Dynamics of senescent cell formation and retention revealed by p14ARF induction in the epidermis

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Running title: Senescent cell formation and retention in the epidermis

Precis: A novel mouse model provides insights into the dynamics of cellular senescence, a central mechanism for tumor suppression.
Abstract

Cellular senescence, a state of cell-cycle arrest accompanied by dramatic morphologic and metabolic changes, is a central means by which cells respond to physiologic stress and oncogene activity. Senescence is thought to play important roles in aging and in tumor suppression, yet the dynamics by which senescent cells are formed, their effects on tissue function, and their eventual fate, are poorly understood. To study cellular senescence within an adult tissue we developed transgenic mice inducibly expressing p14$^{ARF}$ (human ortholog of murine p19$^{ARF}$), a central activator of senescence. Induction of p14$^{ARF}$ in the epidermis rapidly led to widespread apoptosis and cell-cycle arrest, a stage which was transient, and was followed by p53-dependent cellular senescence. The endogenous Cdkn2a products, p19$^{ARF}$ and p16$^{Ink4a}$, were activated by the transgenic p14$^{ARF}$ through p53, revealing a senescence-promoting feed-forward loop. Commitment of cells to senescence required continued p14$^{ARF}$ expression, indicating that entry into this state depends on a persistent signal. However, once formed, senescent cells were retained in the epidermis, often for weeks after transgene silencing, indicating an absence of an efficient rapidly-acting mechanism for their removal. Stem cells in the hair follicle bulge were largely protected from apoptosis upon p14$^{ARF}$ induction, but irreversibly lost their ability to proliferate and initiate follicle growth. Interestingly, induction of epidermal hyperplasia prevented the appearance of senescent cells upon p14$^{ARF}$ induction. Our findings provide basic insights into the dynamics of cellular senescence, a central tumor suppressive mechanism, and reveal the potential for prolonged retention of senescent cells within tissues.
Introduction

Cellular senescence is a coordinated program activated by cells in response to a variety of physiological stresses, including DNA damage and oncogenic signaling (1-3). Senescent cells have been identified in early tumor lesions in mouse models and human samples (4), providing evidence that senescence is an important barrier to cancer formation. Cells entering senescence undergo cell-cycle arrest, which is thought to be irreversible, accompanied by dramatic changes in morphology, chromatin structure and metabolism (1-3). The Rb and p53 tumor suppressor proteins are central in the execution of senescence, and are most often activated in this context by the two products of the \textit{Ink4a} (\textit{Cdkn2a}) locus: p16\textsuperscript{Ink4a} and p19\textsuperscript{ARF} (p14\textsuperscript{ARF} in human) (1, 2, 5). These genes are not expressed in the large majority of tissues in the embryo and the adult, but are transcriptionally activated during stress, tumorigenesis or aging (6-8).

Senescence has been detected \textit{in vivo} in various contexts, including in tissues suffering from DNA damage or shortened telomeres (9, 10), chemotherapy-treated tumors (11, 12), and tissues undergoing wound healing, fibrosis or inflammation (13-15). Senescence is also detected during aging (16-18) and is thought to contribute to this process, potentially through the depletion of functional stem cells (1-3). Secretion of inflammatory cytokines and ECM components, an important aspect of the senescent cell phenotype, could influence tissue aging and exert other non-cell-autonomous effects, including stimulation of proliferation of neighboring cells (19).

While senescent cells can remain viable in culture for months and years, their fate \textit{in vivo} is unclear. The detection of senescent cells in aged tissues suggests that they can accumulate and affect tissue physiology. Consistent with this, experimental removal of senescent cells prevents aging-like phenotypes caused by genetically-induced aneuploidy (20). However, recent studies have shown that immune cells recruited through cytokine secretion rapidly clear senescent cells from cancerous or
fibrotic tissue (13, 21, 22). Whether additional mechanisms act to eliminate senescent cells is not known, and a detailed analysis of the retention rates of senescent cells in tissues is lacking.

Basic study of the senescence program in vivo is essential for understanding its various physiological roles, including tumor suppression. To allow the study of senescent cells in vivo, the dynamics of their formation and removal, and their effects on adult tissues, we sought to develop a system in which senescence could be activated in a direct and controlled manner. We generated transgenic mice carrying a tetracycline-inducible p14ARF gene, a central activator of senescence, whose product activates p53 through Mdm2 sequestration (23). We focused on the skin as a model epithelial tissue, in which senescence is thought to occur during normal aging (16, 24). Our findings reveal basic aspects of the dynamics of senescent cell formation and retention in the epidermis.
Materials and Methods

Transgenic mice

To generate tet-p14 mice we relied on Flp/FRT-mediated recombination into ES cells (25). We cloned the human \textit{p14}^{ARF} coding sequence into the pBS31 vector, which contains an FRT recombination site and a tet-responsive element, and transfected this plasmid together with a Flp/e expression vector into the KH2 ES cells, in which an FRT site was placed in the \textit{Col1a1} gene locus. Recombined cells were injected into tetraploid blastocysts for implantation. Tet-p14 mice (mixed C57Bl6 and 129sv) were crossed with K5-rtTA mice (FVB) (26) and with tet-shp53 mice (mixed C57Bl6 and 129sv) (27). For transgene induction 2mg/ml doxycyclin (dox) was added to the drinking water of double-transgenic mice and sibling control single-transgenics at 3 weeks of age. For BrdU labeling mice were injected with 0.1mg/gr body-weight two hours prior to sacrifice. For hyperplasia induction mice were treated three times a week with 6.5\textmu g TPA in 100ml acetone; untreated skin regions from the same mice were collected as control tissue.

Immunohistology

Upon sacrifice mice were shaved and back skins were dissected, formalin-fixed and paraffin embedded. Immunohistology was performed according to standard procedures, using Peroxidase Substrate kits (Vector) or fluorescently labeled secondary antibodies (Jackson). Antibodies used: \textit{p14}^{ARF} (ab3642, Abcam), p21 (sc-6246, Santa Cruz), p53 (CM5p, Novocastra), \textit{p19}^{ARF} (ab80, Abcam), Ki67 (RM-9106, Labvision), BrdU (MS-1058, Labvision), CC3 (#9661, Cell Signaling), K14 (GP-CK14, Progen), K5 (GP-CK5, Progen), and K15 (sc-56520, Santa Cruz). In Situ Cell Death Detection Kit (Roche) was used for TUNEL. Images were collected using an Olympus BH2 upright or a Nikon Eclipse Ti inverted fluorescent microscope, using DS-Qi1Mc and DS-Fi1 cameras and processed using NIS Elements software (Nikon), in some cases using the EDF function for Z-stacking. Where noted we used a Zeiss LSM710 confocal microscope.
Senescence-associated β-Galactosidase stains

10-12μm cryosections of OCT-embedded mouse skins were fixed in 0.5% glutaraldehyde for 15 minutes, stained overnight at 37°C with 40mM phosphate buffer pH=6 with 5mM K₄Fe(CN)₆, 5mM K₃Fe(CN)₆, 150mM NaCl, 2mM MgCl₂ and 1mg/ml X-gal, washed in PBS, fixed in 95% ethanol for 15 minutes, counterstained with nuclear fast red, dehydrated and mounted. The numbers of SAβGal positive cells among interfollicular epidermal cells were scored from >10 microscopic fields.

RNA extraction and qRT-PCR

Back skin samples were minced and RNA was extracted using the RNeasy Fibrous Tissue kit (Qiagen). Quantitative RT-PCR and Taqman reactions were performed using standard methods.
Results

**p14ARF induction in the skin**

To develop a system for the induction of senescence in the adult skin we generated p14ARF tetracycline-inducible mice (tet-p14). We used the human gene to facilitate the distinction between endogenous p19ARF and the transgene. Tet-p14 mice were crossed with K5-rTA transgenic mice (26) (Figure 1A), which carry the tetracycline-dependent transcriptional activator under the control of the Keratin 5 promoter, directing expression to the basal layer of the epidermis. Mice treated with doxycycline (dox) expressed the transgenic p14ARF in the majority of basal epidermal cells, as detected with a human-specific antibody (Figure 1B and Figure S1). p14ARF appeared in subnuclear puncta, consistent with localization in the nucleoli, the location of the endogenous protein (Figure 1B).

Double-transgenic mice treated with dox for 2 days showed high levels of nuclear p53 expression in the epidermis, and co-localized expression of its target, p21Cip1 (Figure 1C). Expression levels of additional known p53 transcriptional targets were increased (Figure 1D), indicating that p53 was activated. The transgenic p14ARF was thus properly localized and molecularly active.

**p14ARF induces cell-cycle arrest and apoptosis**

p53 activation can lead to several cellular outcomes, including reversible cell-cycle arrest, apoptosis and senescence (28). We examined which of these occurred upon p14ARF induction. Two days of transgene activation in 3-week old mice led to a dramatic decrease in proliferation of basal epidermal cells, as indicated by Ki67 staining (Figure 1E). However, this effect was transient, and, surprisingly, after 1 week we observed hyperproliferation of basal cells, which was driven by cells that did not express the p14ARF transgene; the degree of hyperproliferation decreased after longer induction times (Figure 1E,F). We also observed large numbers of cells undergoing apoptosis after two days of transgene activation, involving ~15% of basal cells (Figure 1G,H). This also was transient: by one
week of induction apoptosis levels returned nearly to normal (Figure 1H), and at two weeks of induction the expression of apoptosis-mediating p53 targets (Noxa and Bax) was similar to that in control mice (Figure 1D).

These findings indicate that p14ARF activation in vivo is sufficient to induce two of the central p53 responses, apoptosis and cell-cycle arrest. This initial synchronous response is transient, and is counteracted by transient hyperproliferation of neighboring transgene-non-expressing cells, most likely acting to maintain tissue homeostasis.

**p14ARF induces p53-dependent cellular senescence and activates the endogenous Ink4a products**

Since p14/19ARF activation often occurs in situations where senescence is the cellular outcome, we tested whether senescent cells appear upon transgene induction. Two or four days after p14ARF activation we did not observe an increase in the numbers of cells showing senescence-associated β-galactosidase (SAβGal) activity, a robust marker of senescence (16, 29) (Figure 2A,B and data not shown). However, starting at 1 week of transgene activation we detected increased SAβGal-positive cell numbers in the epidermis (Figure 2A,B). A small number of SAβGal-positive cells were found also in control mice; we determined that 4-8% of epidermal cells underwent senescence in response to p14ARF activation above this basal level (Figure 2B and Figure S2).

We examined the expression of senescence-associated marker genes in mRNA extracted from whole skins. Dcr2 levels were mildly increased after two weeks of transgene induction, while Pai1 was not significantly induced (Figure 2C). Interestingly, the levels of the two products of the endogenous Ink4a locus – p16Ink4a and p19ARF – were dramatically increased (Figure 2C). This revealed a positive-feedback loop by which p14/19ARF reinforces its own expression as well as of its partner, p16Ink4a, promoting both p53- and Rb-pathway activity. The endogenous p19ARF protein was detectable in a subset of highly-expressing epidermal cells using a mouse-specific antibody (Figure 2D). An increase
in the mRNA levels of \( p16^{\text{Ink4a}} \) and \( p19^{\text{ARF}} \) was detected in some mice already at two days of induction (Figure 2C), suggesting that initiation of senescence occurs early after transgene expression.

\( p19^{\text{ARF}} \) has been shown to execute p53-independent functions, but whether these pertain to senescence is unclear (30, 31). We found that when an inducible transgenic shRNA against p53 (tet-shp53) (27) was co-expressed with the \( p14^{\text{ARF}} \) transgene, eliminating p53 expression, no SA\( \beta \)Gal-positive cells appeared (Figure 2E-G). This indicates that \( p14^{\text{ARF}} \)-induced epidermal cell senescence is dependent on p53. The endogenous \( p16^{\text{Ink4a}} \) and \( p19^{\text{ARF}} \) genes were not activated in shp53-expressing mice (Figure 2H), revealing that the self-amplification of \( p14/19^{\text{ARF}} \) expression occurred through p53.

Consistent with the activation of the Cyclin/CDK inhibitors \( p16^{\text{Ink4a}} \) and \( p21^{\text{Cip1}} \), we observed a dramatic decrease in the levels of phosphorylated Rb in the epidermis, particularly in transgene-expressing cells, after 2 days and 2 weeks of activation (Figure S3). Interestingly, phosphorylation levels of the Rb homolog p130 were also decreased in transgene-expressing cells, although this was less apparent at 2 weeks of activation (Figure S3). These findings indicate that both Rb and p130 are initially activated in response to \( p14^{\text{ARF}} \) induction and may contribute to cell cycle arrest and senescence.

**Commitment to senescence requires continuous \( p14^{\text{ARF}} \) expression**

We next assessed the time required for cells to commit to senescence after \( p14^{\text{ARF}} \) activation. Conceivably, once initiated, the senescence program could be executed independently of the inducing signal. To test whether this is the case, we activated \( p14^{\text{ARF}} \) for four days and then re-silenced the transgene by dox removal for another three days (Figure 3A,B). While SA\( \beta \)Gal-positive cells appeared in mice in which the transgene was continuously expressed for seven days, we did not observe the appearance of senescent cells in mice in which the transgene was silenced (Figure 3C,D). The numbers of cells expressing stable p53 were also decreased in the epidermis of these mice (Figure 3E,F). Three days after transgene silencing the mRNA levels of \( p19^{\text{ARF}} \) and \( p16^{\text{Ink4a}} \) were decreased,
yet remained higher than those observed in control mice (Figure 3G), reflecting a lag in response to p14ARF silencing. Together these results indicate that cells do not commit to senescence until at least five days of continuous high p14ARF expression levels, despite activating p53, p16Ink4a and p19ARF, and are able to revert to a non-senescent state or be eliminated from the tissue. This suggests that in a physiologic setting only persistent stress leading to continuous p14/19ARF expression will cause cells to commit to senescence.

**Senescent cells are retained in the epidermis**

We next assessed whether, once formed, senescent cells are retained in the epidermis or are rapidly eliminated. We activated p14ARF for two weeks to generate senescent cells, and then removed dox to re-silence the transgene for an additional four weeks (Figure 4A,B). Strikingly, we found that high numbers of SAβGal-positive cells remained in the epidermis four weeks after transgene silencing (Figure 4C,D). Furthermore, p53-positive cells were also detected in the epidermis after transgene silencing, indicating that, indeed, senescent cells were retained in the tissue (Figure 4E,F). Many of the retained SAβGal-positive and p53-positive cells were located in the basal layer, despite the high cell turnover in this compartment (Figure 4C,E). These findings indicate that, once formed in the epidermis, senescent cells are capable of retaining their phenotype and position for weeks, independently of the initiating signal.

The numbers of SAβGal-positive and p53-positive cells, were, however, reduced in the transgene-silenced mice relative to mice continuously expressing p14ARF for six weeks (Figure 4D,F). This could be a result of the reduced formation of new senescent cells upon transgene-silencing, but also due to gradual clearance of senescent cells and/or a partial loss of the senescent phenotype. Surprisingly, the levels of p19ARF and p16Ink4a were greatly reduced in transgene-silenced mice (Figure 4G), indicating that their expression depended on a continuous signal, and that independent mechanisms must support the observed p53 activity and senescence.
p14\textsuperscript{ARF} induces hair-follicle stem cell dysfunction

Despite the various effects of transgene induction, skin morphology remained largely intact (Figure S4). Epidermal thickness was increased in some mice, a potential result of the observed hyperproliferation; however this increase was limited in significance and was variable among mice and skin regions (Figure S4).

The most striking change in the skin was a dramatic decrease in the numbers of developed hair follicles (HFs), and progressive hair loss (alopecia), which was noticeable 10 days after induction and pronounced by six weeks (Figure 5A,B). The growth stage of hair follicles, anagen, is initiated by entry into the cell cycle of HF stem cells residing in the bulge region; this occurs synchronously in all HFs at 3 weeks of age (32). We found that the transgenic p14\textsuperscript{ARF} was expressed in bulge cells, identified by K15 expression, and that p53 was concomitantly stabilized in these cells (Figure 5C). Activation of p14\textsuperscript{ARF} at 3 weeks of age blocked the entry of bulge stem cells into the cell cycle and the initiation of anagen, and follicles remained in a telogen-like (resting) state (Figure 5B,D). Silencing of p53 at the time of p14\textsuperscript{ARF} induction allowed normal entry into anagen (Figure S5).

We noted that at two days of activation, despite their high levels of p53 expression, much fewer HF bulge cells underwent apoptosis than other epidermal cells (Figure 5E,F); in contrast, after 2 weeks of induction many bulge cells expressed high levels of endogenous p19\textsuperscript{ARF} (Figure 5G). These stem cells thus display a qualitatively distinct response to p53 from that of other epidermal cells, primarily activating the circuitry of senescence rather than apoptosis. Together, these findings indicate that p14\textsuperscript{ARF} activation prevents HF stem cells from initiating growth through blockage of their proliferative potential.
**p14ARF re-silencing does not allow hair follicle growth**

We next assessed whether the re-silencing of p14ARF would allow HF stem cells to regain function, and initiate anagen. We found that in mice in which p14ARF was activated for 2 weeks and then re-silenced for 4 weeks, only 18% of hair follicles contained proliferating cells in the bulge or the hair germ region below it, in contrast with 66% of follicles in control mice (Figure 6A,B). This indicates that the dysfunction of HF stem cells is largely irreversible. As in the interfollicular epidermis, p53 was detected in bulge cells of transgene-silenced mice (Figure 6C). p19ARF was detectible up to 2 weeks after transgene shutoff (Figure 6D).

To directly test whether transgene re-silencing would allow hair growth, we activated p14ARF for 6 weeks to induce more widespread alopecia, and then re-silenced the transgene for another 4 weeks; this did not result in hair re-growth (Figure 6E). These findings indicate that the majority of bulge cells attain a non-proliferative state that is not reversed after transgene silencing.

**p14ARF activation in skin hyperplasia**

To assess the effects of p14ARF on cells in a pre-neoplastic state we induced epidermal hyperplasia by treatment with the phorbol ester TPA (33, 34). We hypothesized that the competitive advantage of transgene-non-expressing cells may allow hyperplasia to develop also in p14ARF-induced mice, but that senescence formation could be affected by this different tissue context. We treated mice with TPA for four days and then activated p14ARF for two weeks, maintaining TPA treatment. Indeed, hyperplasia occurred in both control and p14ARF-induced mice, manifested by epidermal thickening and high basal layer proliferation rates (Figure 7A-C). Strikingly, in TPA-treated mice, p14ARF induction did not increase the fraction of epidermal SAβGal-positive cells, nor the absolute number of SAβGal-positive cells per field (Figure 7D,E and Figure S6); this, despite the presence of transgene expressing cells (Figure 7F and Figure S6). We also did not detect an increase in SAβGal-positive cells after one week of p14ARF induction in TPA-treated mice (Figure S6). These findings indicate that upon p14ARF...
induction within TPA-induced hyperplasia senescent cells are either very rapidly eliminated or are not formed at all.

TPA is known to induce HF stem cell activation (34); indeed, control TPA-treated mice displayed a dramatic increase in HF growth (Figure 7A). In contrast, HFs of p14ARF-induced mice did not enter anagen but instead developed into thickened epidermal growths containing proliferating cells (Figure 7G,H). K15-positive cells were no longer detected in most follicles, and where present were surrounded by these growths (Figure 7I). Thus, TPA cannot reverse the inactivation of HF stem cells by p14ARF induction, and these cells are mostly lost in this context.
Discussion

Recent studies have convincingly established that cellular senescence is a stress-response program functioning in vivo in diverse physiological contexts, and acting prominently in tumor suppression (2-4). Nevertheless, most basic aspects of this program remain poorly characterized. Here we describe an experimental system that allows the generation and study of senescent cells in tissues of choice, relying on p14ARF, a central activator of this program.

p14ARF induction in the adult epidermis led to p53 activation, and caused apoptosis, growth arrest, and senescence, all seemingly initiated in parallel in different cell subsets. The rapidly occurring stage of apoptosis and proliferation arrest was transient, suggesting tissue adaptation. An above-basal level of apoptosis may persist also during longer transgene induction times, yet may be less apparent, since it does not occur synchronously as upon initial induction. Interestingly, proliferative inhibition was followed by epidermal hyperproliferation, most likely representing a non-cell-autonomous compensatory response to cell loss. Whether this is induced by factors secreted by the p14ARF-expressing cells remains to be studied.

Senescent cells, as defined by SAβGal activity, were first detected only one week after transgene activation. This is consistent with previous reports, which demonstrated that p19ARF and p16Ink4a are activated during keratinocyte senescence and functionally contribute to this process (35-37).

Our finding that exogenous p14ARF expression activates the endogenous p16Ink4a and p19ARF genes reveals a novel positive-feedback loop promoting entry into senescence, and involving both the p53 and Rb pathways. The molecular circuitry mediating this loop, and the potential role played in it by known regulators of the Ink4a locus such as Polycomb, p63 and E2F proteins (5), require further analysis. Our finding that p53 mediates this positive loop stands in contrast to previous studies reporting suppression of p16Ink4a/p19ARF by p53 (38-40). Compensatory activation of these genes in situations of p53 loss may occur through a distinct pathway.
The parameters determining the different outcomes of p53 activation remain largely unknown (28). p14ARF and p53 protein levels varied between epidermal cells, and this variability most likely contributed to outcome determination. However, the resistance of bulge cells to apoptosis indicates that cell identity also plays a central role in choice of outcome.

Our detection of increased p16ink4a and p19ARF levels in some mice early after transgene activation suggests that the initiation of the senescence program occurs rapidly in a subset of cells. We found that continuous p14ARF expression was required for these cells to complete the entry into senescence. This finding has interesting implications: when stress is lifted, damage is repaired, or potentially oncogenic signals are silenced, cells that have already activated the senescence program appear to be able to abort its completion and avert this irreversible fate.

Once formed, many senescent cells were retained in the epidermis for weeks after the silencing of p14ARF. This was surprising due to the high cell turnover in the tissue; senescent basal cells are thus capable of “holding on” to their position within the proliferating compartment. Furthermore, although the numbers of SAβGal- and p53-positive cells did decrease over time, the retention of many of these cells indicates that senescent epidermal cells do not trigger a rapidly-acting efficient system for their removal. This stands in contrast to other previously described settings in vivo, including liver fibrosis and Ras-induced senescence, in which inflammatory cytokines recruit immune cells to rapidly remove senescent cells (13, 21, 22). We did not observe overt inflammation in the skins of p14ARF-activated mice (Figure S4), and only small increases in cytokine expression were detected in the tissue (data not shown). It has been shown that p53 in fact suppresses the senescence associated secretory phenotype (SASP), and that p16ink4a function does not influence it (41, 42). Whether the retention of senescent cells is due to the absence of a SASP or to a mechanism overriding it requires a detailed dissection of the signaling interactions in the tissue.
In the context of TPA-induced hyperplasia, despite the presence of p14<sup>ARF</sup>-expressing cells, we did not detect increased senescence. There are several possible explanations for this interesting finding: TPA could affect keratinocytes in a manner preventing their senescence, increased epidermal cell turnover during hyperplasia could lead to the rapid elimination of transgene-expressing cells prior to their entry into senescence, or a rapid mechanism for directed senescent cell removal could be activated in these conditions, potentially mediated by TPA-induced inflammation (34). Additional study is required to distinguish between these possibilities. Interestingly, we noted increased epidermal thickness in TPA-treated p14<sup>ARF</sup>-expressing mice due to basal-cell expansion (Figure S6), further emphasizing the complex tissue dynamics induced by this transgene.

Hair follicle stem cells have been shown to be resistant to DNA damage-induced apoptosis due to reduced levels of p53 activation (43). Here we show that very little apoptosis of these cells occurs even upon high p53 expression. The resistance to apoptosis thus appears to be an inherent trait of these stem cells. In contrast, p19<sup>ARF</sup> protein levels were abundant in the bulge, indicating that the positive feedback loop activated by p14<sup>ARF</sup> is more powerful than in the interfollicular epidermis. Potentially, the non-proliferating status of these stem cells at telogen contributes to the apparent local accumulation of senescence. The persistence of non-functional HF stem cells in the tissue may mimic hair follicle aging.

Together, our findings provide novel insights into the workings of cellular senescence within the living tissue, and indicate that senescent cells can be retained in organs for prolonged periods, affecting their physiology.
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The authors declare that they have no conflict of interest.
References


Figure Legends

Figure 1. p14\textsuperscript{ARF} induces p53 activation, cell-cycle arrest and apoptosis. A. Diagram of transgenic mouse lines crossed to generate inducible p14\textsuperscript{ARF} expression in the epidermis. B. Top: Immunohistochemical stain of human p14\textsuperscript{ARF} (brown) in the skins of 3 week-old double-transgenic mice (K5-rtTA/tet-p14) and control single-transgene mice (tet-p14) treated with doxycycline (dox) for 2 days (2d). Bottom: Immunofluorescent stain of p14\textsuperscript{ARF} (white) in the same tissues. K14 (green) labels the basal epidermis. DNA (DAPI) is labeled blue in all images. C. Co-stain of p53 (green) and p21 (red) in the same tissues. Merged images are shown on bottom. D. Expression levels of indicated p53 target genes as assessed by qRT-PCR on skins of mice in which p14 was induced for 2 days; graph on right shows expression of apoptosis-associated p53 targets following 14 days of induction. Values indicate mean across individual mice ±S.E.M. E. Stain of the proliferation marker Ki67 in skins of indicated mice, treated with dox for 2 or 7 days. Bottom images show co-stain of BrdU (red) and p14\textsuperscript{ARF} (white) upon 7 days of activation; absence of co-stained cells indicates that proliferating cells do not express the transgene. F. Fraction of Ki67-positive basal epidermal cells following p14\textsuperscript{ARF} activation for the indicated times. Dots indicate individual mice, bars indicate mean across mice ±S.E.M. G. Staining of apoptotic cells in skins of mice treated with dox for 2 days, detected by cleaved Caspase 3 (CC3, top, brown) or TUNEL (bottom, red). H. Fraction of TUNEL-positive epidermal cells in mice treated with dox for the indicated times. * P<0.05, ** P<0.005, t-test. NS – non-significant.

Figure 2. p14\textsuperscript{ARF} induces p53-dependent senescence and activates the endogenous \textit{Ink4a} products. A. SAβGal stain of skin sections from double-transgenic and control mice treated with dox for the indicated times starting at 3 weeks of age (d – day, wk – week). B. Fraction of epidermal SAβGal-positive cells in control and p14\textsuperscript{ARF}-induced mice treated with dox for the indicated times. *
$P<0.05$, ** $P<0.005$. Bottom graph shows values after subtraction of SAβGal-positive fractions in matched control mice (gray dots in top graph), to correct for batch and normal-aging effects. **

**C.** Expression levels of indicated senescence-associated genes in mice induced for p14ARF for the indicated times, assessed by qRT-PCR. **D.** Confocal image of endogenous p19ARF expression (red, punctate) in mice expressing p14ARF for 2 weeks. Red in top image is autofluorescence. **E.** Stain of p14ARF (top) and p53 (bottom) in double transgenic mice expressing the transgene for 2 weeks (left) and in sibling triple-transgenic mice co-expressing an inducible p53 shRNA (tet-shp53). **F.** SAβGal stain of skin sections from the same mice. **G.** Fraction of SAβGal-positive cells in the epidermis of control, double- and triple-transgenic mice. **H.** Expression levels of p19ARF and p16<sub>ink4a</sub> in the skins of the same mice.

**Figure 3. Commitment to senescence requires continuous p14ARF expression.** **A.** Stain of p14ARF (white) in skins of mice in which it was activated for 7 days (7d) or for 4 days followed by 3 days in which dox was removed (4d on + 3d off). **B.** Relative p14ARF expression levels in indicated mice, showing transgene shutoff. **C.** SAβGal stain of skins of indicated mice. **D.** Fraction of SAβGal-positive cells in the epidermis. **E.** Stain of p53 (brown) in skins of the same mice. **F.** Fraction of p53-positive cells in the epidermis. **G.** p19ARF and p16<sub>ink4a</sub> expression levels in the indicated samples. * $P<0.05$, ** $P<0.005$, NS – non significant.

**Figure 4. Senescent cells are retained in the epidermis.** **A.** Stain of p14ARF in skins of mice in which it was activated for 2 or 6 weeks (2, 6wk), and in mice in which it was activated for 2 weeks and then silenced for 4 weeks (2wk on + 4wk off). Control (Cont) mice received dox either for 6 weeks or for 2 weeks followed by 4 weeks without treatment. **B.** Relative p14ARF expression levels in indicated mice.
C. SAβGal stain of skins of indicated mice. D. Fraction of SAβGal-positive cells in the epidermis. E. Stain of p53 in indicated mice. F. Fraction of p53-positive cells in the epidermis, normalized to the mean values of control mice in two independent experiments. G. Expression levels of *p19^ARF* and *p16^{ink4a}* in the indicated samples. * P<0.05, ** P<0.005, NS – non significant.

**Figure 5. p14^ARF causes dysfunction of hair-follicle bulge stem cells.** A. Control and double-transgenic mice following 6 weeks of p14^ARF induction. Shown are two induced mice with different degrees of alopecia. B. Skins of control and double-transgenic mice (hematoxilin and eosin stained) treated with dox for 8 days starting at 3 weeks of age, the time of synchronous entry into anagen. Control mice show large follicles in anagen. C. Left: stain of p14^ARF* (white) in the hair follicle bulge labeled with K15 (red) 2 days after induction. Right: expression of p53 in the bulge (different follicle). D. Stain of Ki67 (white) in 3-week-old mice treated with dox for 4 days. K5 (green) labels the basal epidermis. Follicles of control mice show proliferating bulge cells (arrowhead) and progenitor cells (arrow); p14^ARF*-induced hair follicle stem cells do not express Ki67 and do not give rise to the hair germ. Insets show region of bulge cell proliferation. E. TUNEL stain (red) of mice two days after p14^ARF* induction, demonstrating lack of apoptosis in the bulge (K15, green) and apoptosis in the epidermis. Insets magnify the bulge and the epidermis. F. Fraction of TUNEL-positive cells in K15-positive bulge cells 2 and 7 days after p14^ARF* activation; gray-shaded area shows the apoptosis levels in the epidermis (epi) of the same 2d-induced mice, as shown in Figure 1H. G. Left: expression of endogenous p19^ARF* (red) in the bulge in mice after 2 weeks of p14^ARF* induction; Right: p53 expression (white) and K15 expression (red) in a consecutive section of the same follicle.

**Figure 6. Hair-follicle stem cells remain dysfunctional after p14^ARF shutoff.** A. Ki67 stain of hair follicles of mice in which p14^ARF* was activated for 2 or 6 weeks (2wk, 6wk), or activated for 2 weeks and then re-silenced for 4 weeks (2wk on + 4wk off). Also shown are matched single-transgene
controls. **B.** Fraction of hair follicles containing Ki67-positive cells in the bulge or hair germ. **C.** Stain of p53 (white) in the bulge region of indicated mice. **D.** Stain of endogenous p19\(^{ARF}\) (red) in follicles of mice in which p14\(^{ARF}\) was activated for 2 weeks, and then silenced for 1, 2 or 4 weeks. **E.** Images of mice in which p14\(^{ARF}\) was activated for 6 weeks (left) or activated for 6 weeks followed by 4 weeks in which dox was removed (right). Matched control mice are shown on left in each image. Bottom images show close up magnification of skins (shaved).

**Figure 7.** p14\(^{ARF}\) activation during hyperplasia. **A.** Hematoxilin and eosin (H&E) stained skin sections of TPA-treated (TPA+) or untreated (TPA-) control and p14-induced mice (2 weeks). Top: epidermis, bottom: whole skin. **B.** Ki67 expression in same skins. **C.** Fraction of Ki67-positive cells in basal layer. **D.** SA\(\beta\)Gal stain. **E.** Fraction of SA\(\beta\)Gal-positive epidermal cells. **F.** p14\(^{ARF}\) expression (white) in the epidermis of TPA-treated and untreated p14-induced mice. **G.** H&E stain showing hair follicles in the same mice. **H.** Ki67 stain showing proliferation throughout the follicle of TPA-treated mice, contrasting with lack of Ki67 in the bulge of untreated mice (arrow). **I.** K15-positive cells are eliminated from many follicles in TPA-treated mice; shown is an example of a follicle in which remaining K15 cells are placed within a thickened follicle. Red stain in dermis is non-specific.
Figure 2

A

SA-βGal

B

Control

p14

C

2d

2wk

Relative expression

K5-rtTA / tet-p14

D

2wk

p19

K5-rtTA / tet-p14

E

K5-rtTA / tet-p14

K5-rtTA / tet-p14 / tet-shp53

F

K5-rtTA / tet-p14

K5-rtTA / tet-p14 / tet-shp53

G

SA-βGal-positive fraction

H

Relative expression

Control

p14

p14 + shp53

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Figure 3

A

K14 p14

Cont 7d 4d on + 3d off

B

Relative expression

Cont 7d 4d on + 3d off

C

SA-βGal

Cont 7d 4d on + 3d off

D

SA-βGal positive fraction

Cont 7d 4d on + 3d off

E

p53

Cont 7d 4d on + 3d off

F

p53 positive fraction

Cont 7d 4d on + 3d off

G

p19

Cont 7d 4d on + 3d off

p16

Cont 7d 4d on + 3d off
Figure 4

A) K14 p14

B) Relative expression

C) SA-βGal

D) SA-βGal positive fraction

E) p53

F) p53 positive fraction

G) p19

H) p16

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Figure 5

A  
6wk  
tet-p14  
K5-rtTA / tet-p14

B  
8d  
tet-p14  
K5-rtTA / tet-p14

C  
2d  
K5-rtTA / tet-p14  
K15 p14  
K15 p53

D  
4d  
tet-p14  
K5-rtTA / tet-p14

E  
K5-rtTA / tet-p14  
K15 p14 TUNEL

F  
TUNEL-positive fraction

G  
2wk  
K5-rtTA / tet-p14  
K5 p19  
K15 p53
Figure 6

A

Cont (2wk) 2wk Cont (2+4wk) 6wk 2wk on + 4wk off

K14 K16 Ki67

B

% Follicles with Ki67+ cells in bulge or below

Cont p14 Cont p14 Cont p14

n=3 n=7 n=4 n=7

C

Cont 2wk on 6wk on 2wk on + 4wk off

K14 K15 p53

D

2wk + 1wk off + 2wk off + 4wk off

K5 p19

E

6wk 6wk on + 4wk off

...
Figure 7

A) TPA -
   - tet-p14
   - K5-rtTA/tet-p14

   TPA +
   - tet-p14
   - K5-rtTA/tet-p14

B) Ki67

C) Ki67
   - Ki67 positive fraction of basal cells
   - n=3, n=5, n=3, n=6
   - Cont, p14, Cont, p14
   - TPA -
   - TPA +

D) SA-βGal

E) SA-βGal
   - SA-βGal positive fraction
   - n=3, n=6
   - Cont, p14, Cont, p14
   - TPA -
   - TPA +

F) K5-rtTA/tet-p14
   - TPA -
   - TPA +

G) K14

H) Ki67

I) SA-βGal
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