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## Increasing *p16<sup>INK4a</sup>* expression decreases forebrain progenitors and neurogenesis during ageing

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### Abstract

Mammalian ageing is associated with reduced regenerative capacity in tissues that contain stem cells<sup>1,2</sup>. It has been proposed that this is at least partially caused by the senescence of progenitors with age<sup>3,4</sup>; however, it has not yet been tested whether genes associated with senescence functionally contribute to physiological declines in progenitor activity. Here we show that progenitor proliferation in the subventricular zone and neurogenesis in the olfactory bulb, as well as multipotent progenitor frequency and self-renewal potential, all decline with age in the mouse forebrain. These declines in progenitor frequency and function correlate with increased expression of *p16<sup>INK4a</sup>*, which encodes a cyclin-dependent kinase inhibitor linked to senescence<sup>5</sup>. Ageing *p16<sup>INK4a</sup>*-deficient mice showed a significantly smaller decline in subventricular zone proliferation, olfactory bulb neurogenesis, and the frequency and self-renewal potential of multipotent progenitors. *p16<sup>INK4a</sup>* deficiency did not detectably affect progenitor function in the dentate gyrus or enteric nervous system, indicating regional differences in the response of neural progenitors to increased *p16<sup>INK4a</sup>* expression during ageing. Declining subventricular zone progenitor function and olfactory bulb neurogenesis during ageing are thus caused partly by increasing *p16<sup>INK4a</sup>* expression.

Stem cells must persist throughout adult life in numerous tissues, including the central nervous system (CNS)<sup>6</sup>, in order to replace the mature cells that are lost to turnover, injury, or disease. However, the function of stem cells and other progenitors declines with age in diverse tissues including the haematopoietic system<sup>7–9</sup>, muscle<sup>10,11</sup> and brain<sup>6,12,13</sup>. Consistent with this, ageing tissues exhibit reduced repair capacity and an increased incidence of degenerative disease<sup>1,4</sup>. However, the mechanisms responsible for the age-related decline in the function of stem cells and other progenitors remain uncertain.

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**Author Contributions** A.V.M. studied the effect of age on forebrain progenitors, *p16<sup>INK4a</sup>* expression and function during ageing in the subventricular zone (Figs 1 and 2), and *Bmi1* expression during ageing (Supplementary Fig. 3). S.G.S. contributed to studies of *p16<sup>INK4a</sup>* expression during ageing, and the effect of *p16<sup>INK4a</sup>* on proliferation and neurogenesis in the subventricular zone and hippocampus (Figs 2 and 3 and Supplementary Fig. 1). N.M.J. studied neurogenesis in the olfactory bulb and hippocampus (Fig. 3 and Supplementary Fig. 1). S.H. and R.P. examined *p16<sup>INK4a</sup>* expression in the ageing enteric nervous system and its effect on neural crest stem cells (Supplementary Fig. 2). J.K. and N.E.S. provided ageing *p16<sup>INK4a</sup>*-deficient and control mice for some of the experiments and discussed results throughout the project. S.J.M. helped to design and interpret experiments and wrote the manuscript with help from A.V.M., S.G.S. and N.M.J.

*p16<sup>INK4a</sup>* gene expression increases with age in a variety of tissues<sup>14–16</sup>. Although induction of *p16<sup>INK4a</sup>* expression can cause the senescence of a variety of cell types in culture<sup>5,17</sup> and *in vivo*<sup>18</sup>, some cells (including some neural progenitors) are unaffected by increased *p16<sup>INK4a</sup>* expression or *p16<sup>INK4a</sup>* deletion<sup>19–21</sup>. It is thus unclear whether increased *p16<sup>INK4a</sup>* expression causes declines in progenitor function during ageing *in vivo*. We have addressed this question by examining progenitor frequency, proliferation and neurogenesis in the forebrain lateral ventricle subventricular zone (SVZ) of ageing wild-type and *p16<sup>INK4a</sup>*-deficient mice. The SVZ contains a mixed population of stem cells and other progenitors that engage in neurogenesis throughout adult life<sup>6,13,22</sup>. The rate of neurogenesis is known to decline in ageing mammals<sup>13</sup>, but the physiological mechanisms responsible for this decline have not been identified.

We compared various measures of progenitor function in the SVZ of 60-day-old, 1-yr-old and 2-yr-old mice to determine the effects of ageing. Consistent with a previous study<sup>6</sup>, we found a twofold reduction with age in the frequency of SVZ cells that formed multipotent neurospheres in culture (Fig. 1a; asterisk,  $P < 0.05$ ). This was associated with an approximately twofold reduction in the self-renewal potential of these multipotent neurospheres (Fig. 1b; asterisk,  $P < 0.05$ ). *In vivo*, we observed an approximately threefold reduction in the rate of proliferation in the SVZ with age (Fig. 1c, d; asterisk,  $P < 0.05$ ). These data suggest that stem cell frequency and self-renewal potential, as well as overall proliferation rate, decline with age in the SVZ.

Adult *Bmi1*-deficient mice also exhibit reduced stem cell frequency and self-renewal potential, as well as reduced proliferation in the SVZ<sup>20</sup>. These effects are largely caused by increased *p16<sup>INK4a</sup>* and *Arf* expression in the absence of *Bmi1* (refs 19–21). We therefore wondered whether the age-related changes observed in wild-type mice (Fig. 1) were associated with increased *p16<sup>INK4a</sup>* or *Arf* expression in neural progenitors. *p16<sup>INK4a</sup>* and *Arf* expression increase with age in some tissues<sup>15,16</sup>, although these studies did not examine neural progenitors. We did not detect any age-related increase in *Arf* expression in uncultured SVZ cells at the RNA or protein levels (data not shown). In contrast, *p16<sup>INK4a</sup>* expression increased substantially with age in uncultured SVZ cells (Fig. 1e). *p16<sup>INK4a</sup>* expression was not detectable by polymerase chain reaction (PCR) in the SVZ of 60-day-old mice, but became detectable by 1 yr of age and further increased by 2 yr of age. We were not able to detect *p16<sup>INK4a</sup>* protein in the SVZ by western blot, consistent with previous studies that have also not been able to detect this protein in most uncultured mouse tissues that express *p16<sup>INK4a</sup>* mRNA<sup>15,16</sup>, presumably due to its low expression level and the limited sensitivity of available antibodies.

To test the function of *p16<sup>INK4a</sup>* in ageing neural progenitors we examined young (60-day-old) and old (2-yr-old) *p16<sup>INK4a</sup>*-deficient mice as well as littermate controls. *p16<sup>INK4a</sup>* deficiency did not grossly affect the composition of the SVZ, with similar proportions of SVZ cells staining positively for the glia marker GFAP or the neuroblast marker doublecortin in wild-type and *p16<sup>INK4a</sup>*-deficient mice irrespective of age (data not shown). Similarly, apoptotic SVZ cells (activated caspase-3<sup>+</sup>) were rare in all treatments (data not shown). Deletion of *p16<sup>INK4a</sup>* in young mice did not significantly affect the frequency of SVZ cells that formed multipotent neurospheres in culture (Fig. 2a), or the self-renewal potential of these cells upon subcloning (Fig. 2b). This is consistent with our failure to detect *p16<sup>INK4a</sup>* expression in SVZ cells from young mice *in vivo*, as well as with previous studies<sup>19,20</sup>. In contrast, *p16<sup>INK4a</sup>* deficiency in old mice significantly increased the frequency of SVZ cells that formed multipotent neurospheres in culture (Fig. 2a; hash,  $P < 0.05$  relative to old wild-type mice), as well as the self-renewal potential of these cells (Fig. 2b; hash,  $P < 0.05$  relative to old wild-type mice). Consistent with this finding, the rate of proliferation among SVZ cells *in vivo* was also significantly increased by *p16<sup>INK4a</sup>* deficiency in old but not young mice (Fig. 2c; hash,  $P < 0.05$  relative to old wild-type mice). Nonetheless, the percentage of proliferating cells in

old  $p16^{INK4a}$ -deficient mice was still significantly less than observed in normal or  $p16^{INK4a}$ -deficient young mice (Fig. 2c; asterisk,  $P < 0.05$  relative to young mice). Like old wild-type mice, old  $p16^{INK4a}$ -deficient mice also exhibited enlarged lateral ventricles due to cortical atrophy (Fig. 1c, data not shown). These observations indicate that  $p16^{INK4a}$  deficiency partially rescued the age-related declines in progenitor activity in the SVZ but did not prevent cortical atrophy.

$p16^{INK4a}$  deficiency seemed to rescue completely the age-related decline in cells that can form stem cell colonies in culture (Fig. 2a), while only partially rescuing the overall decline in SVZ proliferation (Fig. 2c). One possibility that would be consistent with previous studies of  $p16^{INK4a}$  in neural progenitors<sup>19–21</sup> is that  $p16^{INK4a}$  expression had a greater effect on SVZ stem cells than on downstream progenitors. A number of studies have argued that stem cells can be identified within the SVZ based on their ability to retain the DNA replication label 5-bromodeoxyuridine (BrdU; stem cells divide infrequently and are retained within the SVZ while other progenitors divide continuously before migrating out of the SVZ)<sup>22–25</sup>. Therefore, we examined the effects of age and  $p16^{INK4a}$  deficiency on the frequency of BrdU label-retaining cells. We administered BrdU for 8 days, followed by a 4-week chase with no BrdU, then killed the mice.  $p16^{INK4a}$  deficiency had no effect on the frequency of label-retaining cells in the young adult SVZ (Fig. 2d). The frequency of label-retaining cells declined significantly in old wild-type mice but not in old  $p16^{INK4a}$ -deficient mice (Fig. 2d). Thus, the frequencies of label-retaining cells *in vivo* exhibited similar trends as observed for the frequencies of cells that could form multi-lineage colonies in culture. These data suggest that  $p16^{INK4a}$  deficiency can largely rescue the age-related decline in the frequency of early progenitors within the SVZ.

SVZ progenitors form neuroblasts throughout life that migrate into the olfactory bulb and differentiate into neurons<sup>13,22</sup>. To test whether the increase in  $p16^{INK4a}$  expression within the SVZ also affects neurogenesis, we examined the effects of age and  $p16^{INK4a}$  deficiency on the generation of new neurons in the olfactory bulb. We administered BrdU to mice for 8 days to mark dividing progenitors, followed by a 4-week chase period with no BrdU (during which neurons could migrate and differentiate), then killed the mice to analyse sections through the olfactory bulb by confocal microscopy. As we observed for progenitor function,  $p16^{INK4a}$  deficiency had no effect on neurogenesis in young mice, but neurogenesis significantly decreased with age, and  $p16^{INK4a}$  deficiency significantly increased the frequency of newly generated olfactory bulb neurons in 15–19-month-old mice (Fig. 3i). Notably,  $p16^{INK4a}$  deficiency had no effect on the frequency of non-neuronal cells within the olfactory bulb (Fig. 3j). This may at least partially reflect our previous observation that changes in  $p16^{INK4a}$  expression do not appear to affect gliarestricted progenitors<sup>20</sup>.  $p16^{INK4a}$  deficiency thus partially rescued the age-related decline in neurogenesis in the olfactory bulb in addition to rescuing partially the decline in progenitor function in the SVZ.

To test whether these effects of  $p16^{INK4a}$  occurred throughout the CNS, we also examined the effect of  $p16^{INK4a}$  deficiency on progenitor activity and neurogenesis in the dentate gyrus<sup>26</sup>. The rates of progenitor proliferation and neurogenesis in the dentate gyrus decline markedly with age<sup>12</sup>. To test whether this is affected by  $p16^{INK4a}$ , we administered BrdU for 8 days to mark proliferation in the subgranular layer, followed by a 4-week chase period without BrdU to examine neurogenesis in the granular layer.  $p16^{INK4a}$  deficiency did not significantly affect the rate of proliferation among progenitors in the subgranular layer, or the frequency of BrdU<sup>+</sup>NeuN<sup>+</sup> newly generated neurons or BrdU<sup>+</sup>NeuN<sup>-</sup> non-neuronal cells in the granular layer of the dentate gyrus (Supplementary Fig. 1). Thus, although  $p16^{INK4a}$  deficiency consistently increased all measures of progenitor activity and neurogenesis in the ageing subventricular zone/olfactory bulb, it did not detectably affect proliferation or neurogenesis in the dentate gyrus.

We also examined the effect of age and  $p16^{INK4a}$  deficiency on the neural crest stem cells that persist throughout adult life in the enteric nervous system (in the gut wall)<sup>19,20,27</sup>. The frequency of these neural crest stem cells declined with age, and  $p16^{INK4a}$  expression increased with age in  $p75^+$  (neurotrophin-receptor expressing) gut cells enriched for neural crest stem cells (Supplementary Fig. 2). However,  $p16^{INK4a}$  deficiency had no effect on neural crest stem cell frequency in young or old mice. These results indicate that although the age-related increase in  $p16^{INK4a}$  expression causes a decline in SVZ progenitor function and neurogenesis, other mechanisms are more important in the ageing of stem and progenitor cells in the hippocampus and peripheral nervous system.

The mechanisms that account for the increase in  $p16^{INK4a}$  expression with age remain unclear. In principle,  $p16^{INK4a}$  expression could be developmentally programmed to increase with age to counter the increasing incidence of cancer that is observed in the ageing nervous system. Alternatively,  $p16^{INK4a}$  expression may reflect the induction of senescence in ageing cells in response to damage that accumulates from oxidative metabolism or other stresses. Although *Bmi1* is important for  $p16^{INK4a}$  repression<sup>19,20,28</sup>, we did not detect any change in *Bmi1* expression with age at the RNA or protein levels in SVZ cells (Supplementary Fig. 3). Nonetheless, subtle reductions in *Bmi1* expression or activity cannot be excluded and could be functionally important, as even the twofold reduction in *Bmi1* expression in *Bmi1*<sup>-/+</sup> mice leads to increased  $p16^{INK4a}$  expression (data not shown). Additional work will be required to resolve the potentially multifactorial mechanisms that regulate age-related changes in  $p16^{INK4a}$  expression.

The simplest interpretation of our data is that  $p16^{INK4a}$  acts cell autonomously within neural stem cells, because  $p16^{INK4a}$  deficiency increased the self-renewal of single neural stem cells in culture. However, we cannot exclude the possibility of non-cell-autonomous effects by  $p16^{INK4a}$  *in vivo*. In addition to investigating this issue further, it will also be of interest to determine whether circulating humoral factors<sup>11</sup> influence  $p16^{INK4a}$  expression during ageing.

One study found that moderately increased expression of  $p16^{INK4a}$  and  $p19^{ARF}$  in transgenic mice does not detectably accelerate gross measures of ageing or change lifespan, despite reducing cancer incidence<sup>29</sup>. However, that study did not examine neural progenitor function or neurogenesis. We also examined  $p16^{INK4a}$  bacterial artificial chromosome (BAC) transgenic mice that exhibited a moderate increase in  $p16^{INK4a}$  expression and found no effect of  $p16^{INK4a}$  overexpression on stem cell frequency or self-renewal potential, although old transgenic mice did exhibit a modest reduction in SVZ proliferation (data not shown). It seems that the levels of  $p16^{INK4a}$  that are required to deplete stem cells are qualitatively higher than the levels that are required to inhibit carcinogenesis. Together, the data suggest that although physiological increases in  $p16^{INK4a}$  expression during ageing reduce neural progenitor function and neurogenesis in the SVZ/olfactory bulb, modest overexpression of  $p16^{INK4a}$  does not substantially amplify these effects or accelerate gross measures of ageing.

Although physiological  $p16^{INK4a}$  expression reduces stem/progenitor cell function and neurogenesis with age, how this relates to overall ageing or lifespan remains unclear. It is difficult to examine the effect of  $p16^{INK4a}$  deficiency on mouse lifespan because  $p16^{INK4a}$  deficiency increases cancer incidence<sup>30</sup> in addition to affecting age-related changes in stem/progenitor cell function. Declining stem/progenitor cell function may be a major cause of the decline in regenerative capacity and the increase in degenerative disease that is observed in ageing tissues. On the other hand, increases in the death or dysfunction of mature cells in ageing tissues also contribute to age-related morbidity. It is similarly unknown whether physiological differences in the rate at which stem/progenitor cell activity declines with age has a detectable impact on longevity. The fact that  $p16^{INK4a}$  did not affect progenitors in certain regions of the nervous system suggests that  $p16^{INK4a}$  is not likely to promote generically the ageing of all

cells. Indeed, it is likely that there will be important differences between tissues, and perhaps between progenitor cells and differentiated cells, in terms of the genes that regulate the ageing process. Although these questions remain unanswered, it may be possible to gain important new insights into the ageing process at a cellular level by studying individual cell types that have known physiological functions *in vivo*, such as haematopoietic or neural stem cells.

Our data suggest that stem cell function is regulated by a balance between proto-oncogenes, like *Bmi1* (that promote stem cell maintenance and regenerative capacity but can also contribute to neoplastic proliferation), and tumour suppressors, like *p16<sup>INK4a</sup>* (that reduce regenerative capacity and promote ageing but also reduce cancer incidence). This balance changes with age and is influenced by other proto-oncogenes and tumour suppressors that also regulate *p16<sup>INK4a</sup>* expression. The networks of proto-oncogenes and tumour suppressors that regulate stem cell self-renewal, cancer cell proliferation and stem cell ageing may have evolved to balance the need for regenerative capacity while guarding against the increasing risk of neoplasms with age. *p16<sup>INK4a</sup>* thus reduces cancer incidence<sup>5,30</sup> but also contributes to ageing by reducing progenitor function and neurogenesis in at least certain regions of the nervous system.

## METHODS

### Mice

*p16<sup>INK4a</sup>±* mice were backcrossed at least six times onto a C57BL background. *p16<sup>INK4a</sup>* genotyping was performed by PCR as described<sup>30</sup>.

### Isolation of CNS progenitors

Adult SVZ was obtained by microdissecting the lateral walls of the lateral ventricles, then dissociating for 20 min at 37 °C in 0.025% trypsin/0.5 mM EDTA (Calbiochem) plus 0.001% DNase1 (Roche). After quenching the enzymatic dissociation with staining medium (L15 medium containing 1 mg ml<sup>-1</sup> BSA (Sigma A-3912), 10 mM HEPES (pH 7.4) and 1% penicillin/streptomycin (BioWhittaker)) containing 0.014% soybean trypsin inhibitor (Sigma) and 0.001% DNase1, the cells were washed and re-suspended in staining medium, triturated, filtered through nylon screen (45µm, Sefar America), counted by haemocytometer, and plated.

### Cell culture and self-renewal assay

Cells were plated at clonal density (1.3 cells per µl) on ultra-low-binding plates (Corning) to grow neurospheres. Culture medium was based on a 5:3 mixture of DMEM-low glucose as described previously<sup>19</sup>: neurobasal medium (Gibco) supplemented with 20 ng ml<sup>-1</sup> human bFGF (R&D Systems), 20 ng ml<sup>-1</sup> EGF (R&D Systems), 1% N2 supplement (Gibco), 2% B27 supplement (Gibco), 50µM 2-mercaptoethanol, 1% penicillin/streptomycin (BioWhittaker), and 10% chick embryo extract. Cultures were maintained at 37 °C in 6% CO<sub>2</sub>/balance air. To measure self-renewal, individual neurospheres were dissociated by trituration then replated at clonal density as above. Secondary neurospheres were counted 5–10 days later to determine the number of secondary neurospheres formed per primary neurosphere. CNS neurospheres were tested for multipotency by replating one neurosphere per well into 48-well plates and then culturing adherently for 3–5 days before triple staining for oligodendrocytes (O4), neurons (TuJ1) and astrocytes (GFAP). See Supplementary Methods for details.

### BrdU incorporation/proliferation assays

To quantify SVZ proliferation, mice were injected intraperitoneally with 50 mg kg<sup>-1</sup> of BrdU (Sigma), and killed 2 h after BrdU injection. To quantify proliferation in the dentate gyrus, mice received a single injection of 50 mg kg<sup>-1</sup> BrdU, followed by 1 mg ml<sup>-1</sup> BrdU in their



drinking water for 8 days before being killed and analysed. To quantify neurogenesis in the dentate gyrus and the olfactory bulb and BrdU retention in the SVZ, mice were treated with BrdU for 8 days. However, the animals were then taken off of BrdU for 4 weeks before the animals were killed. The brains were dissected, fixed in 4% paraformaldehyde overnight, then cryo-protected in 15% sucrose, embedded in 7.5% gelatin/15% sucrose, and flash frozen. Twelve-micrometre sections were cut on a Leica cryostat.

For detection of BrdU in the tissue sections, DNA was denatured in 2 M HCl for 30 min at room temperature and neutralized with 0.1 M sodium borate. Sections were pre-blocked for 1 h at room temperature in goat serum solution (PBS containing 5% goat serum, 1% BSA and 0.3% Triton X-100 (Sigma)). Primary rat anti-BrdU (1:500, Accurate Chemical) diluted in goat serum solution was incubated overnight at 4 °C, followed by fluorescein isothiocyanate (FITC)-conjugated anti-rat Fc fragment (Jackson Labs) for 3–4 h at room temperature. Slides were counter stained in 2.5  $\mu\text{g ml}^{-1}$  DAPI for 10 min at room temperature, then mounted using ProLong antifade solution (Molecular Probes). Numbers of BrdU-labelled cells were divided by total DAPI<sup>+</sup> or NeuN<sup>+</sup> nuclei.

To ensure that BrdU incorporation results were not skewed by dividing endothelial or haematopoietic cells, we double-labelled SVZ sections with antibodies against BrdU and either anti-CD45 (to identify blood cells) or anti-VE-cadherin (to identify endothelial cells). We did not detect any BrdU<sup>+</sup> haematopoietic or endothelial cells.

Western blots and quantitative RT-PCR were performed as described in Supplementary Information.

### Statistical analysis

Experiments that involved multiple treatments were initially analysed by analysis of variance. Statistically significant results were followed up with Student's *t*-tests.

### Confocal analysis of neurogenesis in the olfactory bulb

A Zeiss LSM 510 confocal laser-scanning microscope was used to obtain 25–30 random fields of view throughout all regions of one entire olfactory bulb of each mouse with a  $\times 40$  or  $\times 63$  objective lens. For each image, 1- $\mu\text{m}$ -thick optical sections were scanned with three different lasers through a 12- $\mu\text{m}$  sagittal tissue section creating a Z-series stack with three distinct channels of fluorescence. An ultraviolet enterprise laser was used to detect the DAPI signal labelling all nuclei. An argon laser detected FITC (BrdU), and a HeNe laser detected Cy3 (NeuN). Channels were merged together to determine whether DAPI, BrdU and NeuN signals co-labelled at every 1- $\mu\text{m}$  slice of the Z-series. Three-dimensional projections were made using LSM 510 software.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

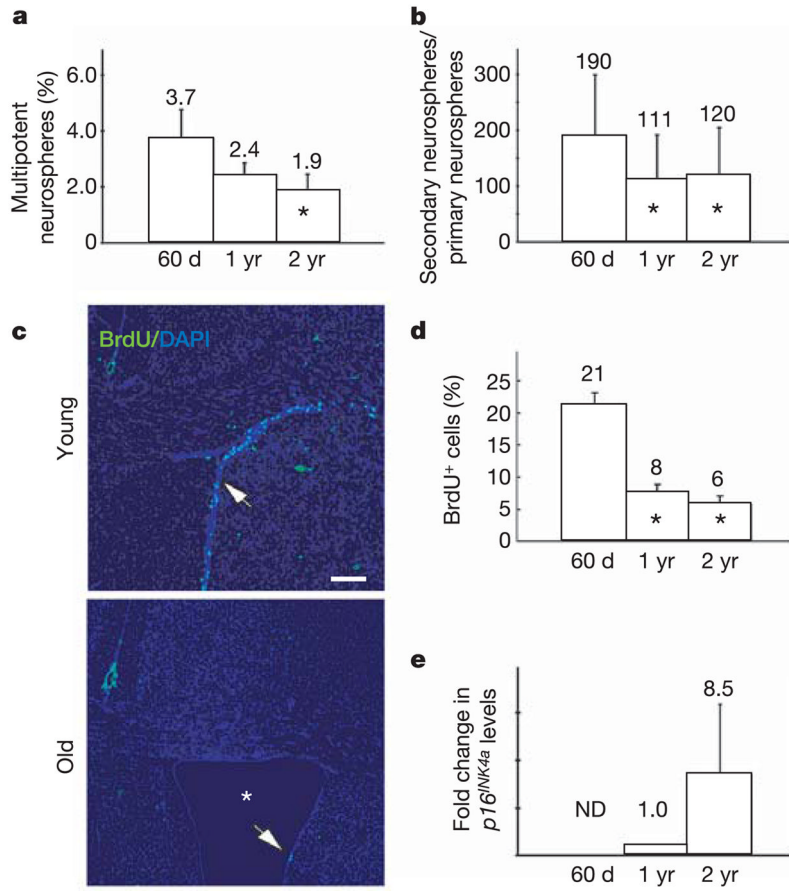
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## References

1. Chien KR, Karsenty G. Longevity and lineages: toward the integrative biology of degenerative diseases in heart, muscle, and bone. *Cell* 2005;120:533–544. [PubMed: 15734685]
2. Geiger H, Van Zant G. The aging of lympho-hematopoietic stem cells. *Nature Immunol* 2002;3:329–333. [PubMed: 11919569]
3. Lombard DB, et al. DNA repair, genome stability, and aging. *Cell* 2005;120:497–512. [PubMed: 15734682]
4. Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* 2005;120:513–522. [PubMed: 15734683]
5. Lowe SW, Sherr CJ. Tumor suppression by *Ink4a–Arf*: progress and puzzles. *Curr Opin Genet Dev* 2003;13:77–83. [PubMed: 12573439]
6. Maslov AY, Barone TA, Plunkett RJ, Pruitt SC. Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice. *J Neurosci* 2004;24:1726–1733. [PubMed: 14973255]
7. Morrison SJ, Wandycz AM, Akashi K, Globerson A, Weissman IL. The aging of hematopoietic stem cells. *Nature Med* 1996;2:1011–1016. [PubMed: 8782459]
8. Chen J, Astle CM, Harrison DE. Genetic regulation of primitive hematopoietic stem cell senescence. *Exp Hematol* 2000;28:442–450. [PubMed: 10781902]
9. de Haan G, Nijhof W, Van Zant G. Mouse strain-dependent changes in frequency and proliferation of hematopoietic stem cells during aging: correlation between lifespan and cycling activity. *Blood* 1997;89:1543–1550. [PubMed: 9057635]
10. Conboy IM, Conboy MJ, Smythe GM, Rando TA. Notch-mediated restoration of regenerative potential to aged muscle. *Science* 2003;302:1575–1577. [PubMed: 14645852]
11. Conboy IM, et al. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* 2005;433:760–764. [PubMed: 15716955]
12. Kuhn HG, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci* 1996;16:2027–2033. [PubMed: 8604047]
13. Enwere E, et al. Aging results in reduced epidermal growth factor receptor signaling, diminished olfactory neurogenesis, and deficits in fine olfactory discrimination. *J Neurosci* 2004;24:8354–8365. [PubMed: 15385618]
14. Nielsen GP, et al. Immunohistochemical survey of p16<sup>INK4A</sup> expression in normal human adult and infant tissues. *Lab Invest* 1999;79:1137–1143. [PubMed: 10496532]
15. Krishnamurthy J, et al. *Ink4a/Arf* expression is a biomarker of aging. *J Clin Invest* 2004;114:1299–1307. [PubMed: 15520862]
16. Zindy F, Quelle DE, Roussel MF, Sherr CJ. Expression of the p16<sup>INK4a</sup> tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene* 1997;15:203–211. [PubMed: 9244355]
17. Sharpless NE, Ramsey MR, Balasubramanian P, Castrillon DH, DePinho RA. The differential impact of p16<sup>INK4a</sup> or p19<sup>ARF</sup> deficiency on cell growth and tumorigenesis. *Oncogene* 2004;23:379–385. [PubMed: 14724566]
18. Michaloglou C, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* 2005;436:720–724. [PubMed: 16079850]
19. Molofsky AV, et al. Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16<sup>INK4a</sup> and p19<sup>Arf</sup> senescence pathways. *Genes Dev* 2005;19:1432–1437. [PubMed: 15964994]
20. Molofsky AV, et al. Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* 2003;425:962–967. [PubMed: 14574365]
21. Bruggeman SWM, et al. *Ink4a* and *Arf* differentially affect cell proliferation and neural stem cell self-renewal in *Bmi1*-deficient mice. *Genes Dev* 2005;19:1438–1443. [PubMed: 15964995]
22. Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 1999;97:703–716. [PubMed: 10380923]

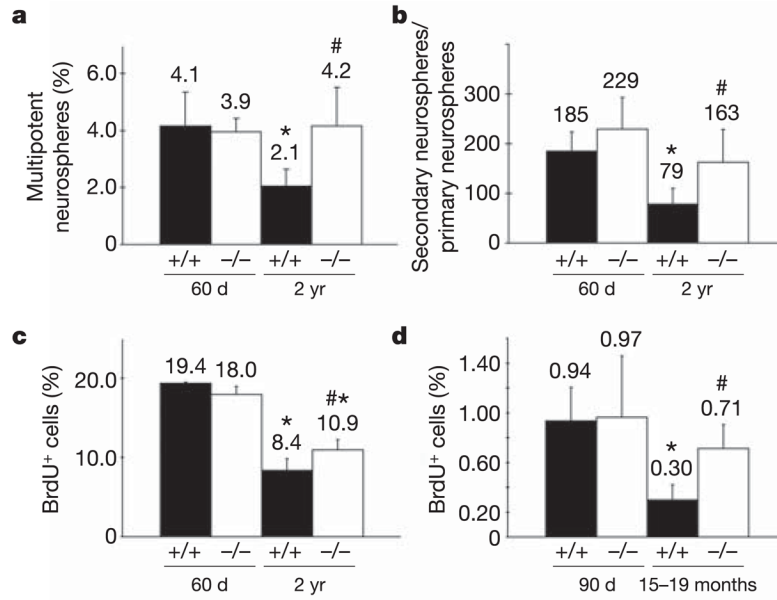
23. Capela A, Temple S. LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. *Neuron* 2002;35:865–875. [PubMed: 12372282]
24. Chiasson BJ, Tropepe V, Morshead CM, Kooy D. v d Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cell characteristics. *J Neurosci* 1999;19:4462–4471. [PubMed: 10341247]
25. Johansson CB, et al. Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* 1999;96:25–34. [PubMed: 9989494]
26. van Praag H, et al. Functional neurogenesis in the adult hippocampus. *Nature* 2002;415:1030–1034. [PubMed: 11875571]
27. Kruger GM, et al. Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. *Neuron* 2002;35:657–669. [PubMed: 12194866]
28. Jacobs JLL, Kieboom K, Marino S, DePinho RA, Lohuizen M. v The oncogene and polycomb-group gene *bmi-1* regulates cell proliferation and senescence through the *ink4a* locus. *Nature* 1999;397:164–168. [PubMed: 9923679]
29. Matheu A, et al. Increased gene dosage of *Ink4a/Arf* results in cancer resistance and normal aging. *Genes Dev* 2004;18:2736–2746. [PubMed: 15520276]
30. Sharpless NE, et al. Loss of p16<sup>INK4a</sup> with retention of p19<sup>Arf</sup> predisposes mice to tumorigenesis. *Nature* 2001;413:86–91. [PubMed: 11544531]



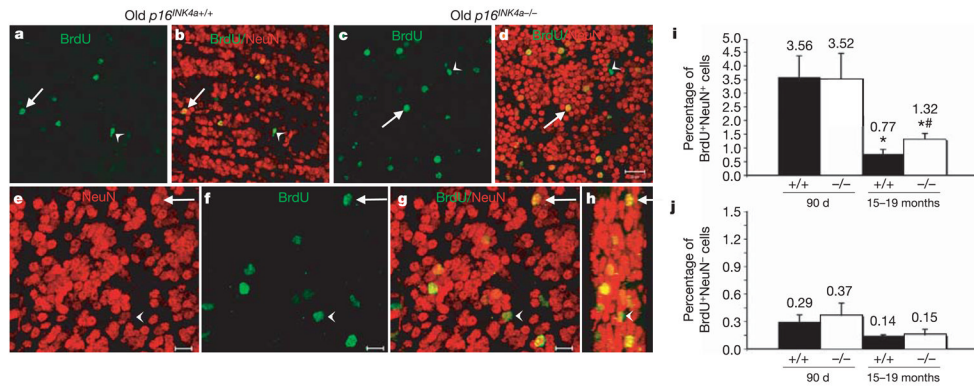


**Figure 1. Neural progenitor function declines with age**

**a**, The percentage of SVZ cells that formed multipotent neurospheres in culture declined with age (asterisk,  $P < 0.05$  relative to 60-day-old mice; three independent experiments; 5–6 mice per age; error bars for all panels are  $\pm$ s.d.). **b**, The self-renewal potential of these primary neurospheres also declined with age (asterisk,  $P < 0.05$ ; three independent experiments; 5–6 mice per age). **c**, **d**, Proliferation in the SVZ (percentage of BrdU<sup>+</sup> cells after a 2-h pulse) also declined significantly with age (three mice per age; 5–7 sections per mouse). The SVZ thinned in old mice (**c**, arrows), and the lateral ventricle expanded (asterisk) due to cortical atrophy (**c**, lateral ventricle is not visible at this magnification in young mice; scale bar, 200 $\mu$ m). **e**, *p16<sup>INK4a</sup>* mRNA expression increased with age as detected by quantitative (real-time) PCR in uncultured SVZ cells (three independent samples per age). Note that 1-yr-old samples were set to 1.0 as the reference sample. ND, not detected.



**Figure 2. *p16<sup>INK4a</sup>* causes age-related declines in stem and progenitor cell function in the SVZ**  
**a**, The percentage of SVZ cells that formed multipotent neurospheres in culture significantly declined in 2-yr-old wild-type mice as compared with 60-day-old mice (asterisk,  $P < 0.01$ ) but significantly increased in old mice with *p16<sup>INK4a</sup>* deficiency (hash,  $P < 0.01$  relative to old wild-type mice). **b**, Self-renewal potential (the number of secondary neurospheres generated per subcloned primary neurosphere) significantly declined in old wild-type mice as compared to young mice (asterisk,  $P < 0.05$ ). *p16<sup>INK4a</sup>* deficiency significantly increased the self-renewal of neurospheres from old but not young mice (hash,  $P < 0.05$  relative to old wild-type mice). **c**, The percentage of SVZ cells that incorporated a 2-h pulse of BrdU significantly declined in old as compared to young mice (asterisk,  $P < 0.01$ ), but significantly increased in old mice with *p16<sup>INK4a</sup>* deficiency (hash,  $P < 0.01$  relative to old wild-type mice). **d**, The frequency of BrdU label-retaining cells in the SVZ significantly declined in old wild-type mice as compared with young wild-type mice (asterisk,  $P < 0.05$ ), but significantly increased in old mice with *p16<sup>INK4a</sup>* deficiency (hash,  $P < 0.05$  relative to old wild-type mice). All values are mean  $\pm$  s.d. for at least three independent experiments. All mice were histologically negative for intracranial neoplasms.



**Figure 3. *p16<sup>INK4a</sup>* causes age-related declines in olfactory bulb neurogenesis**  
**a–d**, Low-magnification images (scale bar, 20µm) from sagittal sections of the olfactory bulb of old wild-type (**a, b**; same field of view) and *p16<sup>INK4a</sup>*-deficient (**c, d**; same field of view) mice. Arrows point to new neurons in the granular layer (BrdU<sup>+</sup>NeuN<sup>+</sup>) whereas arrowheads point to new non-neuronal cells (BrdU<sup>+</sup>NeuN<sup>-</sup>). **e–h**, Higher magnification images (scale bar, 10µm) from one field of view from an old *p16<sup>INK4a</sup>*-deficient mouse. Arrow indicates BrdU<sup>+</sup>NeuN<sup>+</sup> neuron; arrowhead indicates BrdU<sup>+</sup>NeuN<sup>-</sup> non-neuronal cell. Panel **h** is a three-dimensional, reconstructed side view (80° turn in the z-axis) of panel **g**. **i**, Neurogenesis significantly (asterisk,  $P < 0.05$ ) declined with age (BrdU<sup>+</sup>NeuN<sup>+</sup> neurons as a percentage of all NeuN<sup>+</sup> neurons). *p16<sup>INK4a</sup>* deficiency did not affect the level of neurogenesis in young mice, but significantly (hash,  $P = 0.02$  relative to old wild-type mice) increased neurogenesis in old mice. **j**, The frequency of BrdU<sup>+</sup>NeuN<sup>-</sup> non-neuronal cells was not significantly affected by *p16<sup>INK4a</sup>* deficiency (also as a percentage of NeuN<sup>+</sup> neurons). The same trends were observed when the counts were expressed per unit area (not shown). Values are mean ± s.d. from 25 to 30 fields of view per mouse, three mice per treatment.