Pleiotropic age-dependent effects of mitochondrial dysfunction on epidermal stem cells

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Tissue homeostasis declines with age partly because stem/progenitor cells fail to self-renew or differentiate. Because mitochondrial damage can accelerate aging, we tested the hypothesis that mitochondrial dysfunction impairs stem cell renewal or function. We developed a mouse model, *Tg(KRT14-cre/Esr1)$^{20Efu/J}$* × *Sod2^{tm1Smel}*, that generates mitochondrial oxidative stress in keratin 14-expressing epidermal stem/progenitor cells in a temporally controlled manner owing to deletion of *Sod2*, a nuclear gene that encodes the mitochondrial antioxidant enzyme superoxide dismutase 2 (Sod2). Epidermal *Sod2* loss induced cellular senescence, which irreversibly arrested proliferation in a fraction of keratinocytes. Surprisingly, in young mice, *Sod2* deficiency accelerated wound closure, increasing epidermal differentiation and reepithelialization, despite the reduced proliferation. In contrast, at older ages, *Sod2* deficiency delayed wound closure and reduced epidermal thickness, accompanied by epidermal stem cell exhaustion. In young mice, *Sod2* deficiency accelerated epidermal thinning in response to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, phenocopying the reduced regeneration of older *Sod2*-deficient skin. Our results show a surprising beneficial effect of mitochondrial dysfunction at young ages, provide a potential mechanism for the decline in epidermal regeneration at older ages, and identify a previously unidentified age-dependent role for mitochondria in skin quality and wound closure.

cellular senescence | oxidative stress | skin aging | stem cell proliferation | superoxide dismutase 2

Stem and progenitor cells are crucial for tissue homeostasis, repair, and regeneration. In response to injury, they proliferate and differentiate to replace damaged or dysfunctional cells (1, 2). In the skin, epidermal basal cells differentiate to form distinct epidermal layers: the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC) (3). In the SB layer, epidermal basal cells are identified by nuclei that stain strongly with hematoxylin and eosin (H&E). These cells differentiate to form the SS layer, composed mainly of cells with lightly stained nuclei. SS cells differentiate into the SG layer, identified by cells with prominent cytoplasmic granules, which terminally differentiate to form the SC layer containing acidophilic anucleated cells.

Tissue homeostasis declines with age partly because stem/progenitor cells fail to self-renew or differentiate (4). Oxidative damage can contribute to this decline in compartments such as the hematopoietic system (5, 6). Aging is caused by intrinsic and extrinsic factors that cooperate to drive aging phenotypes (7). Mitochondrial dysfunction has been suggested to play a major role in intrinsic aging (8). Furthermore, mitochondrial damage is associated with extrinsic aging, particularly ultraviolet (UV) radiation-induced photoaging in the skin (9). Thus, mitochondrial damage may be a common link between intrinsic and extrinsic aging.

Mitochondrial stress can decrease life span and health span. Superoxide dismutase 2 (SOD2) scavenges mitochondrial superoxide to protect against oxidative damage. *Sod2* deficiency decreases life span in several species. Mice with constitutive *Sod2* deficiency are neonatally lethal on multiple genetic backgrounds, presenting with neurodegeneration, spongiform encephalopathy, cardiomyopathy, hepatic fat accumulation, and failure to thrive; cells from these mice exhibit impaired spare respiratory capacity, genomic instability, and mitochondrial functional defects (10–17). *Sod2*$^{-/-}$ cells also have a reduced proliferative capacity (17), consistent with the finding that constitutive *Sod2* deficiency induces cellular senescence, a tumor-suppressive mechanism that irreversibly arrests cell proliferation (18), in mouse skin (19). Conversely, overexpression of mitochondrial antioxidants can partly rescue age-related pathologies (14, 20), increase organismal life span (21), and prolong stem cell replicative life span (22).

Interestingly, some studies have suggested that mild mitochondrial stress can be beneficial (23). Here we show that mitochondrial stress owing to *Sod2* deficiency in epidermal cells can have positive or negative effects on skin regeneration, and that these effects depend on age. We show that epidermal *Sod2* deficiency induces cellular senescence, which reduces proliferative capacity in the skin but stimulates the differentiation of epidermal stem/progenitor cells. This stimulation accelerates wound closure in young mice, but the proliferative decline drains stem cell pools with aging and retards wound closure. Our findings extend the concept of antagonistic pleiotropy, which stipulates that gene action can be beneficial at young ages but deleterious at older ages, to mitochondrial function in the skin.

Results

Mice with a Keratinocyte-Specific Mitochondrial Defect. Because constitutive *Sod2*$^{-}$ deficiency is neonatal lethal (12), it is not possible to study age-specific effects in these mice. Consequently, we constructed an inducible, temporally regulated, tissue-specific mouse model of *Sod2* deficiency. We created mice carrying a *Sod2* gene into which we inserted *LoxP* sites (*Sod2^{tm1Smel}*) into sequences flanking a region required for catalytic activity. We then crossed these animals to mice carrying a tamoxifen (TAM)-activated

Significance

Mitochondrial damage can accelerate features of aging, including impaired tissue regeneration, but little is known about how aging and this damage interact to impair tissue renewal. We show that mitochondrial oxidative stress in the epidermis alters wound healing depending on age. Epidermal mitochondrial damage accelerated wound closure in young mice; however, in older mice, this damage limited epidermal cell proliferation and reduced epidermal stem cell numbers, leading to delayed wound closure. Our findings uncover a surprising beneficial effect of mitochondrial dysfunction at young age (accelerated wound closure), and a potential mechanism for the reduced epidermal regeneration at older ages (stem cell depletion).

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Cre recombinase (Cre-ERT) under control of the keratinocyte-specific keratin 14 promoter. This mouse, *Tg(KRT14-cre/Esr1)*$^{20Efu/J}$ *x Sod2*^{tm1Smel}, herein designated *K14S*, allows deletion of critical *Sod2* sequences in keratinocytes after TAM treatment. TAM, but not estrogen, causes nuclear translocation of Cre-ERT, a fusion protein comprised of Cre and a mutant estrogen receptor ligand-binding domain, thus allowing Cre to excise sequences between the *LoxP* sites (Fig. S1*A*). Because the *K14* promoter is active primarily in epidermal stem/progenitor cells (24, 25), TAM deletes *Sod2* mainly in epidermal, but not dermal, cells (Fig. S1 *B* and *C*). As expected, TAM deleted *Sod2* in dorsal skin and skin-containing tails and toes, but not in the heart, liver, intestine, or lungs, of *K14S* mice (Fig. S1*D*). TAM did not delete *Sod2* in *Sod2*^{tm1Smel} mice (designated *S*), which lack *Cre* recombinase (Fig. S1*D*).

We measured the copy numbers of recombined and total *Sod2* alleles by quantitative PCR (qPCR) (Fig. S1*E*). After TAM treatment, >92% of *Sod2* genes showed a deletion in the epidermis, but not the dermis, of *K14S* mice. No recombination occurred in the epidermis or dermis of *Tg(KRT14-cre/Esr1)*$^{20Efu/J}$ × B6-*Gt(ROSA)26Sor*^{tm1Sor}/J mice (designated *K14R*), which lack *LoxP* sites. Accordingly, qPCR showed that *Sod2* mRNA levels were substantially lower in the epidermis (Fig. S1*F*), but not the dermis (Fig. S1*G*), of TAM-treated, but not vehicle-treated, *K14S* mice. As expected, *Sod2* mRNA levels were comparably high in the epidermis and dermis of vehicle- and TAM-treated *K14R* mice. This finding indicates that TAM reduced *Sod2* expression in epidermal cells of *K14S* mice, confirming successful generation of an inducible keratinocyte-specific *Sod2*-deficient mouse and overcoming the impediment of neonatal lethality caused by constitutive *Sod2* deficiency.

To determine the SOD2 expression pattern in skin, we used immunohistochemistry on whole mounts of tail epidermis from vehicle- or TAM-treated *K14S* mice, counterstaining for CD49f, a keratinocyte marker to visualize epidermal compartments. SOD2 was detectable in the sebaceous glands and epidermal layers, with highest expression in the middle of the hair follicle or isthmus region (Fig. 1*A*) in which stem/progenitor cells reside (26). Cells in the bulb area expressed lower SOD2 levels. TAM reduced epidermal SOD2 levels markedly, consistent with *Sod2* deletion in the targeted tissue.

Constitutive *Sod2* loss in mice severely reduces mitochondrial complex II activity, but not complex IV activity, and decreases mitochondrial spare respiratory capacity without altering the basal respiration rate (12, 19). To determine whether keratinocyte-specific *Sod2* loss has similar effects, we stained *K14S* skin for succinate dehydrogenase (SDH) (complex II) and cytochrome *c* oxidase (COX) (complex IV) activities. TAM-treated, but not vehicle-treated, *K14S* mice had significantly less SDH activity in the hair follicles, but not in the underlying skeletal muscle (Fig. 1*B* and Fig. S1*I*). As expected (19), mitochondrial complex IV (COX) activity was similar in the TAM- and vehicle-treated *K14S* mice (Fig. S1 *H* and *I*). These results confirm that the mitochondrial dysfunction was confined to epidermal keratinocytes in TAM-treated *K14S* mice.

Epidermal *Sod2* deficiency did not significantly increase morbidity in all cohorts tested up to ∼16 mo after TAM treatment of 4-mo-old mice. Thus, epidermal deletion of *Sod2* did not noticeably compromise the health of mice up to 20 mo of age.

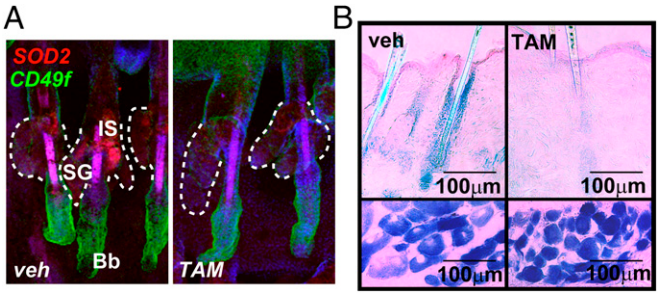

Fig. 1. Keratinocyte-specific mitochondrial dysfunction in mice. (*A*) Coimmunostaining for SOD2 (red) and the keratinocyte marker CD49f (green) and nuclear staining by DAPI (blue) on tail whole mounts of 2-y-old *K14S* mice treated with (*Left*) corn oil (vehicle; veh) or (*Right*) tamoxifen (TAM) at age 4 mo. (*B*) Representative photomicrographs of skin of 8-mo-old *K14S* mice treated with veh or TAM at age 4 mo, stained for succinate dehydrogenase (SDH; blue). (*Upper*) Hair follicles outlined by dashed lines. (*Lower*) Skeletal muscle beneath the SC layer.

Epidermal *Sod2* Deficiency Accelerates Wound Closure in Young Mice. To determine the consequences of epidermal *Sod2* deficiency, we assessed vehicle- and TAM-treated *K14S* mice for wound healing after a skin biopsy. We treated 4-mo-old mice, and assessed wound healing 4 mo later (at 8 mo of age). Surprisingly, TAM-treated, but not vehicle-treated, mice showed accelerated wound closure (Fig. 2*A*). TAM-treated mice also had an unusually thick layer of epidermal cells near the wound edges (Fig. 2*B*), but no difference in collagen formation (Fig. S2*A*), suggesting rapid epithelialization. Indeed, the number of epidermal cells near the wound edges was greater in TAM-treated *K14S* mice compared with vehicle-treated *K14S* mice. There was a trend toward a lower percentage of proliferating cells in the wound areas of TAM-treated mice, but this difference was not statistically significant (Fig. 2*C* and Fig. S2 *B* and *C*).

To explore the mechanism behind the accelerated wound closure in young mice, we isolated RNA from wound areas of vehicle- and TAM-treated *K14S* mice at 2 and 6 d after injury, and quantified mRNA levels of genes associated with cell proliferation, differentiation, and inflammation. Genes associated with cell cycle progression (*Ccna2*, *Ccnb1*, and *Ccnd1*, encoding cyclins A2, B1, and D1), and growth inhibition (*Lrig1*, encoding leucine-rich repeats and immunolobin-like 1 domains) (27), were expressed at similar levels in vehicle- and TAM-treated *K14S* wounds (Fig. S2*D*), consistent with comparable PCNA staining (Fig. 2*C* and Fig. S2*B*). Genes expressed predominantly in epidermal basal cells, such as delta-like 1 (*Dll1*) and integrin-alpha 6 (*Itga6*), were also at comparable expression levels (Fig. S2*D*). Genes associated with epidermal stem/progenitor cells, such as leucine-rich repeat-containing G protein-coupled receptor 6 (*Lgr6*) (28), and with hyperproliferation, such as keratin 6B (*Krt6b*) (29), showed significantly lower expression in TAM-treated *K14S* mice at 2 d after wounding (Fig. 2*D*). Thus, it is unlikely that increased proliferation is responsible for the accelerated wound closure.

We also assessed apoptosis in the wound sites. Both cohorts had few TUNEL-positive cells, suggesting that apoptosis does not contribute to the difference in wound closure (Fig. S2*E*). Finally, because transient inflammation is important for wound healing (30), we assessed mRNA levels of genes encoding the proinflammatory cytokines IL-1α (*Il1a*), IL-1β (*Il1b*), and tumor necrosis factor (*Tnf*), which remained similar in vehicle- and TAM-treated *K14S* wounds (Fig. S3*A*). Thus, epidermal loss of *Sod2* does not have a significant affect on the inflammatory response during wound healing.

Increased Epidermal Differentiation in Young Mice. In contrast to proliferation- and inflammation-associated genes, genes associated with differentiation, expressed predominantly in suprabasal cells, were elevated at wound sites of TAM-treated *K14S* mice (Fig. 2*E*). These genes include loricrin (*Lor*), S100 calcium-binding protein A3 (*S100a3*), and, to a lesser extent, Krüppel-like factor 9 (*Klf9*), keratin 10 (*Krt10*), and CD36, suggesting that epidermal *Sod2* deficiency enhances keratinocyte differentiation. Therefore, we examined the healing wounds by histology. At 6 d after injury,

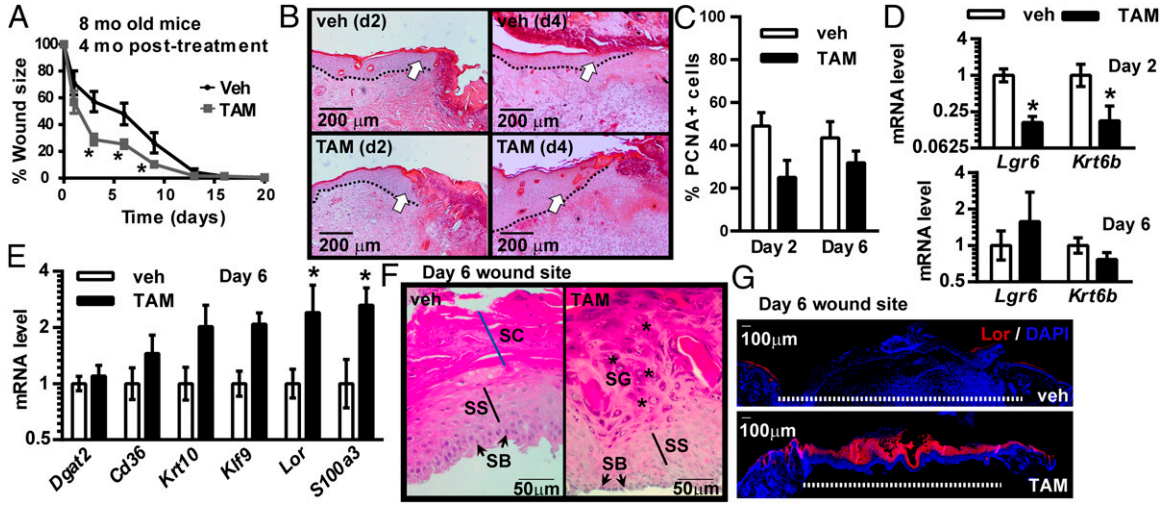

Fig. 2. Wound closure in 8 mo old *K14S* mice treated with veh or TAM at age 4 mo. (*A*) Average wound size (mean ± SEM) after 8-mm punch biopsy in *K14S* mice (veh, $n = 11$; TAM, $n = 8$). Percent wound size refers to the wound area relative to the initial wound area × 100. Asterisks indicate differences at $P < 0.05$ by Student's *t* test. (*B*) Representative photomicrographs of H&E staining of wound areas in *K14S* mice at 2 d and 4 d after injury ($n = 3$ each). Layers above the dashed lines indicate the epidermis. White arrows show area of reepithelialization. (*C*) Quantification of PCNA immunofluorescence in wound areas of *K14S* mice at 2 d ($n = 3$ each) and 6 d ($n = 5$ each) after skin injury. (*D* and *E*) mRNA levels determined by qPCR of genes associated with proliferation (*D*), expressed predominantly in epidermal basal cells, and differentiation (*E*), expressed predominantly in epidermal suprabasal cells, in wound areas of *K14S* mice at 2 d (veh and TAM, $n = 3$) or 6 d (veh, $n = 5$; TAM, $n = 7$) after injury. Mean ± SEM values with asterisks indicate differences at $P < 0.05$ by two-way ANOVA followed by Bonferroni post hoc analysis. (*F*) Representative photomicrographs of H&E staining of wound areas of *K14S* mice at 6 d after injury (veh and TAM, $n = 5$). Stratum basale (SB; arrows) cells are identified by strong nuclear staining (blue) at the wound bottom. Stratum spinosum (SS; black line) are cells with low nuclear staining above the SB layer. Stratum granulosum (SG; asterisks) are cells with granules in the cytoplasm. Stratum corneum (SC; blue line) is the outermost layer. (*G*) Representative photomicrographs of loricrin (LOR; red) staining by immunofluorescence and nuclear staining by DAPI (blue) in wound areas of *K14S* mice at 6 d after injury. Dashed lines indicate the wound site. Multiple photomicrographs of wound sections were taken under 10× magnification and tiled in Adobe Photoshop.

wounds in vehicle-treated mice had numerous cells in the SB layer and a negligible SG layer (Fig. 2*F* and Fig. S3*B*). In contrast, wounds in TAM-treated mice had few cells in the SB layer and a substantial SG layer (Fig. 2*F* and Fig. S3*B*). There were no apparent differences in the SC and SS layers.

To confirm the prominent SG layer in TAM-treated *K14S* mice, we immunostained wounds for loricrin (LOR), which was more prominent in the TAM-treated animals (Fig. 2*G*). These wounds also contained higher *Lor* mRNA levels (Fig. 2*E*). Because SB cells differentiate into SS and SG layers, the increased SG layer in wounds of TAM-treated *K14S* mice suggests that *Sod2* deficiency accelerates epidermal differentiation during wound healing. This acceleration was transient; at 10 d after wounding, epidermal stratification was similar in the TAM- and vehicle-treated mice (Fig. S3*C*). Thus, increased differentiation, rather than cell proliferation, appears to drive the more rapid wound closure in young TAM-treated *K14S* mice.

Delayed Wound Closure and Rapid Epidermal Thinning in Old Mice. The accelerated wound closure in young keratinocyte-specific *Sod2*-deficient mice suggests that mitochondrial dysfunction improves skin repair, contradicting the free radical theory of aging. To explore this possibility, we monitored wound closure in older (age 11 and 14 mo) mice treated with TAM or vehicle at age 4 mo. Although keratinocyte-specific *Sod2* deficiency accelerated wound closure in young mice (Fig. 2*A*), this acceleration was lost in 11-mo-old mice (Fig. S4*A*). Furthermore, *Sod2* deficiency delayed wound closure in 14-mo-old mice (Fig. 3*A*). Thus, the *Sod2* deficiency promoted epidermal differentiation and wound closure in young mice, but delayed wound closure in older mice. We also monitored epidermal thickness, which declines with age in humans and mice (31, 32). We treated *K14S* mice with vehicle or TAM at age 4 mo, and measured epidermal thickness 4, 7, and 10 mo later (at age 8, 11, and 14 mo). Vehicle-treated mice showed a significant decline in epidermal thickness at 10 mo after treatment, whereas TAM-treated animals exhibited this decline at 7 mo after treatment (Fig. 3*B* and Fig. S4*B*). Thus, epidermal *Sod2* deficiency accelerated age-associated thinning of the epidermis.

Depletion of Epidermal Stem Cells in Old Mice. One possible reason for the phenotypes of older TAM-treated *K14S* mice is a reduced capacity of stem/progenitor cells to repopulate the tissue. We quantified mRNA levels of genes expressed in K14-positive stem cells, involucrin (Ivl)-committed progenitor cells, total basal interfollicular epidermal (IFE) cells, and suprabasal IFE cells, as described previously (33). Epidermal *Sod2* deficiency did not significantly alter gene expression associated with K14-positive stem cells or Ivl-committed progenitor cells in young mice (Fig. 3*C* and Fig. S4 *C* and *D*), but significantly decreased the expression of genes associated with K14-positive stem cells in older mice (Fig. 3*C*). These genes include kinesin family member 11 (*Kif11*), *Ccnb1*, centromere protein E (*Cenpe*), cell division cycle 20 (*Cdc20*), *Cdca5*, and *Kif14*. Because expression of these proproliferative genes declined in the TAM-treated, but not vehicle-treated, *K14S* epidermis at the older age but not the younger age, our data suggest that aging exacerbates the effects of the *Sod2* deficiency.

mRNAs associated with Ivl-committed progenitor cells increased with age, but only the ceramide synthase 4 (*Lass4*) mRNA significantly declined in the old *Sod2*-deficient epidermis (Fig. S4 *C* and *D*). mRNA levels of genes expressed in basal and suprabasal cells showed no change (Fig. S4 *C* and *D*). Our data indicate that the *Sod2* deficiency depletes K14-positive stem cells, but not Ivl-committed progenitor or total basal and suprabasal IFE cells, in aged mice.

To verify these phenotypes, we analyzed epidermal stem cell populations by flow cytometry. Using well-characterized markers (CD49f, CD34, and Sca1), as described previously (34), we found

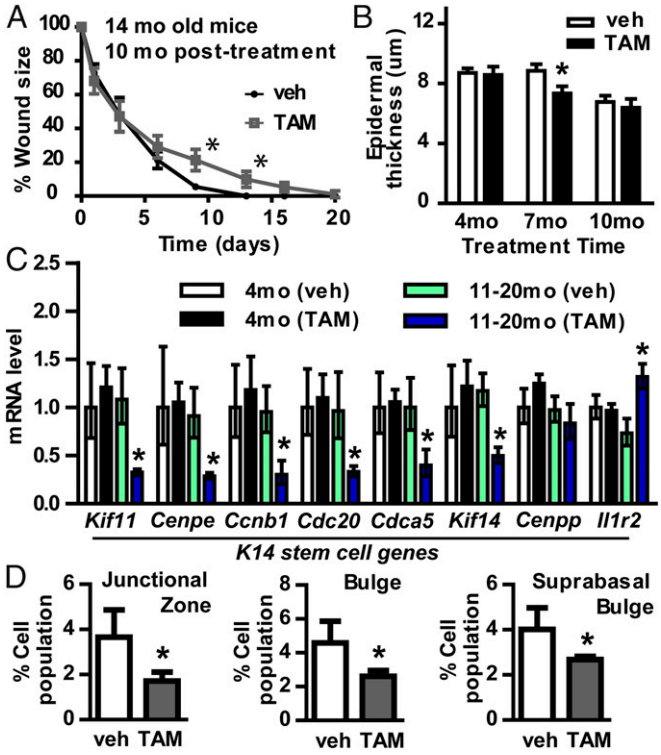

Fig. 3. Wound closure in old *K14S* mice. (*A*) Average wound size (mean ± SEM) after injury by 8-mm punch biopsy in 14-mo-old *K14S* mice treated with veh or TAM (veh, $n = 10$; TAM, $n = 11$) at age 4 mo. Percent wound size refers to the wound area relative to the initial wound area × 100. (*B*) Quantification of epidermal thickness (in μm) in H&E-stained *K14S* mouse skin at 4, 7, and 10 mo after veh (*Left*) or TAM (*Right*) treatment ($n = 5$ each). (*C*) mRNA levels of genes expressed predominantly in $K14^+$ stem cells, analyzed by qPCR, in skin from *K14S* mice at 4 or 11–20 mo after treatment with veh or TAM. Mean ± SEM values with asterisks indicate differences at $P < 0.05$ relative to vehicle control by two-way ANOVA, followed by Bonferroni post hoc analysis. (*D*) Quantification of flow cytometry histographs of epidermal cells stained for CD49f, CD34, and Sca1, isolated from the skin of 14-mo-old *K14S* mice treated with veh or TAM at age 4 mo ($n = 5$ for each). Populations of bulge stem cells ($CD49f^{hi}/CD34^+$), suprabasal bulge stem cells ($CD49f^{lo}/CD34^+$), and junctional zone stem cells ($CD49f^{hi}/CD34^-/Sca1^-$) were quantified as the average percent cell population (mean ± SEM) in the epidermis. Asterisks indicate differences at $P < 0.05$ by Student's *t* test.

a marked reduction in K14-positive stem cells in the junctional zone and bulge area in 14-mo-old TAM-treated, but not vehicle-treated, *K14S* skin (Fig. 3*D* and Fig. S5). Because junctional zone and bulge area stem cells are partly responsible for repopulating the epidermis after injury, their depletion in TAM-treated *K14S* mice suggests that mitochondrial dysfunction exhausts these cells. This exhaustion takes time and manifests as delayed wound healing only in older mice.

Cellular Senescence and Reduced Regeneration. Mitochondrial dysfunction can induce cellular senescence (19, 35). We have shown that constitutive *Sod2* deficiency causes the chronic presence of senescent keratinocytes in the epidermis (19). We also have shown that cutaneous wounding transiently induces senescence in fibroblasts and endothelial cells, which promotes wound healing (36). To determine whether senescent cells reside in the skin of keratinocyte-specific *Sod2*-deficient mice, we treated young (4 mo old) *K14S* mice with TAM or vehicle and stained the skin for senescence-associated β-galactosidase (SA-βgal), an established senescence marker (37), 4 mo later. Relative to vehicle-treated mice, TAM-treated mice had ∼2.5-fold higher SA-βgal activity in the epidermis (Fig. 4*A* and Fig. S6*A*). Interestingly, the staining was most prominent in the stratum corneum or more differentiated layers; staining in hair follicles was nonspecific, as reported previously (37). We used qPCR to assess the expression of $p16^{INK4a}$, an additional senescence marker (36). Untreated 8-mo-old *K14S* mice have low to undetectable levels of epidermal $p16^{INK4a}$ mRNA. After TAM treatment, however, $p16^{INK4a}$ mRNA was detectable in most (>70%) *K14S* skin samples (Fig. 4*B*). $p16^{INK4a}$ mRNA persisted even 10 mo after TAM treatment (Fig. S6*B*), confirming that *Sod2* deficiency induced persistent senescence in the epidermis.

Platelet-derived growth factor-A (*Pdgfa*) is secreted by senescent cells during wound healing (36), but showed no difference in wounds of 8-mo-old vehicle- and TAM-treated *K14S* mice, treated at age 4 mo (Fig. S6*C*). We also assessed mRNA levels of other factors that could contribute to wound healing, including *Pdgfb*, transforming growth factor-beta 1–3 (*Tgfb1*, *Tgfb2*, and *Tgfb3*), fibroblast growth factors 2 and 7 (*Fgf2* and *Fgf7*), and vascular endothelial growth factor (*Vegf*), none of which differed between the two groups (Fig. S6*C*). Thus, it is unlikely that growth factors contribute to the altered wound healing in epidermal *Sod2*-deficient mice.

The senescence growth arrest could reduce the ability of tissues to regenerate. To determine the effect of *Sod2* deficiency on epidermal proliferative capacity, we treated *K14S* mice with the proproliferative agent 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA promoted epidermal thickening (arrows) in vehicle-treated, but not in TAM-treated, *K14S* mice (Fig. 4*C*). Furthermore, it caused epidermal lesions and SC layer separation in TAM-treated *K14S* mice (Fig. 4*C*), which occurs when old mice are treated with TPA (38). Moreover, whereas TPA enhanced epidermal proliferation in vehicle-treated *K14S* mice, as determined by Ki67 staining, it induced less proliferation in the TAM-treated epidermis (Fig. 4*D* and Fig. S6*D*). Thus, forced epidermal proliferation by TPA accelerated epidermal thinning in young *Sod2*-deficient mice, phenocopying the reduced proliferative capacity observed at older ages.

Mitochondrial Dysfunction in Human Keratinocytes. To test the idea that mitochondrial dysfunction reduces proliferation and increases differentiation in epidermal cells, we treated primary human keratinocytes with the mitochondrial electron transport chain complex I inhibitor rotenone. We previously showed that these cells senesce in response to rotenone (19). Rotenone also increased $p16^{INK4a}$ mRNA (Fig. 4*E*) and cell size (Fig. S6*E*), a characteristic of senescent cells, and decreased mRNA levels of the proproliferation genes *CCNA2* and *CCNB1* (Fig. 4*F*), supporting the idea that persistent mitochondrial dysfunction halts keratinocyte proliferation by inducing cellular senescence. Notably, rotenone increased the differentiation-associated mRNAs *CD36* and *KLF9* and, to a lesser extent, *S100A3* (Fig. 4*F*), consistent with mitochondrial dysfunction promoting keratinocyte differentiation. We conclude that mitochondrial dysfunction can accelerate wound closure in young *Sod2*-deficient mice by increasing keratinocyte differentiation, whereas persistent mitochondrial dysfunction during aging can deplete dividing keratinocytes.

Discussion

Our results demonstrate a previously unidentified age-dependent contribution of mitochondria to stem cell and skin function. The use of *K14S* mice allowed us to study mitochondrial dysfunction in $K14^+$ epidermal cells in young and old animals, leading to important and surprising conclusions. First, mitochondrial dysfunction (owing to *Sod2* deficiency) can accelerate wound closure in young mice. This unexpected response was associated with increased reepithelialization and epidermal differentiation. Second, mitochondrial dysfunction can deplete the stem cell reservoir in older mice, leading to decreased epidermal thickness and delayed wound closure. Third, mitochondrial dysfunction induces cellular senescence in many $K14^+$ epidermal

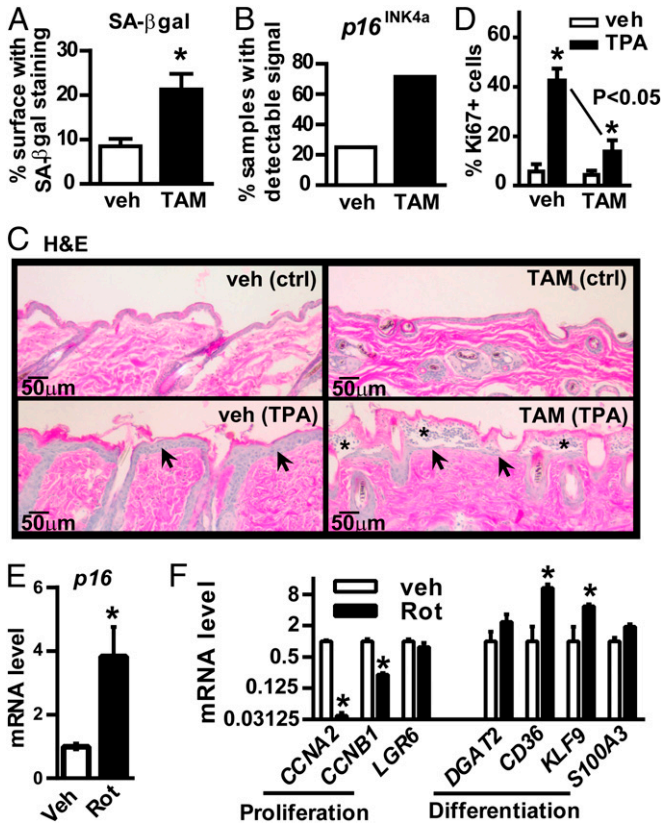

Fig. 4. Cellular senescence in mouse and human keratinocytes with mitochondrial dysfunction. (*A*) Average percentage of SA-βgal$^+$ cells quantified from the surface with blue staining relative to the total epidermal surface. Mean ± SEM values with asterisks indicate differences at $P < 0.05$ by Student's *t* test. (*B*) Average percentage of 8-mo-old *K14S* mice with detectable levels (Ct <40) of *p16^{INK4a}* mRNA in epidermal samples at 4 mo after treatment with veh ($n = 4$) or TAM ($n = 7$). (*C*) Representative photomicrographs of H&E-stained skin of acetone-treated (ctrl) or 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated 8-mo-old *K14S* mice 48 h after treatment with veh or TPA ($n = 3$ for both). In both cases, animals were pretreated with veh or TAM at age 4 mo. Arrows indicate epidermal thickness and asterisks indicate disrupted epidermis after TPA treatment. (*D*) Quantification of Ki67$^+$ cells in the epidermis from acetone- or TPA-treated 8-mo- old *K14S* mice, treated with veh or TAM at age 4 mo. Mean ± SEM values with asterisks indicate significant differences at $P < 0.05$ relative to vehicle control by two-way ANOVA, followed by Bonferroni post hoc analysis. (*E* and *F*) Human keratinocytes treated with 100 nM rotenone (Rot) for 7 d ($n = 3$) and analyzed for mRNA levels of p16^{INK4a} (*E*) and proliferation- and differentiation-associated genes (*F*). Mean ± SEM values with asterisks indicate differences at $P < 0.05$ by Student's *t* test and two-way ANOVA, followed by Bonferroni post hoc analysis.

keratinocytes. Finally, forced epidermal cell proliferation by TPA limited the ability of keratinocytes to proliferate and populate the epidermis in young *Sod2*-deficient mice, mimicking the decline in tissue regenerative potential at older ages. To our knowledge, this is the first report demonstrating markedly disparate age-dependent effects of mitochondrial dysfunction.

Mitochondrial oxidative stress can potentiate stem/progenitor cell differentiation in *Drosophila* hematopoietic cells (39). Our findings suggest that this is also true in murine skin. The signaling pathways associated with this phenomenon are incompletely understood. However, mitochondrial oxidative stress was shown to promote epidermal differentiation in mice (40) and in culture (41), as did uncoupled mitochondria (42). Our studies of constitutive *Sod2*-deficient mice also showed increased keratinocyte differentiation (19), but early lethality complicated interpretation of this result. An interesting question for future study is whether a mitochondrial signal is needed for wound closure in humans.

Mitochondrial dysfunction caused by epidermal *Sod2* deficiency depleted epidermal stem cells, but with phenotypic effects only later in life. Some stem cells, including those in the epidermis, reportedly are resistant to oxidative stress and refractory to age-related decline in mice (43, 44). Because rapidly dividing transit-amplifying (TA) cells arising from stem cells are responsible for epidermal expansion (45), we speculate that the senescence of TA cells increases the demand for stem cells. Thus, whereas epidermal stem cells can resist oxidative damage, the increased senescence resulting from mitochondrial oxidative stress can exhaust the stem cell pool (46), causing epidermal thinning and delayed wound healing but only at late ages. Therefore, although epidermal stem cells might not be a direct target of oxidative damage, senescence in TA cells can drive stem cell exhaustion with aging.

Constitutive whole-body *Sod2* deficiency decreased epidermal thickness by postnatal day 17 (19), yet keratinocyte-specific *Sod2* deficiency caused epidermal thinning after age 7 mo. This milder phenotype suggests that other tissues might contribute to epidermal thinning in constitutive whole-body *Sod2*-deficient mice. Likewise, the numerous tissues that are affected in constitutively *Sod2*-deficient mice (10, 12) might synergize through cell nonautonomous and/or systemic factors to accelerate the aging phenotypes in these mice.

Finally, senescent cells accumulate with age in multiple tissues, including the skin, where they are thought to contribute to impaired tissue homeostasis and regeneration (18, 47, 48). On the other hand, senescent cells are induced after skin or liver injury; in the skin, they appear to accelerate wound closure, and in both tissues they appear to limit fibrosis (36, 49, 50). Senescent cells also occur during embryonic development, where they apparently fine-tune morphogenesis (51, 52). We speculate that mitochondrial signals might contribute to the pleiotropic effects of senescent cells, but these effects might depend strongly on age.

Materials and Methods

Animal Experiments. All animal studies complied with protocols approved by the Institutional Animal Care and Use Committee of the Buck Institute for Research on Aging. *Sod2^{tm1Smel}* mice were generated by inserting *LoxP* sites 3′ of exon 1 and 5′ of exon 4 (Fig. S6*F* and *SI Materials and Methods*). *Tg (KRT14-cre/Esr1)^{20EfuJ}* × *Sod2^{tm1Smel}* (*K14S*) mice were generated by crossing *Sod2^{tm1Smel}* (*S*) mice with *Tg(KRT14-cre/Esr1)20Efu* (*K14*) mice (Jackson Laboratory). *K14R* mice were generated by crossing B6-*Gt(ROSA)26Sor^{tm1Sor}/J* mice (Jackson Laboratory) with *K14* mice. Genotyping was performed by PCR (*SI Materials and Methods*; primer sequences listed in Table S1). TAM (50 mg/kg body weight) or vehicle (corn oil) was given to 4-mo-old male mice by i.p. injection. Wounds were delivered to dorsal skin using an 8-mm biopsy punch and measured with a caliper. Wound areas were collected after biopsy as described and processed for staining and/or qPCR. Skin samples were also collected after topical treatment with TPA (9.87 μg/mouse/3 mm^2) or vehicle (acetone) for 48 h.

Tissue Staining. Tissues were fixed in 10% (vol/vol) buffered formalin, embedded in paraffin, cut into 7-μm-thick sections, and processed for immunofluorescence (19), H&E (12), and picrosirius red staining (*SI Materials and Methods*). Epidermal thickness was measured using ImageJ software. Skin samples were also embedded in frozen optimal temperature cutting medium (OCT), cut, and processed for immunofluorescence, SA-βgal activity (19), and SDH or COX activity staining (12). For mouse tail whole mounts, epidermal sheets were isolated, fixed, permeabilized, and stained as described previously (53). Antibody conditions are described in *SI Materials and Methods*.

Keratinocyte Analysis. Mouse keratinocytes were isolated from dorsal skin and processed for genotyping, RT-PCR and flow cytometry (*SI Materials and Methods*). PCR primer sets are listed in Tables S2 and S3. For flow cytometry, cell types were identified by gating procedures as described previously (34) and analyzed using FlowJo analysis software.

Cell Culture. Human keratinocytes (AG21837) from the Coriell Cell Repository were cultured in keratinocyte growth medium (CnT-07; Zenbio) with penicillin-streptomycin (Invitrogen) in 20% O_2. Media were replaced every 2 d. Cells were treated with vehicle (DMSO) or rotenone (Sigma-Aldrich) at 100 nM for 7 d, then collected for PCR analysis (*SI Materials and Methods*).

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