

Age-associated inflammation connects RAS-induced senescence to stem cell dysfunction and epidermal malignancy

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Aging is the single biggest risk factor for malignant transformation. Among the most common age-associated malignancies are non-melanoma skin cancers, comprising the most common types of human cancer. Here we show that mutant H-Ras activation in mouse epidermis, a frequent event in cutaneous squamous cell carcinoma (SCC), elicits a differential outcome in aged *versus* young mice. Whereas H-Ras activation in the young skin results in hyperplasia that is mainly accompanied by rapid hair growth, H-Ras activation in the aged skin results in more dysplasia and gradual progression to *in situ* SCC. Progression is associated with increased inflammation, pronounced accumulation of immune cells including T cells, macrophages and mast cells as well as excessive cell senescence. We found not only an age-dependent increase in expression of several pro-inflammatory mediators, but also activation of a strong anti-inflammatory response involving enhanced *IL4/IL10* expression and immune skewing toward a Th2 response. In addition, we observed an age-dependent increase in the expression of *Pal1*, encoding an immune suppressive ligand that promotes cancer immune evasion. Moreover, upon switching off oncogenic H-Ras activity, young but not aged skin regenerates successfully, suggesting a failure of the aged epidermal stem cells to repair damaged tissue. Our findings support an age-dependent link between accumulation of senescent cells, immune infiltration and cancer progression, which may contribute to the increased cancer risk associated with old age.

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The convergence between aging and cancer harbors a unique dichotomy: whereas uncontrolled cell proliferation is one of cancer hallmarks, aging is associated with a gradual decay in tissue regeneration and overall compensatory proliferative capacity. Yet, aging remains the strongest single risk factor for cancer development.^{1,2} One example of an age-related cancer is cutaneous squamous cell carcinoma (SCC).³ SCC, the second most common skin cancer after basal cell carcinoma, commonly develops in sun-exposed skin and is prevalent in two main human groups, the elderly and organ transplant recipients.⁴ Both of these are paradigms of reduced immune competence, underscoring the role of the immune system in SCC biology. The immune system has a key role in eliminating incipient tumor cells, a process known as immune surveillance,⁵ demonstrated by the susceptibility of *Tcrδ*^{-/-} mice, lacking $\gamma\delta$ T cells, to DMBA/TPA-induced skin cancer.⁶ As such, the gradual deterioration in immune response with advanced age, also known as immunosenescence,⁷ is one of several theories aiming to explain the convergence between aging and cancer.^{8,9} Adaptive immunity is considered to be more affected by aging than innate immunity, as reflected by the inability of old

individuals to mount an effective humoral response.¹⁰ This is partially due to reduced lymphopoiesis, associated with events such as thymic involution¹¹ and age-dependent skewing of hematopoietic stem cell commitment toward myeloid lineage differentiation.^{12,13} Age-dependent dysfunction of adaptive immunity can also result from intrinsic changes within different lymphocytic lineages. Senescence of immune cells was reported in both CD4⁺ and CD8⁺ lymphocytes,^{14,15} and is mainly characterized by loss of CD28 expression.¹⁶ This outcome is mostly associated with chronic exposure to viral infections like CMV or EBV,¹⁵ but exposure of normal T cells to cancer cells can also induce a senescence-like phenotype in T cells, characterized by loss of CD28 expression and upregulation of p53, p21 and p16.¹⁷

Although the immune system has an important role in preventing tumor growth, it can also promote cancer development and support tumor proliferation, invasion and metastasis.¹⁸ Several adverse conditions characterized by chronic inflammation, such as inflammatory bowel disease,¹⁹ and chronic liver inflammation²⁰ are known risk factors for cancer development. In addition, dermal atrophy accompanied by chronic stromal inflammation, caused by

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Abbreviations: SCC, squamous cell carcinoma; 4-OHT, 4-hydroxytamoxifen; OIS, oncogene-induced senescence; GSEA, gene set enrichment analysis; SA-beta-gal, senescence-associated beta galactosidase; IHC, immunohistochemistry

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mesenchyme-dependent inhibition of Notch signaling, was found to be a driver for multifocal SCC development.²¹ In fact, chronic inflammation promoted by immune cells was recently recognized as one of the cancer hallmarks.²² Increased inflammation can occur in a variety of normal-aged tissues, a phenomenon dubbed inflammaging.²³ This is partly attributed to accumulating senescent cells, observed in aged tissues.²⁴ Such cells secrete a plethora of pro-inflammatory molecules, in a process termed senescence-associated secretory phenotype (SASP).²⁵ This is believed to promote age-related diseases as well as support malignant growth.²⁴ Age-related inflammation is also believed to contribute to the dwindling regenerative capacity of aged adult stem cells.^{12,26} SCC can originate in hair follicle stem cells (HF-SC),²⁷ raising the question whether inflammation can promote the neoplastic transformation of these adult epidermal stem cells.

Here we describe the use of a transgenic mouse strain expressing activated mutant H-Ras in the epidermis to elucidate the differential response of young *versus* aged animals to such oncogenic trigger.

Results

Differential response of aged skin to H-Ras overexpression.

To investigate age-dependent tissue response to oncogenic RAS activation, we utilized mice expressing a conditional ER: H-Ras^{G12V} transgene under regulation of the K14 promoter.²⁸ In these mice, oncogenic H-Ras activity in keratinocytes is regulated reversibly by 4-hydroxytamoxifen (4-OHT). We compared 2–4-month-old mice (young) to 18–22-month-old mice (old). 4-OHT, or ethanol (EtOH) as control, was applied daily on the shaved back skin of the mice. After 1 month of treatment, massive changes were

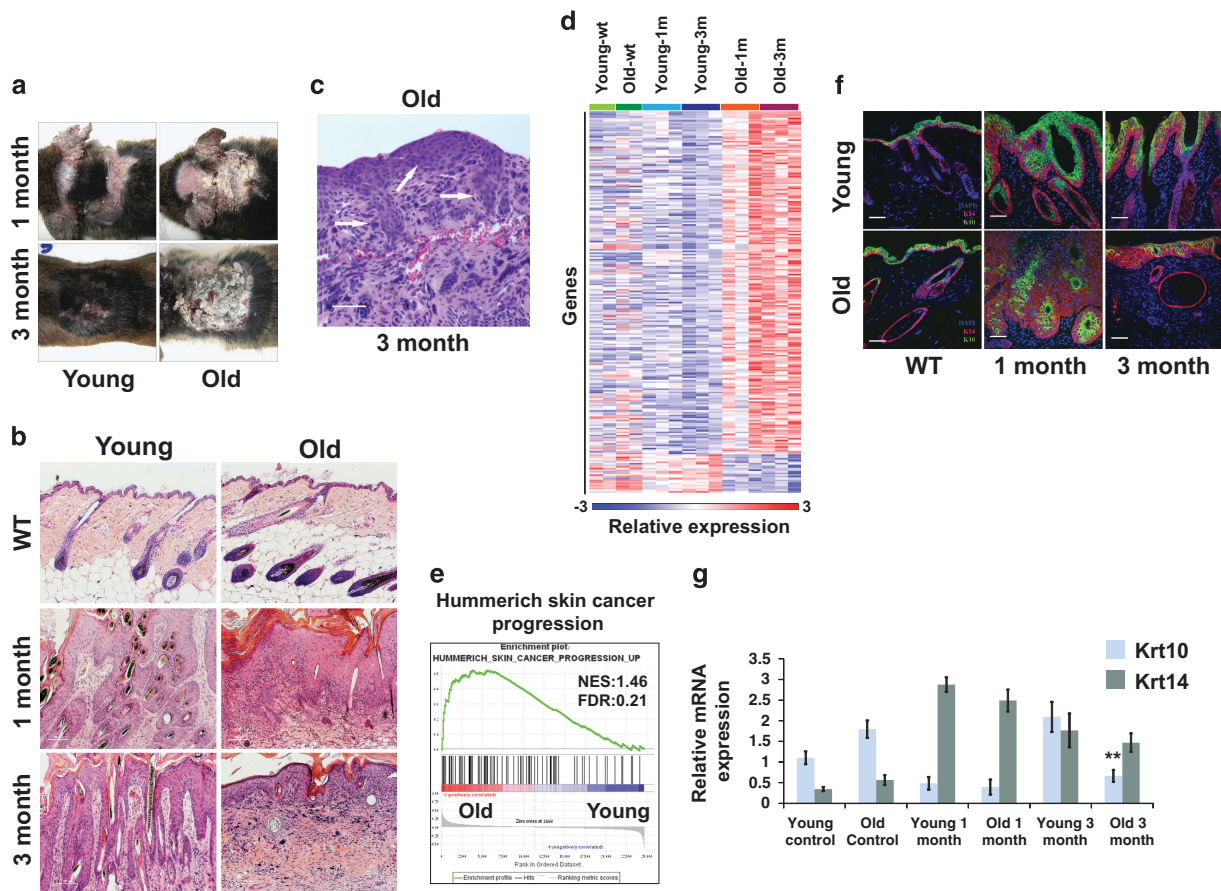


Figure 1 Conditional H-Ras activation in the skin of old but not young mice results in *in situ* SCC. 4-OHT was applied on the shaved back skin of both wt and transgenic mice for the duration of either 1 or 3 months. (a) Representative picture of young and old transgenic mice following 1 and 3 month of H-Ras activation. For the 1 month procedure, a total of 10 mice in two independent experiments were examined. For the 3-month procedure, a total of 14 mice in three independent experiments were examined. (b) H&E staining of sections of the back skin of young and old control wild-type mice (WT, upper) or H-Ras transgenic mice after 1 or 3 months of 4-OHT treatment. (c) Representative image of *in situ* SCC in 3-month-treated-old mice. White arrows indicate full thickness atypia and focal area of invasion. Scale bar = 50 μ m. (d) RNA was extracted from whole skin (epidermis+dermis) and subjected to expression array analysis. Heat map of differential gene expression between WT and H-Ras transgenic young (Y) and old (O) mice, treated with 4-OHT for 1 or 3 months, Fold change > 1.5, *P*-value (permutation test) < 0.05. (e) GSEA plot depicting SCC progression-related genes (MSigDB C2 CGP)³¹ in old *versus* young H-Ras activated mice. (f) Representative immunofluorescence images of back skin sections of WT and H-Ras-transgenic young and old mice treated with 4-OHT for 1 or 3 months, stained with DAPI (blue) and antibodies against K10 (green) and K14 (red). Scale bar = 50 μ m. (g) qRT-PCR analysis of *K10* and *K14* mRNA, amplified from whole skin RNA of the indicated mice. Values represent relative expression normalized to *Gapdh* mRNA \pm SEM; *n* = 3 for WT and *n* = 5, 6, 4 and 6, respectively, for H-Ras activated mice. ***P* < 0.01 (Student's *t*-test)

observed in the 4-OHT-treated skin in both age groups (Figure 1a). In the young mice, rapid hair regrowth was observed as early as 10–14 days from the onset of 4-OHT treatment, yet the skin patches of the old mice remained hairless (Figure 1a). Histological examination revealed markedly acanthotic epidermis together with hyperparakeratosis in both young and old skin. Notably, at variance with young mice, the skin of old mice displayed mild-to-moderate dysplasia; furthermore, extensive inflammation was seen in the old skin when compared with that of young mice (Figure 1b, middle panel). However, no carcinoma was detected in either age group. We therefore prolonged H-Ras activation to 3 months. Owing to the relatively high mortality of old mice under the 1 month protocol (data not shown), the volume of administered 4-OHT was reduced to cover only a smaller patch of skin. The prolonged H-Ras activation revealed pronounced differences between the response of old and young mice. Whereas excessive hair growth was evident in all young mice, no hair regrowth was seen in the old animals. Importantly, development of *in situ* SCC could now be observed in about half of the old mice, but in none of the young animals (Figures 1b and c, white arrows). To determine whether higher levels and/or activity of H-Ras in the old skin might account for the observed differences, we assessed H-Ras activity by IHC staining for phosphorylated ERK (p-ERK). No significant differences were discernible between young and old mice who were treated with 4-OHT for 3 months (Supplementary Figure S1A). In both cases, p-ERK staining was almost exclusively confined to the epidermis (Supplementary Figure S1B), providing further evidence for epidermal-specific H-Ras activation.

To explore the underlying molecular processes, RNA from corresponding skin samples as well as from young and old non-transgenic control mice was subjected to expression array analysis. To identify genes expressed differentially between young and old H-Ras-activated mice, we used the Gene-E comparative marker selection tool (The Broad Institute: <https://www.broadinstitute.org/cancer/software/GENE-E/>). We compared the young *versus* old group, each comprising both the 1- and the 3-month 4-OHT samples together. We selected genes differentially expressed with $P < 0.05$ and fold change above 1.5. Four hundred and sixty genes were deemed differentially expressed between treated young and old mice (Figure 1d). Of those, 395 were upregulated in old skin. Gene set enrichment analysis (GSEA)^{29,30} revealed upregulation in the old skin of a gene set previously described in cutaneous SCC progression (Figure 1e),³¹ lending further support to the conclusion that, in this experimental model, prolonged oncogene activation can progress to a malignant state in old but not young skin.

One trait of high-grade skin dysplasia and SCC is loss of proper skin stratification, which can be visualized by staining of different epidermal layers. To explore a possible differentiation defect, we used two markers of keratinocyte differentiation: keratin 14 (K14), expressed in the basal layer of the epidermis, and keratin 10 (K10), which marks the suprabasal layer (Figure 1f, left). After 1 month of H-Ras activation, overall levels of K14 and K10 were relatively similar in both young and old mice (Figure 1f, middle). Yet, when 4-OHT treatment was extended to 3 months, old mice displayed a significantly higher

K14/K10 ratio relative to the young animals. This included also patches of epidermis lacking almost completely K10 staining, suggestive of full thickness atypia commonly observed in *in situ* SCC (Figure 1f, right). In agreement, *K14* mRNA was elevated by H-Ras activation in both age groups at both time points (Figure 1g), as expected from the observed expansion and thickening of the basal epidermal layer. Surprisingly, although *K10* mRNA was strongly reduced in both age groups after 1 month of treatment (Figure 1g), it recovered by 3 months in the young skin but remained low in the old skin (Figure 1g). Together, these observations suggest that old mice manifest a differentiation defect reminiscent of neoplastic growth.

Oncogene-induced cellular senescence (OIS) is believed to be an important tumor suppressor mechanism.^{32,33} However, cellular senescence is a double-edged sword, which can also promote tumorigenesis through induction of inflammatory factors.³⁴ RAS activation can evoke OIS in cultured keratinocytes³⁵ but little is known about this process *in vivo*. In apparent agreement with the ability of activated RAS to drive OIS, staining for the senescence marker senescence-associated beta galactosidase (SA-beta-gal) confirmed relatively high levels of senescent cells in the skin of 4-OHT-treated transgenic mice (Figure 2a). Surprisingly, the staining was predominantly in the dermis and not in keratinocytes, harboring activated H-Ras. Although dermal SA-beta-gal positive cells were detected in both age groups, they were approximately twofold more abundant in the old skin in both 1-month and 3-month treatments (Figure 2b). mRNA levels of *p16/Cdkn2a*, a CDK inhibitor that enforces senescence via the pRb pathway, were significantly higher in the old mice at both time points (Figure 2c). In agreement, p16 protein levels (determined by IHC) were significantly higher in the dermis of old mice treated for 3 months (Figure 2d and Supplementary Figure S2A), as was also the case for nuclear staining of p21, another cell cycle inhibitor (Figure 2d and Supplementary Figure S2B). Thus, senescent cells accumulate more readily in the stromal compartment of the old skin as a consequence of oncogenic H-Ras activation in the epidermis.

GSEA analysis of mRNA expression data (see Figure 1d) confirmed enrichment for SASP genes in old skin (Figure 2e), based on a previously described set of proteins secreted by cultured human senescent cells.^{36,37} Expression of four SASP genes, *CCR1*, *SPP1*, *IL6* and *IL1 β* , was also validated by reverse transcriptase quantitative real-time PCR (qRT-PCR) (Figure 2f). Expression of all four genes was elevated in both age groups after 1-month treatment, and was only marginally higher in the old group. However, after 3 months of treatment, the old mice displayed significantly higher levels of those mRNAs than the young animals. Of note, IL6 is known to promote tumorigenesis in many settings. It is thus conceivable that the augmented pro-inflammatory pattern may promote the progression of pre-malignant lesions toward cancer in aged animals.

IL4/IL10 pathway activation in aged skin upon H-Ras induction. H-Ras activation elicited a marked accumulation of immune cells in the skin already after 1 month of treatment and more so after 3 months, as evidenced by CD45 immunostaining (Figure 3a). Notably, this accumulation was substantially greater in old skin. Furthermore, GSEA analysis

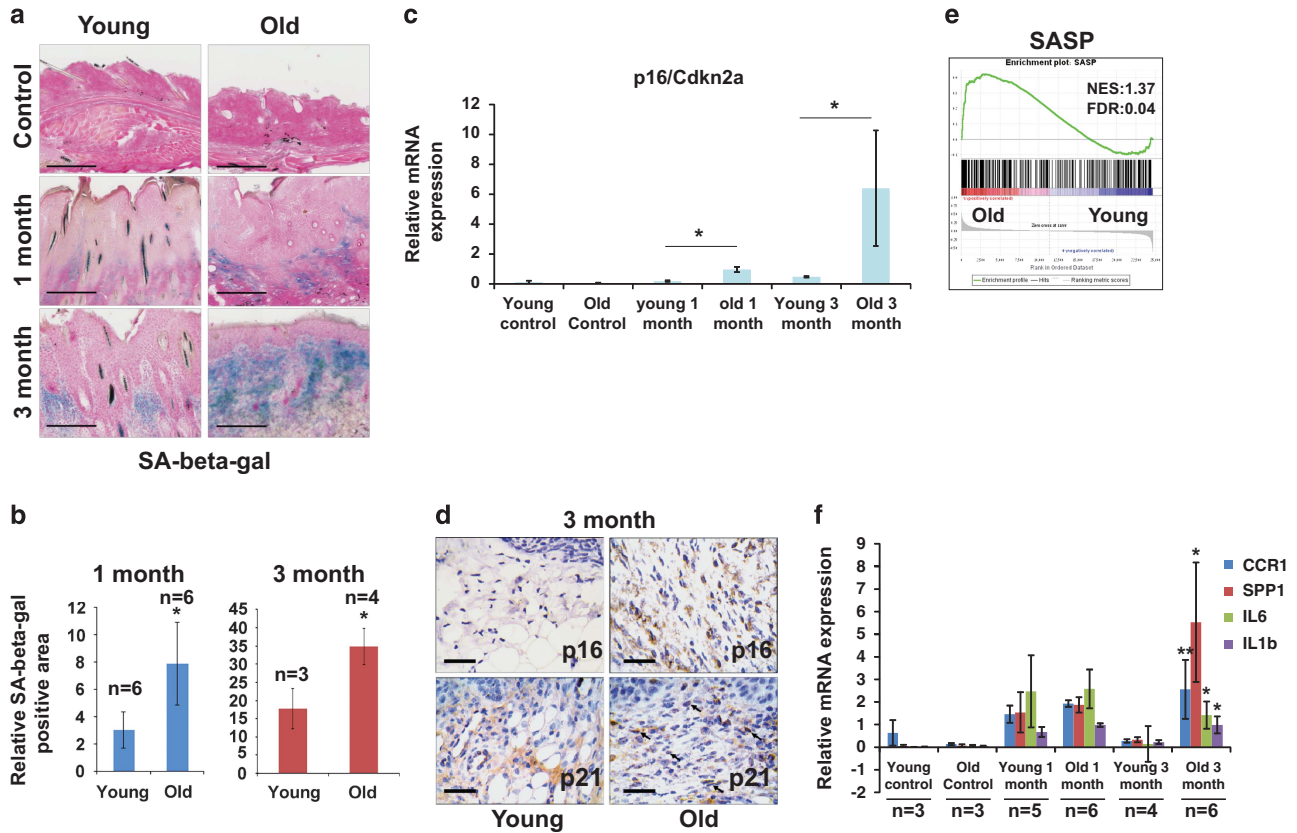


Figure 2 Age-dependent accumulation of senescent cells following H-Ras activation. (a) SA-beta-gal staining (blue) identifies senescent cells in the back skin of young and old WT or H-Ras transgenic mice after 1 or 3 months of 4-OHT treatment. Scale bar = 200 μ m. (b) Quantification of SA-beta-gal staining done with ImageJ (NIH). Values = mean of SA-beta-gal positive area divided by total sample area \pm SD. * P < 0.05. n = number of mice. (c) qRT-PCR analysis of *p16/Cdkn2a* mRNA performed on total skin RNA values represent relative expression normalized to *Gapdh* mRNA \pm SEM; n = 3 for WT and n = 5, 6, 6, respectively, for H-Ras activated samples. * P < 0.05. (d) Representative IHC images of p16 (upper) and p21 (lower). Arrows indicate nuclei with varying degrees of positive p21 staining. Scale bar = 50 μ m. (e) GSEA plot depicting enrichment for SASP-related signature in H-Ras-activated mice. SASP gene set adopted from.^{25,37} (f) qRT-PCR analysis of *CCR1*, *SPP1*, *IL6* and *IL1 β* mRNA in total skin RNA. Values represent relative expression normalized to *Gapdh* mRNA \pm SEM. n = number of mice in each group. * P < 0.05

identified many of the genes upregulated in H-Ras-activated old mice as immune related (Figures 3b–d). Moreover, old skin displayed pronounced TLR signaling and NF- κ B signatures, both implicated in inflammation-driven cancer progression.^{38,39} Indeed, the old H-Ras-challenged skin was markedly enriched for T cells (CD3+, Figure 3e) and macrophages (Figure 3f). NF- κ B is a master regulator of inflammation, whose role in driving tumorigenesis through chronic inflammation is well established.¹⁹ To determine whether NF- κ B might be involved in the inflammatory response observed in the H-Ras-activated skin, we performed IHC for the NF- κ B subunit p65. As seen in Figure 4a and Supplementary Figure S3A, elevated levels of nuclear p65 were present in the old skin relative to young skin, indicating augmented NF- κ B activation. Interestingly, NF- κ B activation was observed in both epidermis and dermis, implying that both epithelial and stromal/immune cells participate in the inflammatory response. Further analysis of the inflammatory response in old skin revealed strong activation of the IL4 and IL10 pathways, both associated with Th2-type immunity (Figures 4b–d). The old mice also displayed elevated expression of *IL10* (Figure 4c, middle panel) and *TNF α* (Figure 4e), both implicated in the Th2 response.^{40,41}

In contrast, young skin displayed elevated expression of *IL20* and several members of the IL20 family, for example, *IL19* and *IL24*, while others like *IL22* were not significantly changed (Figure 4c, lower panel, and Figure 4d). IL4 activity in the skin can elicit a Th2 immune response and inhibit epidermal differentiation via STAT6.^{42,43} Indeed, we observed increased expression of JAK/STAT pathway genes in the treated old mice, including a prominent increase in *Jak3* mRNA (Supplementary Figures S3B and C).

Recruitment of several types of Th2-related innate immunity cells such as macrophages, mast cells and eosinophils can contribute to tumor development and progression.⁴⁴ GSEA analysis confirmed a Th2 expression signature in the old mice (Figure 5a) based on a gene signature identified by Bosco *et al.*⁴⁵ in allergen-induced Th2 cell response implicated in human atopic disease. Furthermore, moderately elevated numbers of CD206-positive cells, indicative of M2-polarized macrophages,⁴⁶ were observed in the dermis and hypodermis of H-Ras-driven skin lesions of the old mice at the 3-month treatment (Figure 5b). Remarkably, pronounced accumulation of mast cells (Toluidine blue positive) was seen in the 3-month treated old skin but to a much lesser extent in young skin (Figure 5c). The augmented presence of mast cells in the aged

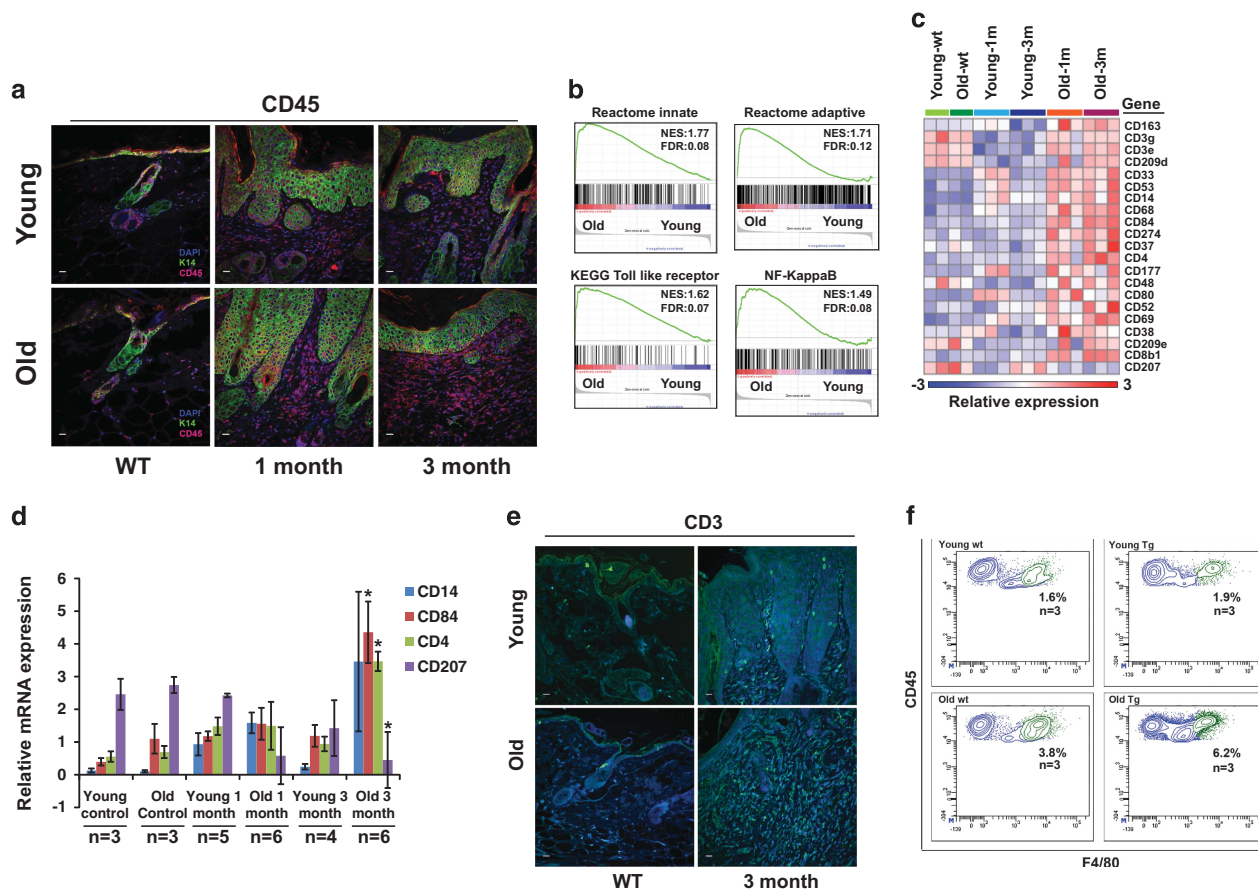


Figure 3 Excessive inflammation in aged skin in response to epidermal H-Ras activation. (a) Immunofluorescence staining as in Figure 1e, except that sections were stained for DAPI (blue), K14 (green) and CD45 (red). Scale bar = 50 μ m. (b) GSEA plot of immune activity in young and old H-Ras-activated mice (MSigDB C2 CP: Reactome, C2 CP: KEGG, C3:TFT). (c) Heat map depicting the differential expression of immune surface markers between WT- and H-Ras-activated young and old mice, fold change > 1.5, P -value (permutation test) < 0.05. (d) qRT-PCR analysis of *CD14*, *CD84*, *CD4* and *CD207* mRNA in total skin RNA. Values represent relative expression normalized to *Gapdh* mRNA \pm SEM. n = number of mice in each group. * P < 0.05. (e) Immunofluorescence staining as in Figure 1e, except that sections were stained for DAPI (blue) and CD3 (green). Scale bar = 50 μ m. (f) Representative FACS analysis of F4/80-positive macrophages extracted from the skin of WT (left) and 3-month 4-OHT-treated transgenic (right) young and old mice. Numbers represent the mean percentage of F4/80-positive cells out of the total CD45-positive cells. n = 3

tissue was also reflected by increased expression of *MCPT2*, encoding a mast cell-specific protease (Figure 5d); the difference between young and old mice was particularly accentuated after prolonged H-Ras activation (Figure 5d). The IL4/IL10 Th2 immune response is suppressive in essence. Although probably mounted to dampen the pro-inflammatory response instigated by oncogene activation in the keratinocytes, it can eventually inflict tissue damage by recruiting ECM modulators such as mast cells and eosinophils. Importantly, the Th2 response can also modulate immune evasion of malignant cells. In particular, *CD274/Pd1*, encoding the PDL1 ligand of the PD1 T cell receptor, can suppress the immune recognition and elimination of numerous types of cancer cells,⁴⁷ including SCC.⁴⁸ Remarkably, expression of *CD274/Pd1* was elevated in old skin after H-Ras activation (Figure 3c, Figures 5e and f and Supplementary Figure S4). This raises the intriguing possibility that augmented immune evasion and suppression of T cell-mediated tumor cell elimination facilitate age-related cancer progression.

The regenerative capacity of old tissues is often compromised. To determine whether this was also true for recovery from oncogene-induced tissue damage, 4-OHT-treated mice

were either killed immediately after 4 weeks of 4-OHT treatment (4 weeks) or allowed to recover for an additional 2 weeks without further 4-OHT treatment (6 weeks). Although young mice showed fast recovery of the skin, including rapid hair regrowth and disappearance of most of the cutaneous aberrations (Figure 6a), regeneration was markedly delayed in old mice. In addition, most of the treated skin remained hairless in the aged mice, suggesting stem cell dysfunction. Histologically, there is almost complete reversal of the skin hyperplasia and hyperkeratosis in the young mice, whereas the aged epithelium remained hyperplastic and inflamed (Figure 6b). After 2 weeks of recovery, numerous Ki67-positive proliferating cells were still evident in both age groups, but were significantly more abundant in the aged, hyperplastic skin (Figure 6c and Supplementary Figure S5A, 6 weeks). Thus, old skin is severely defective in its ability to restore normal tissue homeostasis upon termination of oncogene-induced signaling. The persistent hyperplasia is also reflected by increased accumulation of nuclear p-ERK in the old mice after 4-OHT withdrawal (Supplementary Figure S5B, 6 weeks). The elevated p-ERK signal is evident in the upper layers of the epidermis, suggesting that it is not due to residual K14:H-Ras

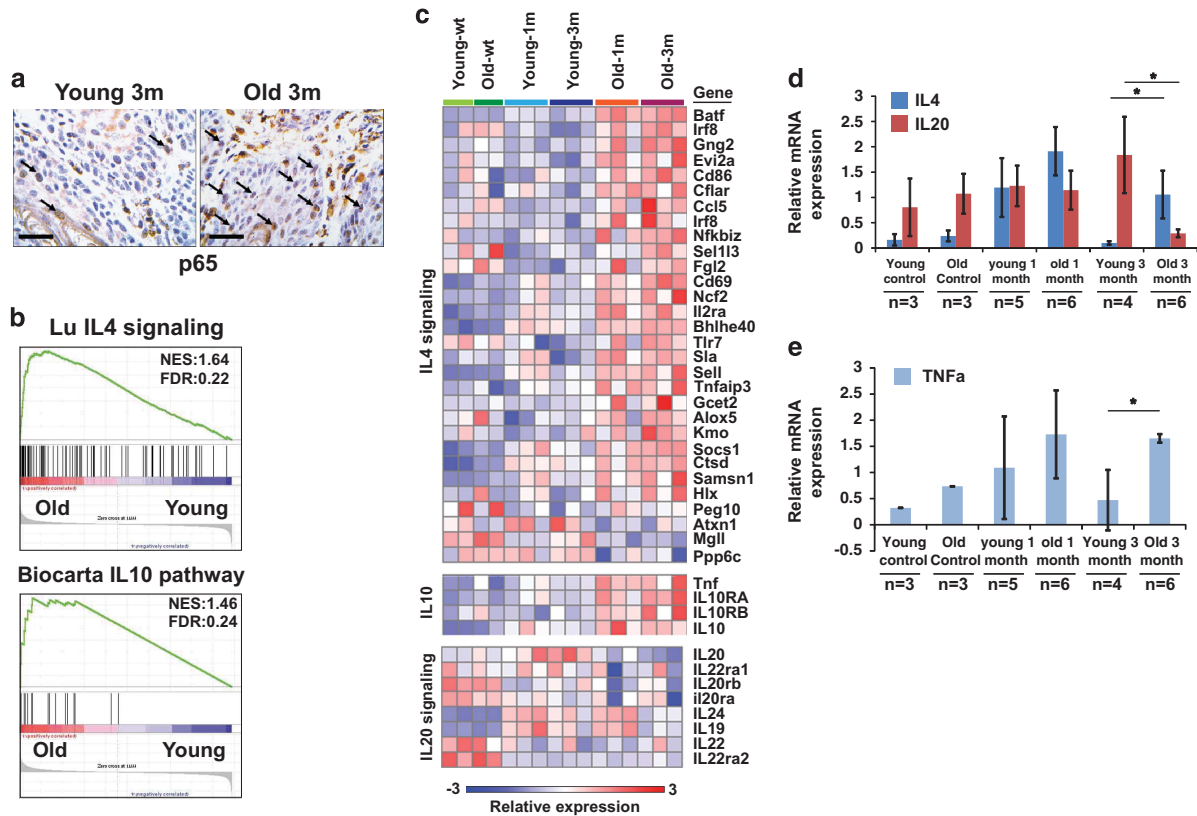


Figure 4 Increased activation of the IL4/IL10 pathway in old skin. (a) Representative IHC images of p65 staining. Arrows indicate nuclear p65 staining. Scale bar = 50 μ m. (b) GSEA plot of IL4/IL10-related signature in young and old H-Ras-activated mice (MSigDB C2 CGP: Lu IL4 signaling, C2 CP:Biocarta IL10). (c) Heat map of differential expression of genes related to the IL4/IL10/IL20 pathways in WT- and H-Ras-activated young and old mice. (d, e) qRT-PCR analysis of IL4, IL20 (d) and TNF α (e) mRNA in total skin RNA. Values represent relative expression normalized to *Gapdh* mRNA \pm SEM. n = number of mice in each group. * P < 0.05 (e)

activity, which is expected to be exerted primarily in the lower, basal layer of the epidermis.

Persistent senescent cells may promote tumor growth and tissue dysfunction. This is presumably why prompt clearance of such cells by the immune system is essential for proper tissue regeneration.⁴⁹ Of note, persistence of senescent cells in immune compromised mice was shown to promote cancer development.⁵⁰ SA-beta-gal analysis (Figure 6d) revealed that whereas dermal senescent cells were hardly detectable in young skin after 2 weeks of recovery, many still persisted at that time in the old mice. Although we cannot exclude the possibility that given a longer recovery period the old mice may eventually achieve full tissue recovery and clearance of senescent cells, our observation suggests that old animals might have a compromised ability to clear senescent cells from their tissues, accompanied by delayed tissue recovery.

The results in Figure 6 suggest that old mice have a defect in skin regeneration after reversible H-Ras activation. Analysis of the expression array data (see Figure 1d) revealed that the major driver of variance in gene expression in this experimental model was H-Ras, accounting for 4046 genes differentially expressed between WT and induced transgenic skin regardless of age (Figure 7a). Of those, 2338 genes were downregulated upon H-Ras activation, whereas 1707 others

were upregulated. HF-SCs are pivotal in skin regeneration upon wound healing; of note, improper wound healing promotes SCC development. To explore possible effects of H-Ras activation on HF-SCs, we compared the H-Ras up- and downregulated genes to a previously described HF-SC signature.⁵¹ Remarkably, almost 20% of the H-Ras downregulated genes overlapped with the HF-SC signature (Figure 7b, upper left). Of particular note, many of those (197 genes) are characteristic of quiescent HF-SC (qHFSC, Figure 7b), whereas only a significantly smaller number⁴⁶ corresponds to active HF-SC (aHF-SC). Moreover, the H-Ras upregulated genes display extensive overlap with transit amplifying signature genes (HF-TAC; Figure 7b, lower right), which is not the case for the H-Ras downregulated genes (Figure 7b, lower left). This effect was also validated by RT-qPCR for representative genes, including *USP18*, *TLE4*, *FGF18*, *LYPD6*, *NPNT*, *LHX2* as well as *KRT15*, whose expression in the skin were strongly downregulated following H-Ras activation for 1 month in both young and old mice (Figure 7c). We further evaluated the HF-SC status following H-Ras activation by staining for two HF-SC markers, keratin 15 (K15) and CD34.⁵² Staining was performed on skin from mice treated for 4 weeks with 4-OHT, followed by 2 weeks of 4-OHT withdrawal (6 weeks). Consistent with the expression

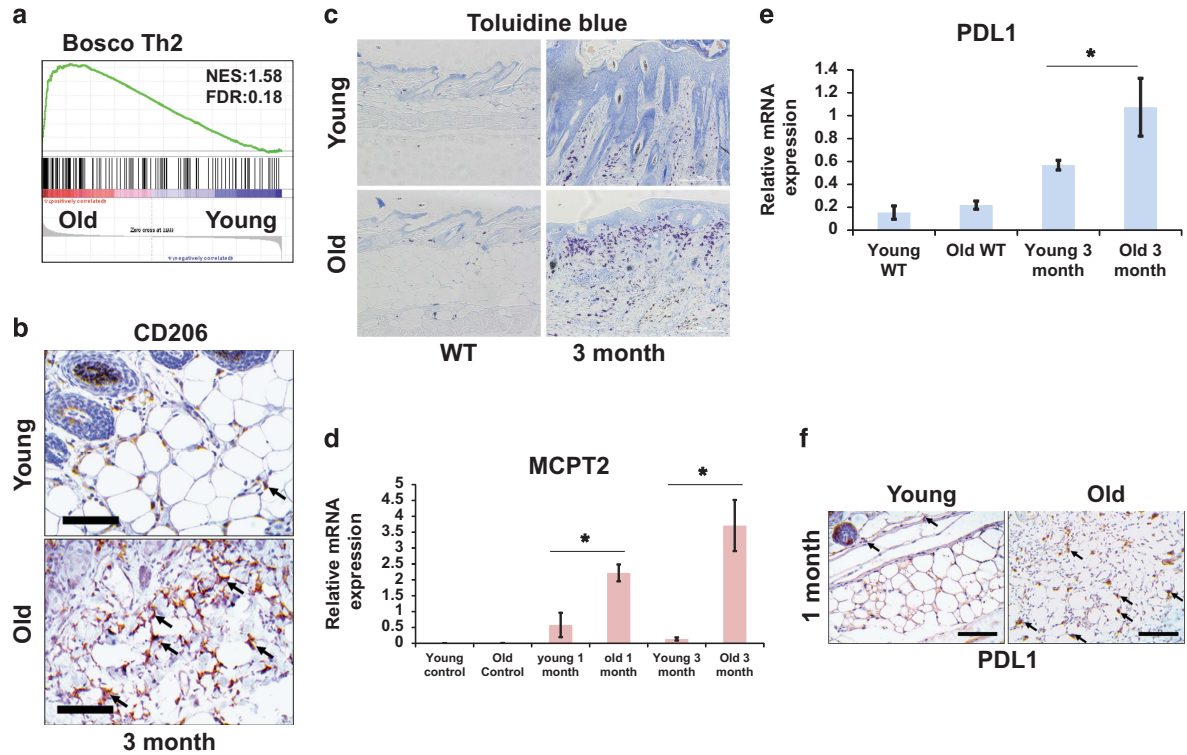


Figure 5 Immune activity in old skin is skewed towards a Th2 response. (a) GSEA plot of Th2-related signature in young and old H-Ras-activated mice (MSigDB C2 CGP: Bosco allergen-induced Th2-associated module). (b) Representative IHC images of CD206 staining. Arrows indicate positive cells. Scale bar = 100 μ m. (c) Toluidine blue staining of back skin sections of WT- and 3-month 4-OHT-treated H-Ras transgenic young and old mice. Scale bar = 100 μ m. (d) qRT-PCR analysis of *MCPT2*. Values represent relative expression normalized to *Gapdh* mRNA \pm SD; $n = 3$ for WT and $n = 4$ for H-Ras-activated samples. $*P < 0.05$. (e) qRT-PCR analysis of *PDL1*. Values represent relative expression normalized to *Gapdh* mRNA \pm SD; $n = 3$ for WT and $n = 15, 14$, respectively, for H-Ras-activated samples. $*P < 0.05$. (f) Representative IHC images of PDL1 staining (arrows). Scale bar = 50 μ m

array results, a marked decrease in K15/CD34-positive cells was observed after 4 weeks of 4-OHT treatment in both age groups (Figure 7d, compare upper panels to middle panel). Remarkably, after 2 weeks of 4-OHT withdrawal, young but not old mice showed considerable numbers of K15/CD34-positive cells (Figure 7d, lower panel). Together, these data suggest that H-Ras activation in the basal epidermis possibly drives HF-SCs differentiation into HF-TAC. Surprisingly, expression of HF-SC-associated genes was markedly recovered by 3 months of H-Ras activation in the skin of young mice (Figure 7c), as observed also after 4-OHT withdrawal (Figure 7d). However, this did not happen in the old mice. Thus, while HF-SC recovery and homeostasis is achieved in young mice, the old animals fail to do so, likely underpinning their defect in skin regeneration.

Discussion

Despite its central role in SCC development,⁵³ activation of H-Ras alone is insufficient to induce invasive carcinoma.⁵⁴ Although other genetic lesions such as p53 mutations are also common in malignant SCC, they are also found in benign lesions that can remain dormant for many years before they develop into SCC.⁵⁵ This raises the question how cells harboring mutations in key oncogenes and tumor suppressors can remain in a state of dormancy for many years, progressing

to cancerous growth only at old age. Here we show that conditional activation of oncogenic H-Ras in the epidermis of aged mice results in *in situ* SCC resembling human Bowen's disease, an established precursor of invasive SCC.⁵⁶ In contrast, the outcome of similar H-Ras activity in young skin results in benign hyperproliferation accompanied by excessive hair growth. We believe that this model better recapitulates the human condition, as most human invasive SCC progress from either actinic keratosis or bowenoid lesions, considered *in situ* or early SCC⁵⁶ and H-Ras mutations can already be found in those lesions.⁵⁷ The pronounced age-dependent differences in neoplastic phenotype are seen in correlation with the level and type of inflammation emerging in the young *versus* old skin. Compared with control skin, both age groups display elevated involvement of both innate and adaptive immunity. However, the immune response in the old skin is far more extensive. Among the most conspicuous phenotypes seen in the H-Ras-activated old skin is increased infiltration of both T and B lymphocytes. B-cells can promote SCC development by enabling recruitment of innate immune cells.⁵⁸ Furthermore, epidermal activation of c-Fos promotes CD4⁺ T-cells recruitment that drives epidermal hyperplasia,⁵⁹ which when combined with a single DMBA treatment gives rise to extremely aggressive tumors.⁵⁹ Moreover, cutaneous infiltration of chronic lymphocytic leukemia predisposes to SCC development,⁶⁰ although CD4⁺ and CD8⁺ T cells can also

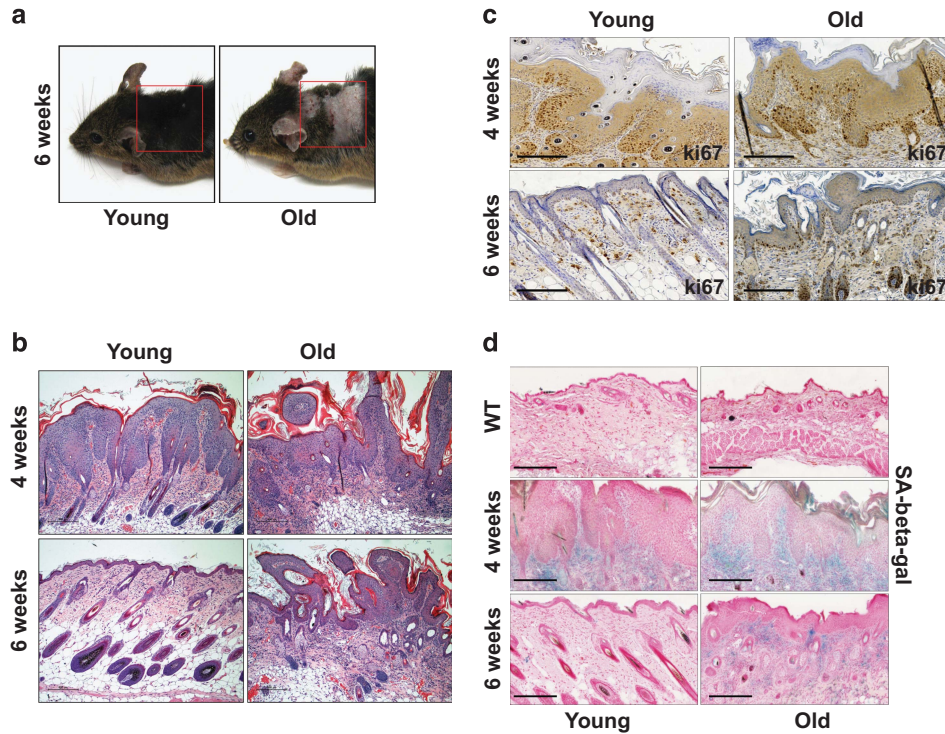


Figure 6 Persistence of senescent cells in aged skin. (a) Representative pictures of young and old transgenic mice 2 weeks after 1 month of 4-OHT treatment followed by 2 weeks of recovery without 4-OHT. Red square marks the region where 4-OHT was applied. (b) H&E staining of sections of the back skin of young and old transgenic mice treated as in (a) and killed either before (4 weeks) or after (6 weeks) 2 weeks of recovery without 4-OHT. Scale bar = 500 μm . (c) Ki67 immunohistochemistry staining of sections prepared as in (b). Scale bar = 200 μm . (d) SA-beta-gal staining (blue) of sections from mice treated as in (b). Scale bar = 200 μm

confer protection against SCC in Notch-deficient mice.⁶¹ Remarkably, even though adaptive immunity is markedly attenuated in old age, we observe massive recruitment of lymphocytes to the old skin following H-Ras activation. The immune response in the old skin is skewed toward a Th2-suppressive mode, including elevated levels of IL10 and IL4 and accumulation of mast cells.⁶² Following TCR activation in aged mice, naive CD4⁺ T cells differentiate toward a Th2 fate.⁶³ Chronic Th2 inflammation is pernicious, as it recruits mast cells and eosinophils, both ECM modulators that can mediate tissue destruction and support tumor growth,^{64,65} although in c-Kit^{-/-} mice mast cells can actually confer tumor protection.⁶⁶ In addition to mast cells and eosinophils, M2-polarized macrophages commonly found in the tumor microenvironment are well documented for their ability to support tumor growth.⁶⁷

Surprisingly, H-Ras activation in the mouse epidermis resulted in massive accumulation of senescent cells in the dermis rather than in the epidermis. It has been proposed that differentiation, not senescence, is the preferred tumor suppressive mechanism in keratinocytes.⁶⁸ This might explain why very little senescence is observed in the epidermis upon H-Ras activation. In contrast, the massive accumulation of senescent cells in the dermis and particularly in the old skin suggests that, following immune recruitment, infiltrating immune cells, possibly lymphocytes, undergo senescence. The rapid clearance of the senescent cells from the skin after termination of H-Ras activity favors the notion that these are indeed immune cells such as lymphocytes, as senescent

epidermal cells were reported to linger in the skin for significantly longer periods.⁶⁹ Senescence of T cells, namely CD4⁺/CD8⁺CD28⁻, is attributed to adaptive immune dysfunction during aging.⁷⁰ Accumulation of CD8⁺CD28⁻ T cells is also quite common in different human cancers, such as lung⁷¹ and colorectal.⁷² Conceivably, infiltrating T cells undergo exhaustion more rapidly in the old skin and so contribute to malignant growth directly via secretion of pro-inflammatory cytokines⁷³ or indirectly by retarding the appropriate immune response to the growing tumor.¹⁷ Moreover, the fact that many senescent cells persist in the old skin even 2 weeks after termination of H-Ras activity might suggest that in aging tissue senescent cells are fast to accumulate but slow to be eliminated, contributing to aging-related pathologies and cancer development. The immune system has a crucial role in clearing senescent cells from damaged tissue.^{50,74} Hence, the diminished competence of the immune system in the aged might be responsible for the accumulation and persistence of senescent cells.

As age-related inflammation is inhibitory to HF-SC function,²⁶ the accumulation of senescent cells that secrete pro-inflammatory cytokines might also contribute to stem cell dysfunction in old tissues. Furthermore, as HF-SC are believed to be among the cells of origin for SCC,⁵⁴ their exposure to chronic inflammation originating from a senescent microenvironment might not only impede their function but also confer neoplastic growth.

Our findings expand our understanding of cancer development in the aged tissue, which is very pertinent to human

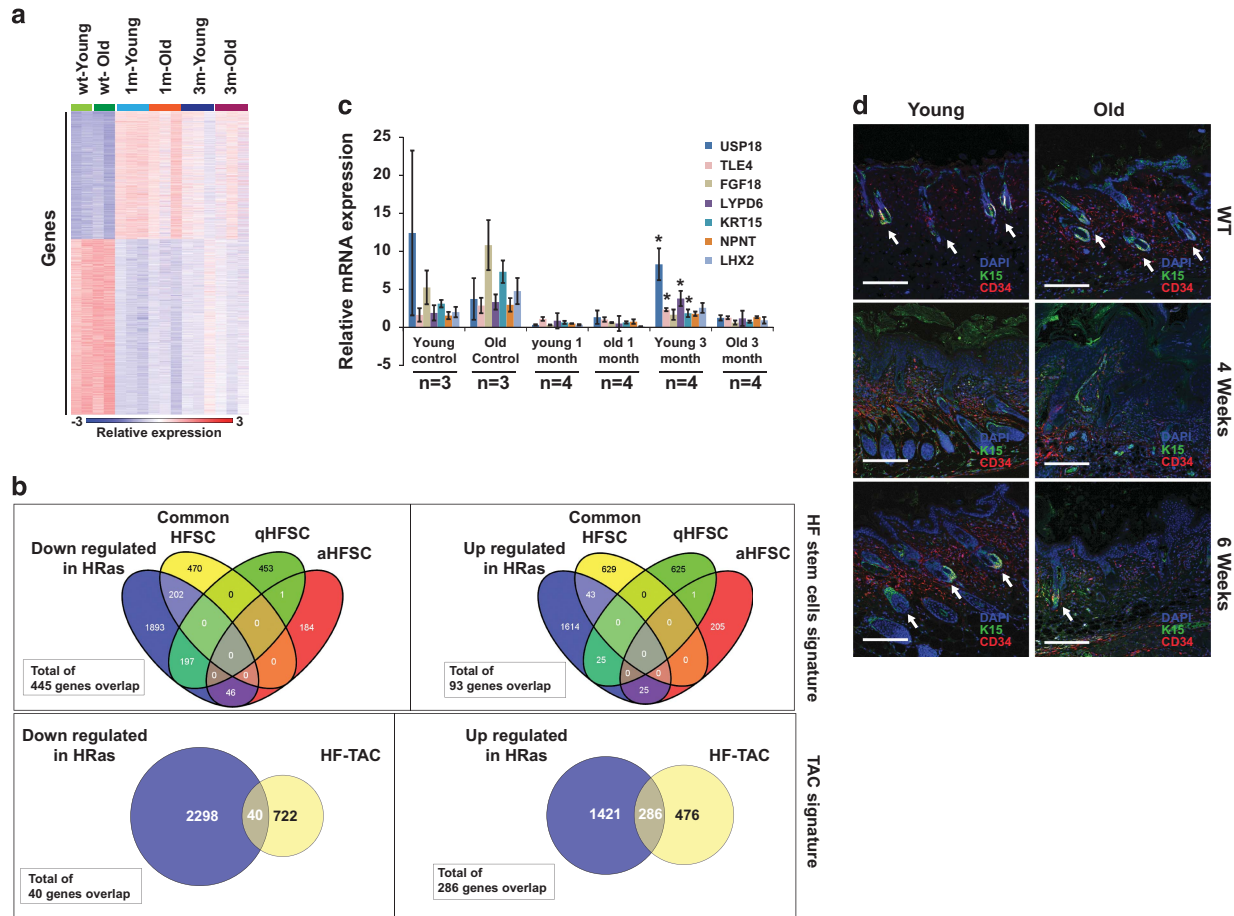


Figure 7 H-Ras activation downregulates many HF-SC-related genes. **(a)** Heat map of differential gene expression in the skin of WT and H-Ras transgenic young (Y) and old (O) mice treated with 4-OHT for 1 or 3 months. Fold change > 1.5 , P -value (permutation test) < 0.05 . **(b)** Venn diagram showing the overlap of genes down- or upregulated in response to H-Ras activation, compared with previously defined HF-SC and TAC gene signatures.⁵¹ **(c)** qRT-PCR analysis of *USP18*, *TLE4*, *FGF18*, *LYPD6*, *KRT15*, *NPNT* and *LHX2*. Values represent relative expression normalized to *Gapdh* mRNA \pm SEM. n = number of mice. $*P < 0.05$. **(d)** Representative immunofluorescence images of back skin sections from WT and H-Ras-transgenic young and old mice treated with 4-OHT for 4 weeks (4 weeks) and after two additional weeks of 4-OHT withdrawal (6 weeks), stained for K15 (green), CD34 (red) and DAPI (blue). Scale bar = 20 μ m

cancer. These findings suggest that aged malignant progression is associated with a conflicting inflammatory response, which on the one hand can facilitate tumor growth and invasion, whereas on the other hand suppressing tumor immune surveillance. Elaborating the dialog between tumor cells and the aged microenvironment might provide clues toward more efficient targeting of the processes that contribute to age-related malignancy.

Materials and Methods

Animals. All mouse experiments were approved by the institutional animal care and use committee (IACUC number 06021211-2) of the Weizmann Institute. 129S. B6-Tg(KRT14-Esr1/H-Ras)1Pkha/J transgenic mice²⁸ were purchased from Jackson Laboratories (Bar Harbor, ME, USA). 4-OHT (Sigma Aldrich Israel, Rehovot, Israel), dissolved in EtOH, was applied topically on the shaved back skin of transgenic mice, once a day, for 1 or 3 months.

Isolation of total RNA and qRT-PCR. Total RNA was isolated with NucleoSpin RNA II kit (Macherey-Nagel, GmbH&Co.KG, Düren, Germany). In all, 1.5 μ g aliquots of RNA were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Madison, WI, USA) and random hexamer primers (Amersham, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). RT-qPCR was performed using SYBR Green Master Mix (Applied Biosystems,

Foster City, CA, USA) in a StepOnePlus instrument (Applied Biosystems). A list of primers used in this study is provided in the Supplementary Material and methods.

Microarray hybridization and analysis. For expression microarray analysis, RNA was extracted as described above. In total, 1 μ g of RNA was used to prepare cDNA that was labeled and hybridized to Affymetrix GeneChip Mouse Gene 2.0 ST arrays. For analysis, the Affymetrix Console was used to normalize all arrays together using the robust multiarray average (RMA) algorithm. Differential gene expression was calculated using gene marker selection, Gene-E (Broad Institute). GSEA was performed using the GSEA tool.^{29,30}

For histopathological analysis, immunohistochemistry and SA- β -gal staining see Supplementary materials and methods.

Conflict of Interest

The authors declare no conflict of interest.

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