest candidate for stem cell regulation (Jacobs et al., 1999; Molofsky et al., 2003, 2005; Park et al., 2003; Dhawan et al., 2009; Bihs et al., 2013). The cyclin-dependent kinase (CDK) inhibitor encoded by p16Ink4a slows down or blocks cell cycle progression by preventing phosphorylation and inactivation of the retinoblastoma tumor suppressor protein (pRb) (Serrano et al., 1993, Hará et al., 1996). Moreover, p16Ink4a expression levels dramatically increase in several tissues with age (Zindy et al., 1997; Krishnamurthy et al., 2004; Yamakoshi et al., 2009), coinciding with the onset of the aging-associated functional decline of adult stem or progenitor cells (Baker et al., 2011; Sousa-Victor et al., 2014). These evidence indicate that dysregulation of the Bmi-1/p16Ink4a pathway likely plays important roles in provoking the aging-associated decline of stem or progenitor cell function and subsequent onset of degenerative diseases. However, because Bmi-1-KO mice die within 20 weeks after birth (van der Lugt et al., 1994), it is difficult to determine exactly where and when dysregulation of the Bmi-1/p16Ink4a pathway occurs during aging in vivo.

To circumvent this problem, in the present study, we used p16−/− mice, in which p16Ink4a expression can be monitored throughout the body using a bioluminescence imaging (BLI) technique (Yamakoshi et al., 2009). This approach, in conjunction with analysis of Bmi-1-KO mice and aged wild-type (WT) mice, uncovered a novel function of the Bmi-1/p16Ink4a pathway in proliferation control of stem or progenitor cells in the submandibular glands (SMGs), which secrete saliva into the oral cavity (Young & van Lennep, 1978). Furthermore, our findings showed that the Bmi-1/p16Ink4a pathway becomes dysregulated in SMGs during the aging process, which leads to induction of p16Ink4a expression and the subsequent decline of SMG function. This unexpected role of the Bmi-1/p16Ink4a pathway in the SMGs will likely provide new insights into the mechanism(s) underlying the aging-associated decline of SMG function and associated salivary gland hypofunction (SGH), which is a serious problem in the elderly population (Scott, 1977; Epstein et al., 1980; Federsen et al., 1985; Yeh et al., 1998; Lenander-Lumikari & Loimaranta, 2000; Sreebny, 2000; van der Maarel-Wierink et al., 2013).
Results

Induction of p16\textsuperscript{ink4a} expression in the SMGs of Bmi-1-KO mice

To unveil the physiological roles of Bmi-1 in the regulation of p16\textsuperscript{ink4a} expression in vivo, we crossed p16\textsuperscript{lac} mice (p16\textsuperscript{ink4a} reporter mice; Yamakoshi et al., 2009) onto a heterozygous Bmi-1-KO mice to produce p16\textsuperscript{lac} mice lacking Bmi-1. When these mice were analyzed using a noninvasive in vivo BLI technique, we observed a significant increase in signals throughout the body compared with control p16\textsuperscript{lac} mice, which was particularly enhanced in the cervical region (Fig. 1A, top right). These signals remained largely unchanged by ablation of Bmi-1 (Fig. S1A). Together, these results suggest that regulation of p16\textsuperscript{ink4a} expression by Bmi-1 may play key roles in SMG function in adult mice.

Dysregulation of the Bmi-1/p16\textsuperscript{ink4a} pathway in the aged SMG

Because p16\textsuperscript{ink4a} expression levels increase in many different tissues during aging (Zindy et al., 1997; Krishnamurthy et al., 2004; Yamakoshi et al., 2009), we assumed that Bmi-1 likely regulates p16\textsuperscript{ink4a} expression in SMGs. Therefore, we investigated the regulation of the p16\textsuperscript{ink4a} promoter region during normal aging in WT mice. The SMGs of aged WT mice expressed significantly higher levels of p16\textsuperscript{ink4a} than those of younger mice (Fig. 2A, left and B). Notably, however, Bmi-1 mRNA and protein levels in SMGs were slightly increased or remained unchanged (Fig. 2A, right and B). However, Bmi-1 binding to the p16\textsuperscript{ink4a} promoter region was significantly reduced in the SMGs of aged WT mice, which is consistent with the reduction in H3K27 me3 levels around the p16\textsuperscript{ink4a} promoter region, as determined by chromatin immunoprecipitation (ChIP) analysis (Fig. 2D). This coincided with the increase of H3K4 me3, an epigenetic mark of active chromatin around the p16\textsuperscript{ink4a} promoter region (Fig. 2D). In addition, it should be noted that the levels of phosphorylated (p)-AKT (AK strain transforming) at Ser 473, a sign of AKT activation, which is known to phosphorylate Bmi-1 and inactivate its ability to bind the p16\textsuperscript{ink4a} locus (Liu et al., 2012), were significantly increased in the SMGs of aged mice compared with those of young mice (Fig. 2B). Collectively, these results indicate that although Bmi-1 mRNA and protein levels were unchanged, the activity of Bmi-1 was reduced, possibly through phosphorylation by AKT during the aging process in vivo. Consequently, the epigenetic silencing of p16\textsuperscript{ink4a} by Bmi-1 is likely to be abolished in SMG parenchymal cells during aging. Similar to the results of Bmi-1-KO SMGs, the expression levels of other cell cycle inhibitors were unchanged during aging (Fig. S1B, C), thus supporting the idea that the Bmi-1/p16\textsuperscript{ink4a} pathway plays key roles in controlling SMG function during aging.

SMG function declines in Bmi-1-KO and aged WT mice

To substantiate this idea, we performed morphometric and salivary secretion analyses using Bmi-1-KO mice or aged WT mice. A lower density of secretory parenchyma (Fig. 3A) and a significant decrease in the number of cells (Fig. 3B) were observed in the SMGs of Bmi-1-KO mice.
structures in young WT mice (Fig. 3I, top). In contrast, expression levels regulate p16Ink4a and dysregulation of this regulatory pathway will lead to upregulation or aged WT mice. p16Ink4a, was strongly expressed in the nuclei of acinar cells and ductal H). Immunofluorescence staining analysis revealed that Bmi-1, but not was significantly reduced in Bmi-1-KO mice and aged WT mice (Fig. 3G, sis revealed that the percentage of proliferating SMG parenchymal cells reduced in Bmi-1-KO mice, as shown by the results of pilocarpine stimulation tests (Fig. 3C). Similar results were observed in aged WT mice compared with young WT mice (Fig. 3D –F), suggesting that the loss of Bmi-1 function during aging possibly reduced the number of secretory parenchymal cells, resulting in an aging-associated decrease in saliva production.

Because Bmi-1 determines proliferative capacity, we tested whether the reduction in parenchymal cell number in the SMGs of Bmi-1-KO and aged WT mice was attributable to the decreased cell proliferation of SMG parenchymal cells. Bromodeoxyuridine (BrdU) incorporation analysis using a three-dimensional (3D) collagen matrix culture revealed that salispheres derived from Bmi-1-KO mice exhibited fewer and shorter branches on day 10 than those derived from WT littermates (Fig. S2G). Furthermore, we determined whether aging affected the function of SMG stem or progenitor cells. In accordance with a previous report of Kit and Msi-1 (Lombaert et al., 2008), which are derived from putative stem cells of ductal origin and comprise cells that express the stem cell markers Sca-1, c-Kit and Msi-1 (Lombaert et al., 2008). The salispheres formed from Bmi-1-KO mice expressed significantly higher levels of p16Ink4a than those formed from WT littermates (Fig. S2B, left). Fewer salispheres were derived from Bmi-1-KO mice, and they incorporated significantly lower levels of BrdU and generated significantly fewer salispheres capable of self-renewal than those derived from WT mice (Fig. S2B, left). Fewer salispheres were derived from Bmi-1-KO mice, and they incorporated significantly lower levels of BrdU and generated significantly fewer salispheres capable of self-renewal than those derived from WT mice (Fig. S2B, left). Fewer salispheres were derived from Bmi-1-KO mice, and they incorporated significantly lower levels of BrdU and generated significantly fewer salispheres capable of self-renewal than those derived from WT mice (Fig. S2B, left).

Putative SMG stem or progenitor cells require Bmi-1 function

To assess whether SMG stem or progenitor cells require Bmi-1, we employed an in vitro model to study SMG stem or progenitor cells that involve the isolation and culture of murine SMG cells as salispheres (Lombaert et al., 2008), which are derived from putative stem cells of ductal origin and comprise cells that express the stem cell markers Sca-1, c-Kit and Msi-1 (Lombaert et al., 2008). The salispheres formed from Bmi-1-KO mice expressed significantly higher levels of p16Ink4a than those formed from WT littermates (Fig. S2B, left). Fewer salispheres were derived from Bmi-1-KO mice, and they incorporated significantly lower levels of BrdU and generated significantly fewer salispheres capable of self-renewal than those derived from WT mice (Fig. S2B, left). Fewer salispheres were derived from Bmi-1-KO mice, and they incorporated significantly lower levels of BrdU and generated significantly fewer salispheres capable of self-renewal than those derived from WT mice (Fig. S2B, left). Fewer salispheres were derived from Bmi-1-KO mice, and they incorporated significantly lower levels of BrdU and generated significantly fewer salispheres capable of self-renewal than those derived from WT mice (Fig. S2B, left).
The Bmi-1/p16ink4a pathway is dysregulated in putative SMG stem or progenitor cells of aged WT mice

To test this idea, expression levels of p16ink4a and Bmi-1, binding of Bmi-1 and histone modifications around the p16ink4a promoter region were examined using salispheres from both young and aged mice. Indeed, the levels of p16ink4a expression were increased in salispheres from aged WT mice compared with those from young WT mice (Fig. 4G, left and H). Note that although Bmi-1 mRNA and protein levels were not affected by aging (Fig. 4G, right and H), Bmi-1 binding and the extent of H3K27me3 modifications around the p16ink4a promoter region were substantially decreased (Fig. 4I). In contrast, the extent of H3K4me3 modifications around the p16ink4a promoter region was significantly increased in salispheres from aged mice (Fig. 4I). Together, these results indicate that the Bmi-1/p16ink4a pathway is dysregulated through histone

self-renewal, and differentiation of putative SMG stem or progenitor cells. These results led us to speculate that the Bmi-1/p16ink4a pathway is associated with the functional decline of SMG stem or progenitor cells during the aging process.

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Figure 4  Aging-associated functional decline of SMG stem or progenitor cells. (A) Phase-contrast images of salispheres formed after 2 days in nonadherent cultures from young and old adult WT mice (left and right panels, respectively). Scale bars, 100 μm. (B) Effect of aging on the number of salispheres. Data are presented as means ± SD, n = 5, ** P < 0.01. (C) Percentage of BrdU-positive cells in 10 representative salispheres from young (7–12 weeks old) or old (25 months old) adult WT mice. Data are presented as means ± SD, n = 3, ** P < 0.01. (D) Percentage salisphere formation of CD24hi/CD29hi cells at self-renewal passage-2 (P2) in enriched medium using from young (7–8 weeks old) or old (25–28 months old) adult WT mice. Data are presented as means ± SD of three independent experiments, * P < 0.05. (E) Phase-contrast images of ductal-like branches on day 10 from young and old adult WT mice (left and right panels, respectively). Salispheres cultured for 2 days were transferred into a 3D collagen matrix. Scale bars, 200 μm. Histogram presenting the average branch numbers (%) of 25 cultured salispheres per mouse, n = 4. (F) Fold increase of Amy1 and Muc19 mRNA levels from 2-day-old salispheres to ductal-like branches cultured for 18 days. Data are presented as means ± SD, n = 3–4. (G) Relative p16\textsuperscript{ink4a} and Bmi-1 mRNA levels (left and right panels, respectively) in salispheres shown in (A) from young (8 weeks old) or old (24 months old) adult WT mice. qPCR results from salisphere RNA samples of individual mice in each group. Mean values of p16\textsuperscript{ink4a} and Bmi-1 mRNA levels in the young WT group were considered controls. Data are presented as means ± SD, n = 4–5, *** P < 0.001. (H) Representative immunoblot of salispheres from young and old adult WT mice for the indicated proteins. β-actin was used as a loading control. (I) ChIP analysis, using the indicated antibodies and salispheres shown in (A), of the p16\textsuperscript{ink4a} locus. Data are presented as means ± SD of three independent experiments, ** P < 0.01; * P < 0.05.
Elevated \( p16^{\text{ink4a}} \) levels inhibit proliferation of salispheres

To further examine the biological role of \( p16^{\text{ink4a}} \) in SMGs, cells derived from the SMGs of young mice were transduced with one of two murine stem cell virus (MSCV) retroviral vectors expressing an internal ribosome entry site (IRES) and green fluorescent protein (GFP) with or without \( p16^{\text{ink4a}} \) (pMSCV–IRES–GFP or pMSCV–\( p16^{\text{ink4a}} \)–IRES–GFP, respectively; Fig. S3A, B) and then subjected to BrdU incorporation analysis and 3D collagen matrix cultures. The proliferation rate of salispheres cultures overexpressing \( p16^{\text{ink4a}} \) was substantially reduced (Fig. S3C), which is consistent with their smaller size (Fig. S3A, D) compared with those of control cells expressing GFP. Furthermore, ectopic \( p16^{\text{ink4a}} \) expression in young WT SMG cells decreased the extent of branching (Fig. S3E) and levels of differentiation markers (Fig. S3F) of salispheres cultured for 7 or 17 days. Collectively, these results indicate that elevated levels of \( p16^{\text{ink4a}} \) limit the proliferation and differentiation potential of putative adult SMG stem or progenitor cells. Thus, \( p16^{\text{ink4a}} \) expression may inhibit the activities of SMG stem or progenitor cells.

Ablation of \( p16^{\text{ink4a}} \) partially rescues the abnormal phenotypes of Bmi-1-KO SMG

Finally, we asked whether the Bmi-1/\( p16^{\text{ink4a}} \) pathway plays an important role(s) in the aging-associated decline of SMG function. The most straightforward approach to address this question would be to use \( p16^{\text{ink4a}} \) KO mice. However, because \( p16^{\text{ink4a}} \)–KO mice die of cancer long before they reach the age at which most normal mice experience a decrease in SMG function, we asked whether \( p16^{\text{ink4a}} \) deficiency can rescue the premature decrease in SMG function in Bmi-1-KO mice. To this end, we generated double-mutant cells lacking both Bmi-1 and \( p16^{\text{ink4a}} \) (Sharpless et al., 2001). Notably, although deletion of \( p16^{\text{ink4a}} \) had no effect on SMG development in young WT mice, defective saliva production by Bmi-1-KO mice was partially rescued by ablation of \( p16^{\text{ink4a}} \) (Fig. 5A). This coincided with a substantial increase in the total number and density of SMG cells and the expression levels of differentiation-specific genes in branched cells (Fig. 5B–D). Taken together, these results indicate that the Bmi-1/\( p16^{\text{ink4a}} \) pathway possibly plays a key role in the aging-associated decline of SMG function, at least to some extent.

Discussion

For many years, aging was believed to be an inevitable and random deterioration of the body, leading to loss of physiological function and increased vulnerability to disease and eventual death. Recent studies, however, reveal that the aging process, similar to other biological processes, is subject to control by various signaling pathways and gene expression patterns. Bmi-1, a PcG epigenetic regulator that blocks expression of the \( p16^{\text{ink4a}} \) CDK inhibitor, plays a key role in aging, and dysregulation of the Bmi-1/\( p16^{\text{ink4a}} \) pathway is believed to cause failure of adult stem cell homeostasis and the onset of aging-associated degenerative diseases (Dhawan et al., 2009; Sousa-Victor et al., 2014). However, because Bmi-1-KO mice die within 20 weeks after birth, it is difficult to determine exactly where and when dysregulation of the Bmi-1/\( p16^{\text{ink4a}} \) pathway occurs during aging in vivo.

To circumvent this problem, we took advantage of using \( p16^{\text{–luc}} \) mice, a recently developed transgenic mouse model that carries the entire human \( p16^{\text{ink4a}} \) locus (Yamakoshi et al., 2009). Note that this human chromosome segment was engineered to express a fusion protein of human \( p16^{\text{ink4a}} \) and firefly luciferase (\( p16^{\text{–luc}} \)) without deleting any genomic DNA sequences of the Ink4a/Arf locus (Yamakoshi et al., 2009). This is very important because Bmi-1 binds not only to the promoter region but also to the intron region of the \( p16^{\text{ink4a}} \) gene locus (Bracken et al., 2007; Kotake et al., 2007). Moreover, expression of the \( p16^{\text{–luc}} \) fusion protein enables us to specifically measure \( p16^{\text{ink4a}} \) expression, but not that of Arf, from this overlapping gene locus.

Using this approach together with Bmi-1-KO mice, we uncovered a novel function of the Bmi-1/\( p16^{\text{ink4a}} \) pathway in the regulation of SMG function. Moreover, we found that this pathway was dysregulated during aging in vivo, leading to the induction of \( p16^{\text{ink4a}} \) expression and subsequent decline of SMG stem or progenitor cell function and saliva...
The obvious remaining question is whether or not our findings can be applied to humans. In mouse models, we have shown that the p16INK4a pathway is responsible for the aging-associated decline of SMG function. However, because defects of saliva production in Bmi-1-KO mice were partially rescued by ablation of p16INK4a (Fig. 5A), it is most likely that the Bmi-1/p16INK4a pathway plays a key role in the aging-associated decline of SMG function to some extent.

Experimental procedures

Animals, BLI and image acquisition

The p16–luc transgenic mice (C57BL/6), BLI technique and image acquisition methods were previously described (Yamakoshi et al., 2009). The p16–luc mice were crossed with Bmi-1+/− mice (C57BL/6) (van der Lugt et al., 1994) to produce p16–luc mice lacking Bmi-1. Aged adult WT mice (C57BL/6) were purchased from the National Centre for Geriatrics and Gerontology Experimental Animal Facility (Obu, Aichi, Japan). Male animals were used for all experiments except for BLI.

Determination of the volume of saliva

Mice were anesthetized and intraperitoneally injected with 1 mg kg⁻¹ of pilocarpine (Nacalai Tesque, Inc., Kyoto, Japan), and 1 min later, saliva was collected from the mouth for 10 min using a micropipette (Ringcaps, Hirschmann Laborgeräte, GmbH & Co. KG, Eberstadt, Germany). The total volume of saliva was measured and calculated per body weight.

Isolation of SMG cells

SMGs were dissected, and cells were isolated, counted and cultured as previously described (Lombaert et al., 2008) with some modifications. In brief, cell suspensions were prepared by first mechanically disrupting the glands followed by enzymatic digestion with collagenase type II, hyaluronidase and CaCl₂ at 37 °C for 40 min and then with 25 U of dispase at 37 °C for 1 h. After filtering, primary cells were further filtered and suspended in Dulbecco’s modified Eagle’s medium/F12 medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with N2, GlutaMAX™, 20 ng ml⁻¹ of epidermal growth factor, 20 ng ml⁻¹ of fibroblast growth factor-2 and 10 µg ml⁻¹ of insulin, penicillin and streptomycin (salisphere medium).

Cell culture

For cell differentiation assays, 2-day-old primary salispheres were cultured in collagen 3D matrix (Cellmatrix Type I-A; Nitta Gelatin, Inc., Osaka, Japan) for 8 or 18 additional days. The average number of branches in 25 cultured salispheres was determined, and the branches were then released from the gelled matrix by depolymerization using collagenase L (Nitta Gelatin, Inc.) and used for quantitative real-time polymerase chain reaction (qPCR) analysis.

The self-renewal of salispheres was performed as previously described (Nanduri et al., 2014). In brief, CD24⁺/CD29⁺ subsets were sorted from primary salispheres and cultured in Matrigel (BD Biosciences, San Jose, CA, USA) with enriched medium (salisphere medium + Rho-inhibitor, Y-27632) for 7 days to induce secondary salisphere formation. Secondary salispheres were passaged two times every 7 days, and self-renewal was evaluated as the percentage of cells capable of forming salispheres at passage 2.

For retroviral infection experiments, isolated SMG cells from WT 7- to 8-week-old mice were allowed to adhere to poly-D-lysine/laminin-coated dishes (BD Biosciences) in salisphere medium. After 24 h, viral supernatant was added to the cells, which were then cultured for an additional 24 h. The cells were harvested, the medium was replaced, and the cells were subjected to nonadherent culture to form salispheres for an additional 1 or 3 days before the differentiation or immunoblot assays, respectively. Images were acquired using an inverted microscope (IX71; Olympus Corp., Tokyo, Japan) equipped with an UPlanFL 10× objective using DP CONTROLLER software.

qPCR and ChIP

Primer sequences and the methodological details of qPCR and ChIP can be found in the Experimental Procedures of Supporting information.

Histology, immunohistochemistry, and immunocytochemistry

Detailed descriptions of the histological, immunohistochemical, and immunocytochemical analyses can be found in the Experimental Procedures of Supporting information.

Immunoblotting

Immunoblotting was performed using the following antibodies: rabbit anti-Bmi-1 (#5856; Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit anti-p16 (sc1207; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).
USA), rat anti-p19Arf (sc32748; Santa Cruz Biotechnology, Inc.), rabbit anti-p15 (sc613; Santa Cruz Biotechnology, Inc.), rabbit anti-phospho-Rb (ser807/811) (#9308; Cell Signaling Technology, Inc.), rabbit anti-phospho-Akt (ser473) (#4060; Cell Signaling Technology, Inc.), rabbit anti-Akt (sc4061; Cell Signaling Technology, Inc.), and mouse anti-Ji-actin (AS5316; Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

Mice were randomly assigned to each group. The sample size (n) of each group is described in the corresponding figure legends. Results are presented as mean ± standard deviation (SD) of a number (n) of independent experiments. Statistical significance was determined using a two-tailed unpaired t-test or Welch's t-test and one-way ANOVA using GRAPHPAD PRISM software (http://www.graphpad.com/scientific-software/prism/).

Acknowledgments

We wish to thank M. van Loohenzi (The Netherlands Cancer Institute) and H. Koseki (RIKEN Centre for Integrative Medical Sciences) for providing the Bmi-1 KO mice, N. E. Sharpless (University of North Carolina) for the p16Ink4a KO mice, and C. J. Sherr (St. Jude Children's Research Hospital) and M. Sugimoto (National Centre for Geriatrics and Gerontology) for the pMSCV–IRES–GFP retroviral vector. We are also grateful to M. Kawashima (Keio University) for explaining how to determine the saliva flow volume and N. Nakagata (Kumamoto University) and N. Ogiso (National Centre for Geriatrics and Gerontology) for their assistance with the mouse experiments.

Funding

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Japan Prize Foundation; the Takeda Science Foundation; Japan Foundation for Aging and Health; and Toyoaki Scholarship Foundation.

Conflict of interest

The authors declare no competing financial interests.

Author contributions

K.Y. conceived the project, designed and performed the experiments, interpreted the results, and wrote the manuscript. S.K. performed the in vitro studies and analyzed the data. M.I. analyzed gene expression and histomorphometry data. H.K. performed the immunoblotting experiments and provided technical support. A.O. performed the BLI experiment. M.U. performed cell sorting and analysis. N.O contributed reagents. E.H. contributed reagents and advice. M.M. helped supervise S.K.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Fig. S1 Analysis of CDKI levels in the SMGs of WT or Bmi-1-KO, young or old adult WT mice.

Fig. S2 Salispheres require Bmi-1 for normal stem or progenitor cell activities.

Fig. S3 Elevated p16INK4a levels inhibit the proliferation of salispheres.

Appendix S1 Complete experimental procedures