Tissue repair and stress responses of aged skin epidermis

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Dedication

I dedicate this work to the figures in my life that at each critical juncture took an interest in my development as a scientist and pushed me to be more curious and embrace the commensurate challenge. My mother, Christina Liu, for always asking me why and challenging me to explain in simple terms the ideas I learned in my science classes. My father, Kevin Wong, for inculcating in me a mechanistic first principles view of the world. My high school chemistry teacher, Katie Thornburg for revealing to me the laws of the molecular world, and gently encouraging me not to make thermite for a project. Finally, Mary Tsai, my fiancée, for her constant encouragement, support, and patience.
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Abstract of Dissertation

Skin is the most conspicuous of organs which both protects and mediates organismal interactions with the environment. The epidermis of the skin is a stratified tissue consisting of basal epidermis which differentiates to form progressively more differentiated layers of keratinocytes. Its regular structure and evolved responses to environmental stresses can be used to study complex stress responses such those caused by aging. The epidermis becomes dysregulated with age, resulting in wrinkling, hair loss and graying, and impaired wound healing. Basal progenitor cells maintain the epidermis, and the regulation of their divisions determine its function and structure as a whole. Cellular energy levels are an important input in the determination of cell fate, and dysregulated energy metabolism occurs during aging. However, the role of bioenergetics on differentiation and homeostasis in the epidermis is poorly understood. This work investigates the effects of interventions targeting cellular metabolism to understand how they may alter the structure and stress responses of aged skin. I reveal the ability of the exercise induced cytokine IL-15 to improve wound healing of aged skin in addition to its previously described rescue effects on skin structure and mitochondria. I further describe the initial characterization of a mouse model harboring bioenergetic disruptions specific to the basal epidermis. Loss of Pgc1a from the adult basal epidermis yields skin repair defects with perturbed NAD+ metabolism and altered differentiation. As organisms like *Acomys* with superior epidermal regeneration possess divergent stress responses from mouse and man, their comparative biology is also investigated. I report in *Acomys* the transient formation of a distinct skin structure which facilitates resistance to the effects of solar radiation by promoting removal of damaged cells by rapid stratification. Taken all together, this body of work is a starting point for more mechanistic investigations into the roles of energy metabolism and cellular differentiation on stress responses in the aged epidermis.
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Chapter 1: The skin epidermis under homeostasis and stress
1.1 The anatomy and development of the skin epidermis

Vertebrates have an exterior body covering of skin which has an inferior mesoderm-derived dermis beneath a connective tissue basement membrane, and a superior layer of neuroectoderm-derived epidermis above (Di-Poi and Milinkovitch, 2016). As a whole, skin refers to stratified multilayered tissue composed of an outermost cornified epithelium with supportive layers of cells beneath: the granular layer, spinous layer, basal layer, basement membrane, and dermis, which has its own layered structure too (Fig. 1). Skin encloses and protects from external environmental hazards such as temperature variation, solar radiation, injury, infection, and dehydration. It is formed when Wnt signaling blocks fibroblast growth factor (FGF) but not bone morphogenetic protein (BMP) signaling within the ectoderm of the gastrula, causing specification of a single layer of multipotent embryonic progenitor keratinocytes (Fuchs, 2007) and the founding of the epidermis. Following specification from the ectoderm, cells in the primitive epidermis rapidly divide, but remain restricted to the basal plane until embryonic day 14 when apical divisions begin to stratify the suprabasal layers (Williams et al., 2011). How the epidermis is stratified, and how of epithelial appendages such as hair follicles and glands are specified during development was the subject of intense study in the last few decades (Benitah and Frye, 2012; Fuchs, 2016; Pispa and Thesleff, 2003; Turing, 1990). It has since been established that local morphogen gradients (such as Wnt, BMP, FGF, Notch, and Sonic Hedgehog/SHH) (Visscher and Narendran, 2014) diffuse and form concentration gradients within the nascent skin and establish the mosaic patterning of hair follicles over the epidermis as initially proposed by Alan Turing as the Chemical Basis of Morphogenesis (Turing, 1990). These morphogen gradients together with the mechanical and structural properties of the underlying dermis designate the placement of hair follicle nucleating structures termed dermal placodes which subsequently recruit the downgrowth.
of epidermal cells to form hair follicles and glands via Wnt signaling and BMP suppression (Dhouailly, 1973; Kobielak et al., 2003). Hair follicles tunnel into the dermis from the skin surface and produce smooth concentric sheaths of epidermal cells and are anchored at the bottom of the tunnel by a small population of dermal cells known as the dermal papilla (Fig. 1). Hair follicles are thus a mixture of ectoderm and mesoderm derived cells, with the primary body of the follicle and accessory sebaceous, eccrine, and apocrine glands specified by the ectoderm-derived fraction and the dermal papilla and outer root sheath specified by the mesoderm-derived fraction (Schneider et al., 2009). Classical tissue recombination experiments combining different types of epidermis and dermis together have revealed an important role for the mesenchyme derived dermis in the types of epidermal appendages which form: for example, feathered or scaled dermis promotes formation of those appendages regardless of the type of epidermis grafted upon it (Lu et al., 2016). The general pattern described for hair follicle formation which requires an interaction between mesenchymal and ectodermal tissue applies to other types of epidermal appendages such as teeth, feathers, eccrine glands like lacrimal and mammary glands, as well as the exocrine glands of skin which include those that produce sweat and sebum (Pispa and Thesleff, 2003). Sebaceous glands are attached to the permanent non-cycling part of hair follicles, and develop during the late stages of hair follicle formation (Niemann and Horsley, 2012). Apocrine sweat glands empty into hair follicles and develop together with them; unlike eccrine sweat glands, their sweat output is unnecessary in organismal heat management. Eccrine sweat glands exist as distinct epithelial structures emptying to the skin surface, and take the form of a straight sweat duct extending from the skin surface to a coiled labyrinth structure in the dermis which forms a secretory lumen (Cui and Schlessinger, 2015).
The epidermis between hair follicles is appropriately called interfollicular epidermis (IFE). Development and elaboration of the IFE is regulated by integration of cell density signals through adherens junctions to form a negative feedback loop inhibiting growth factor and Ras/MAPK pathways; the epidermis undergoes proliferation and expansion based on what is mechanically permitted on a single cell basis within a densely packed cellular environment (Fuchs, 2007). The reliance of HFs and IFE development on opposing signaling pathways for development is key to correct patterning of epidermis. Interfollicular epidermis exists in a state of competition with hair follicles: both BMP and epidermal growth factor (EGF) signaling are active in the IFE, which repress follicle formation through disruption of Wnt and beta-catenin signaling (Kobiela et al., 2003; Mou et al., 2006), while the opposite conditions are required for hair follicle growth. Eventual elaboration of the basal layer into stratified multilayered epithelium is accomplished at embryonic day 14 by a shift in basal cells away from purely parallel symmetric cell divisions to perpendicular asymmetric cell divisions resulting in combinations of differentiated cells and basal cells (Williams et al., 2011). Symmetric cell divisions of non-competitive basal cells possessing diminished integrin bonds to the basement membrane terminally differentiate to also contribute to the stratification of the epithelium (Liu et al., 2019; Miroshnikova et al., 2018).

Interfollicular epidermis is further divided into two types depending on how it responds to hyperkeratinizing events. When provoked to proliferate, epidermal regions can either become orthokeratotic or parakeratotic, depending on the type of cornified envelope which forms in response. Orthokeratotic corneum is anucleate, loose, and flaky, characteristic of squames derived from granular cells, while parakeratotic corneum is condensed and smooth with visible squames, some still with nuclei (Cardoso et al., 2017; Schweizer et al.). The exact appearance of the pattern of hyperkeratosis varies from depending on epithelialized location. Murine dorsal interfollicular
epidermis is orthokeratotic while hair follicles are parakeratotic. These features are easily seen in tail epidermis. Tail skin differs from dorsal skin in that it features clearly defined geometrically tiled domains known as scales, which are hair follicle associated, and interscales which occur between scales and are analogous to dorsal interfollicular epidermis. Scales exhibit parakeratotic and interscales exhibit orthokeratotic epidermal differentiation (Gomez et al., 2013). The underlying stem cell dynamics representing these discrete epidermal regions will be discussed in more detail later.

The dermis, epidermis, and attendant skin layers rest on a sheath of striated muscle tissue. It is important to note that the dermis, epidermis, and all attendant layers of the skin from the subdermal adipose to the cornified envelope all reside on a thin layer of striated musculature known as the panniculus carnosus, which is notable because it plays a dominant role in wound closure but is not present in all animals to the same extent (Wong et al., 2011). The panniculus carnosus is intimately bound to the skin and endows it with twitching and contraction abilities, but its presence varies greatly depending on species. In humans, this muscle is considered vestigial with remnants found within the scalp, auricle, ventral neck, palm, and axilla, but is widespread in other animals such as rodents, cats, dogs, horses, and whales where it covers the majority of the body (Naldaiz-Gastesi et al., 2018).

1.2 The role of skin keratins

The skin epidermis is divided into two main stem cell compartments which reside in the bulge of hair follicles and the basal layer of the interfollicular epidermis. These unique niches are marked by stem cells that express different intermediate filament proteins, which aside from their structural role in skin, have been shown to be reliable markers of cell identity and pluripotent
potential. Keratins of squamous epithelia are filamentous proteins that associate into bundles of filament pairs, and are the primary protein expressed by cells of the epidermis and its appendages (Fuchs, 1995). Keratin proteins are 40-67 kD in size with isoelectric points of 4.5-7.5, with Type I keratins occupying the smaller and more acidic range of the spectrum, and Type II keratins the larger and more basic (Coulombe et al., 1989). Type I and type II keratins have complementary structures which enable the formation of filaments through tandem interaction of their helical domains (Hanukoglu and Fuchs, 1983). Keratins are crucial to skin function as they enable cell-cell anchorage via affixing to intracellular membranous plaques known as desmosomes and hemidesmosomes, which imbue the skin with its mechanical elasticity, toughness, and together with secreted lipids, impermeability (Kouklis et al., 1994). In addition to the aforementioned physical properties, epidermal cells rely on keratin signaling to regulate growth, migration, and other critical cellular functions (Wang et al., 2016). Basal cells express keratin 14 and keratin 5 during homeostasis and activate to express keratin 6 and 16 during wound healing at the wound edge (Paladini et al., 1996). The spinous layers express keratin 10 and keratin 1 and superior layers express the non-keratin filaments, loricrin, filaggrin, and involucrin to form the cornified epithelial envelope (Lopez et al., 2009). These non-keratin intermediate filaments associated with terminally differentiating keratinocytes and modify keratin filaments, rendering them distinct from inferior spinous and granular layer cells and further optimize the mechanical barrier properties of the outermost cornified envelope (Fuchs, 1995). Inside the cells, keratinocytes fill up with keratin proteins and lose their nucleus toward the end of their differentiation process. Hair follicles express a broad spectrum of keratins including keratin 14 and keratin 5 at low levels in the ORS, but uniquely express keratin 17, which is necessary for proper HF development (McGowan et al., 2002). In depth review of keratins and other intermediate filaments relevant to the skin will be excluded.
from this primer for brevity but can be found in the literature (Fuchs, 1995; Jacob et al., 2018; Kouklis et al., 1994; Wang et al., 2016).

1.3 Homeostatic dynamics of the hair follicle

Within hair follicles, a dynamic concentric cylindrical structure is formed which undergoes periodic hair cycling to form the hair or pelage of the organism. Hair follicles are mini-organs formed by epithelial invaginations into the dermis, enclosed by a mesenchymal outer root sheath and anchored by the dermal papilla and interior layers consisting of epidermal cells, matrix cells, and melanocyte progenitors (Fuchs, 2007). Within the bulb of the follicle, multipotent matrix cells reside in close contact within the niche formed by the dermal papilla, and produce rapid dividing transit amplifying cells to sustain hair follicle remodeling and hair shaft synthesis during the growth phase of the hair cycle (Chi et al., 2013). The hair cycle is defined by a few major phases, which can then by more finely delineated. Those major phases are telogen (resting) when the hair follicle is dormant and withdrawn close to the dermal-epidermal junction, anagen (growth) when the hair cycle elongates deep into the dermis and synthesizes a hair, and catagen (destruction) when the hair follicle has completed hair synthesis and begins to transition back to telogen via breakdown of the outer root sheath and resorption of the follicle body back to the depth of the permanent follicle immediately inferior to the basement membrane (the bulge) (Fig. 1) (Chi et al., 2013; Tumbar et al., 2004). Epidermal stem cells from the HF bulge periodically supply the bulb with matrix cells, and study of the cycling HF revealed that broadly, a return of committed ESC lineage cells to the bulge niche upon transition from anagen to catagen does not restore stemness to the returnees. Instead, these committed progeny are critical in enforcing quiescence during telogen through paracrine signaling factors which suppress the DP (Hsu et al., 2011). In mice, the
approximate duration of each of these phases is two weeks for telogen, 1-3 weeks for anagen, and 2-3 days for catagen (Müller-Röver et al., 2001). Hair follicles receive many inputs, and hair growth and biology are closely coordinated with the dermis, as well as the connected vasculature, nerves, and dermal adipose. Recent work has shown that transit amplifying cells derived from matrix cells express SHH to induce proliferation and differentiation of subdermal adipocyte precursors. Subsequent recruitment of adipocytes results in surrounding of the hair follicle in adipose coordinately with onset of anagen, and the blockade of hair follicle SHH signaling prevents proper adipogenesis and hair follicle growth (Zhang et al., 2016a), suggestive of a critical and intimate co-dependence between hair follicles and the dermis. The hypothalamic-pituitary-adrenal axis acting through parasympathetic innervation has also been shown to regulate the proliferation of hair follicle resident melanocyte stem cells. Psychological stress in mice such as by restraint caused hair graying due to overactivation and depletion of melanocyte stem cells (Zhang et al., 2020).

After the first anagen, which is encoded by developmental cues and occurs around postnatal day 28 in mice, subsequent hair cycles progress periodically approximately every 5 weeks on the dorsum of mice through adulthood (Müller-Röver et al., 2001). The dorsal skin of rodents undergo hair cycles which progresses in a synchronized cephalocaudal, midline to flank wave (Müller-Röver et al., 2001). Initiation of the wave depends on hair follicles first transitioning from resting telogen phase to anagen which requires Wnt signaling similar developmental processes (Alonso and Fuchs, 2006). Spatiotemporal synchronization of hair cycling across the dorsal skin has been shown in mice to be coordinated in part through the lymphatic network of epidermal drainage vessels connecting follicles to each other. Moreover, lymphatic vessels associated with
resting telogen skin have been shown to express Wnt inhibitors, revealing how the anagen wave is in part regulated (Gur-Cohen et al., 2019).

Not all hair follicles are equal— the type of hair shaft (there are several- awl, guard, auchene, and zigzag) produced by each follicle is encoded by the cellularity of the dermal papilla, which regulates matrix cell proliferation during the hair cycle. A single follicle can gradually over time change the type of hair shaft it produces due to alterations in the cellularity of the matrix cell population of the dermal papilla, and this is a major determinant of hair thinning and hair loss (guard, awl, auchene, and zigzag in descending order of DP matrix cell abundance) (Chi et al., 2013).

Hair follicles participate in wound healing as a major nexus of epidermal stem cells which contribute to wound re-epithelialization, but are rarely regenerated during adult wound healing except in certain highly regenerative species such as Acomys (Gawriluk et al., 2016). The exception is in cases of extremely large wounds (Ito et al., 2007), where de novo hair follicle regeneration has been reported for some time in animal models as rabbit and mice (Billingham and Russell, 1956; Breedis, 1954; Lacassagne and Latarjet, 1946). Thus, the relation between wound size and severity to the tendency of the skin to regenerate hair follicles has been casually noted for some time, but only recently have we begun to understand why it occurs. The dermis is normally occupied by fibroblasts which maintain the ECM, but during wounding, they can become contractile myofibroblasts with the ability to pull the wound closed. Small wounds are readily dealt with in this manner, but larger wounds require a different approach, presumably because restoration of the subdermal adipose is useful and favored. Pioneering work has shown that regenerated hair follicles in large wounds reprogram proximal myofibroblasts to become normal adipocytes, resulting in normal subdermal adipose. Taken together, hair follicles play a central role
in wound healing and skin remodeling and have the ability to regulate the composition of the dermis and subdermis through recruitment and regulation of adipose. Observation of adult de novo HF formation in large wounds pairs with recent work establishing a role for subdermal adipocyte progenitor cells in sustaining the cellular replenishment of wound beds via differentiation into myofibroblasts, but only in small wounds (Shook et al., 2020). Myofibroblasts have also been shown to be able to transdifferentiate into adipocytes during wound bed remodeling. Taken together with recent evidence demonstrating that adipocytes can additionally recruit myofibroblasts (Plikus et al., 2017; Schmidt and Horsley, 2013), an elegant system of wound bed maintenance wherein hair follicles play a central role begins to emerge. Wounds of any size are closed with the help of contractile myofibroblasts that can later become adipose tissue. Adipose tissue supports HF formation and cycling, and large wounds requiring greater amounts of myofibroblasts to close also regenerate HFs de novo.

1.4 Homeostatic dynamics of interfollicular epidermis

The interfollicular epidermis is the neighborhood of epidermis adjacent to hair follicles. Here, the stem cell niche is made up of a single layer of basal cells under normal homeostatic conditions. The basal layer is also interspersed with a few non-keratinocyte cells such as tissue resident T-cells, antigen presenting resident macrophage Langerhans cells, and photo-responsive pigment producing melanocytes as well as Merkel cells which mediate tactile sensation. The basal layer of the epidermis stratifies upward to form the multilayered spinous layer, so called because first observations in formalin fixed paraffin embedded sections revealed shrunken contracted cells anchored by desmosomes with spiny morphology. Spinous layer keratinocytes terminally differentiate to give rise to the granular layer. The granular layer takes its name from the grainy
appearance of its cells when observed using brightfield microscopy owing to their advance state of epithelial differentiation. The granular layer rapidly gives way after a few cell layers to the cornified epithelial envelope consisting of keratin-laden keratinocytes called squames, with are densely crosslinked as described above by a meshwork of keratins sheathed in additional filament proteins, thus becoming cornified.

The historical model proposed to explain stem cell dynamics of the interfollicular epidermis relied on the concept of transit amplifying cells similar to hair follicles. In this model, infrequently cycling basal stem cells give rise to lineage restricted but rapidly proliferating transit amplifying cells. These slow dividing epidermal stem cells and their progeny transit amplifying cells and resultant cornified epithelium were called epidermal proliferative units. However, the epidermal proliferative unit concept of infrequently dividing basal cells anchoring transit amplifying progeny in the interfollicular epidermis has since been disproven (Clayton et al., 2007). Of course, as discussed in the section on hair follicles, transit amplifying cells do play a role in epidermal biology within hair follicles, and this distinction is important to make note of.

Later work utilizing keratin 14-cre driven transgenic mice continued to suggest a single interfollicular stem cell population, albeit one capable of dividing both symmetrically and asymmetrically with random fate (Clayton et al., 2007; Doupé et al., 2010). Subsequent work with both keratin 14- and involucrin-cre transgenic mice once again reintroduced the concept of slow cycling basal cells feeding a population of more rapidly dividing cells (Mascré et al., 2012). An alternative hypothesis has since been proposed surrounding the observation that the cornified envelope is formed by two different terminal differentiation programs. This includes either orthokeratotic corneum without suprabasal nuclei and parakeratotic corneum with suprabasal nuclei as exemplified by murine dorsal and tail skin (Schweizer et al.), suggesting a more
heterogenous stem cell population composed of long- and short-term label retaining cells (Gomez et al., 2013).

Through recent efforts with live imaging and improved lineage tracing coupled with mathematical modeling (Clayton et al., 2007; Rompolas et al., 2016; Sada et al., 2016), it is becoming apparent that the patterning and topology defined by the dermis in part regulates the maintenance of heterogenous stem cell populations in the epidermis. Microscopic observation of skin cross-sections reveals the epidermis has an undulating topology which contributes to its elasticity (Kruglikov and Scherer, 2018). In human skin and certain epithelial sites in rodents such as the oral palate, the dermis is topologically complex with dermal and corresponding epidermal undulations (known as rete ridges in humans). These undulations have recently been shown to delineate fast and slow cycling basal epidermal stem cell populations which are enclosed by scale and interscales (Changarathil et al., 2019; Sada et al., 2016), and extends to the dorsal skin and other epithelial regions as well as evidenced by the presence of integrin bright label retaining cells (LRCs) coinciding with rete ridges (Watt, 1998). When viewed top-down onto the apical surface of murine tail skin, these undulations encode discrete patches of epidermis characterized by an island of rapidly cycling Slc1a3 expressing non-LRCs in the valleys (scale) of the undulations with a perimeter of slow cycling Dlx1 expressing LRCs in the peaks (interscale) (Sada et al., 2016). Aged epidermis is thinner and flattens leading to a loss of its topological undulations (Mine et al., 2008), and exhaustion of valley Slc1a3 expressing cells, which appears to be partially compensated for by Dlx1 expressing cells (Changarathil et al., 2019). The decline of fast cycling stem cells as marked by Slc1a3 concomitantly with aging related changes in the mechanical properties of the skin is curious. Whether the decline of this population is causes the loss of skin structure, or if the loss of skin structure causes the decline of the population is still unknown. Mechanotransduction
has been seen to determine cell fate of brain (Georges et al., 2006; Lu et al., 2006), mesenchymal (Engler et al., 2006; Winer et al., 2009), and muscle stem cells (Gilbert et al., 2010). Epidermal mechanotransduction is mediated by YAP/TAZ also plays an important role in organogenesis and stratification, as discovered in Drosophila epithelia (Elbediwy and Thompson, 2018). Pregnancy places extraordinary demands on the abdominal epidermis to expand rapidly. In mice, the mechanically strained underlying dermis secretes proteins (Sfrp1 and lgbp2) which induce the differentiation of rapidly proliferating Tbx4 expressing cells from Axin2 expressing basal epidermal cells (Ichijo et al., 2017). Taken together with observations that epidermal stem cells grown on micropatterns in vitro under differential cortical tension undergo delamination, differentiation, and altered cytoskeletal and cell adherence protein expression when compressed by neighboring cells (Miroshnikova et al., 2018), the role of mechanical forces in the maintenance of heterogeneous stem cell populations in the skin warrants further investigation. In summary, the interfollicular epidermal stem cell population is heterogeneous and the development and maintenance of the stratified epidermis can be explained wholly through the existence of both slow cycling and fast cycling stem cells which are defined by the epidermal patterning, topology, and perhaps mechanobiology laid out by the underlying dermis.

1.5 Stem cell competition within the interfollicular epidermis

Competition within the stem cell niche is a major regulatory force in both development and the maintenance of the epidermis (Changarathil et al., 2019; Ellis et al., 2019; Liu et al., 2019). In the developing epidermis after specification from the ectoderm, competition among basal epidermal stem results in the engulfment and clearance of loser cells with weaker basement membrane attachments during the phase of symmetric in-plane expansion (Ellis et al., 2019).
Clonal heterogeneity of stem cells has been documented to decrease over time with aging, in particular within the skin and in the circulating blood. Dominating clonal selection may favor the propagation of proliferative but functionally inferior stem cells, resulting in a net loss of regenerative and development potential in spite of normal or even higher stem cell counts owing to a skewing of cell fate as seen in the case of hematopoietic stem cells (Park and Bejar, 2018; Sudo et al., 2000). In the developed epidermis, multiple factors determine whether an ESC will survive in the basal niche (thus “winning”) or if it will become detached (“losing”), with unfit losers becoming either engulfed by their neighbors or ejected upward through the strata until eventual conversion into cornified envelope squames (Ellis et al., 2019). The basal stem cell layer is spatially confined with the basement membrane below, more differentiated layers above, and neighboring basal cells all around within the plane. In this context, non-competitive cells are those that for whatever reason do not form appropriate contacts with their surroundings, in particular to the basement membrane through expression of certain integrin proteins, such as the hemidesmosome binding filament protein Collagen 17A1 (COL17A1) (Liu et al., 2019). As COL17A1 expression in the epidermis declines with age, Liu et al. tested forced overexpression of COL17A1 in aged murine epidermis and revealed it rescues skin aging. As cells are squeezed due to in-plane cell divisions, the cortical tension across the cell body is decreased, and they become anisotropic in the axis perpendicular to the compressive load, and this action in particular exposes differences in basement membrane attachment (Miroshnikova et al., 2018). Loser cells are thus squeezed perpendicularly upward and concomitantly extruded and induced to differentiate, while basal cells are triggered by the differentiation event to proliferate and fill the free space (Mesa et al., 2018). Taken together, stem cell competition in the interfollicular epidermal niche promotes barrier integrity by selecting for basal stem cells capable of robust
basement membrane attachment, while shunting loser cells toward stratification and augmentation of the skin barrier through cornification. This phenomenon couples mechanical sensing as a surrogate for the crowding and integrity of the epidermal basal layer and superior stratified layers together with proliferation, differentiation, and delamination of the basal epidermal stem cells. An additional benefit of the competitive maintenance of epidermis is a resistance to oncogenic transformation. While the idea of cancerous keratinocytes thriving and outcompeting non-transformed cells in the niche is congruent with observations of cancers in other tissues, skin is distinctly resistant to it. Oncogenic transformation of basal epidermal cells via PI3K overactivation was rapidly suppressed via SH3RF1 dependent differentiation (Ying et al., 2018). While the authors of that study did not go further, SH3RF1 has predicted Rac (a Rho GTPase) binding since it has a RING-finger domain, which suggests that the differentiation is downstream of cytoskeletal remodeling (McMullan et al., 2003; Sit and Manser, 2011). Taken together, the epidermis functions like a biomechanical “state machine” to efficiently maintain the quality and function of the epidermis until old age.

1.6 The role of bioenergetics and energy sensing within the skin epidermis in determination of cell fate

The role of bioenergetics in the regulation of epidermal stem cell biology of the skin is largely unknown at the time of writing, but comparisons with findings made in other cell types may provide useful starting points for investigation. The epidermis is a highly proliferative tissue which replenishes all of its cells on average every 4 weeks through the aforementioned processes of basal stem cell divisions coupled with stratification. In addition to competition via physical interactions with surrounding cells, cell fate is also regulated in part by the mitochondrial network.
within each cell (Zhang et al., 2018). Mitochondria are capable of rapidly integrating the cellular energy landscape, and are positioned to direct stem cell fate through epigenetic and metabolic reprogramming in response (Bahat and Gross, 2019). Cell fate decisions such as whether to divide parallel or perpendicularly with their resident layer have differential energy costs due to the types of physical forces that must be overcome in the process of mitosis. Symmetric cell divisions resulting in two equal daughter cells within the original layer increases the compressive forces on all cells within that layer. It was found that when epidermal stem cells were cultured in vitro on confining micropatterns, cells squeezed by other cells on all sides preferentially delaminated in a predictable manner (Miroshnikova et al., 2018). Indeed, in silico modeling of the skin with epidermal and dermal compartments required accounting for substrate deformability together with the energy costs associated in order to accurately model the dynamics of the interfollicular epidermal stem cell compartment correctly (Kobayashi et al., 2018). Asymmetric cell divisions producing one stem and one differentiating daughter cell must overcome the forces exerted by the spinous, granular, and other suprabasal epidermal layers. Selecting these modes of division require intracellular reconfiguration of the mitotic spindle and associated mitotic apparatus. During the process of mitosis, the quantity and quality of mitochondria partitioned into each daughter cell is associated with the mode of division and direction of division relative to the tissue axis, as well as daughter cell fate. Differentiating daughter cells resulting from asymmetric divisions are fated for stratification and eventual shedding as cornified epithelium, and their energy requirements differ from stem daughters fated to remain in the basal layer as quiescent stem cells.

The importance of mitochondria and their central role in bioenergetics is highlighted by the mitochondrial bottleneck, a quality control system which ensures oocytes contain functional pristine mitochondrial organelles through regulating their heteroplasmy (Khrapko, 2008). The so-
called mitochondrial bottleneck is well established as occurring during the process of oogenesis, whereby only mitochondria of low mutational burden are permitted to participate in oocyte formation (Cao et al., 2007), but now also been shown to occur in somatic tissues such as blood and hair follicles (Barrett et al., 2020). Although how somatic cells are able to partition a mixed mitochondrial population during division in such a way remains unknown in vertebrates, the process has been described in other systems. In budding yeast which undergo asymmetric cell divisions, active cell transport mechanisms ensure the precise delivery of only a subset of the mother cell’s mitochondria to the daughter cell (Rafelski et al., 2012). While reasonable to speculate that active partitioning processes exist in higher organisms, evidence is lacking. However, the theory of mitochondrial fission prior to division coupled with partitioning of organelles proportionally to cell volume does not seem to account for the drastic mtDNA allelic frequency distributions that first turned researchers on to partitioning of mitochondria and the bottleneck (Mishra and Chan, 2014). What is clear is that nature has refined effective mechanisms to provision daughter cells with the quantity and quality of mitochondria necessary for their eventual cell fate, underscoring the critical role of mitochondria as arbiters of cell outcomes by integrating bioenergetic cues.

Mitochondria integrate intracellular energy state signals, and use the resulting information to determine energetically feasible cell fates, but are also vulnerable to damage (Bahat and Gross, 2019). Naturally occurring forms of progeria occur in mice and humans and uniformly derive from cellular defects in DNA repair or detoxification of reactive species which subsequently promote DNA damage. Mitochondrial DNA is particularly susceptible to genotoxic insults as they are constantly in a state of metabolic flux which produces a high quantity of ROS (Cline, 2012), thus deficiencies in detoxification measures can have profound effects due to genomic damage (Kujoth...
et al., 2005). While mitochondria have some DNA repair capabilities, their high mutational burden necessitating complementation between organelles suggests that this capability is not as robust as that of the nucleus. The precise complement of mtDNA repair capabilities is still the subject of active research. Indeed, initial reports of DNA polymerase in mitochondria were greeted with skepticism that they might simply be contaminants from the nucleus (Kazak et al., 2012). Thus, inferior repair of mtDNA together with a local source of abundant ROS from the electron transport chain yields a situation wherein mutations often persist and become codified into clonal germlines (Trifunovic et al., 2004). The skin additionally must compensate with an additional genotoxic input in the form of ultraviolet radiation from exposure to sunlight (Ouhtit et al., 2000). The effects of solar radiation specifically on epidermal mitochondria results in a number of stereotyped and well characterized photoaging associated mtDNA mutations (Birch-Machin and Swalwell, 2010). It is interesting to note that consequently, the majority of cellular turnover within the skin epidermis is coordinated to occur at night in synchronization with circadian rhythms (Janich et al., 2013).

In recent decades, significant advances have been made toward understanding the regulation of mitochondrial biogenesis, fusion, and fission, and their combined role in the homeostatic maintenance of a variety of tissues (Palikaras and Tavernarakis, 2014). Peroxisome proliferator-activated receptor gamma coactivator-1a (Pgc1a), commonly termed the master regulator of mitochondrial biogenesis, has been implicated as a negative regulator of senescence brought on by aging in some tissues such as the vasculature (Xiong et al., 2013). Forced overexpression of Pgc1a in muscle tissue is capable of rescuing it from atrophy due immobilization (Kang et al., 2015). Specifically in the skin, mitochondrial biogenesis defects linked to Pgc1a have been ascribed to vitiligo (Dell’Anna et al., 2017), aging associated deterioration of skin barrier function (Macias et al., 2014; Wong et al., 2019), and circadian arrhythmic of the epidermal stem
cell niche (Janich et al., 2013; Liu et al., 2007; Solanas et al., 2017). Pgc1a (and Pgc1b) is a transcriptional co-activator of genes involved in energy metabolism, and it exerts its effects through the stabilization of transcription factors operating within the NRF-TFAM pathway (Lin et al., 2005). The net effect on cellular energy output by Pgc1a co-activation of these genes is increased mitochondrial fatty acid beta oxidation, oxidative phosphorylation and attendant increases in Krebs cycle activity (Lin et al., 2005).

Fusion and fission are critical processes regulating all aspects of mitochondria including metabolic efficiency, stress resilience, trafficking and intracellular localization, network topology, organelle maintenance, and calcium buffering (Held and Houtkooper, 2015). Changes in the balance of mitochondrial fusion and fission have been shown to have a direct impact on stem cell fate (Chen and Chan, 2017) and promote metabolic dysfunction and senescence in epithelial cells (Tezze et al., 2017). In addition to biogenesis of new organelles, existing mitochondrion are recycled, and the overall intracellular mitochondrial network constantly undergoes dynamic reconfiguration to meet cellular needs. Mitochondrion can be divided into two or more discrete organelles via fission and combined together again with their inner and outer membranes joined to form elongated complex single organelles via fusion. Cytosolic Drp1 specifies fission sites together with endoplasmic reticulum contacts on mitochondria and recruits additional factors such as dynamin-1, MFF, FIS1, and MiD51 to execute scission (Sharma et al., 2019). Expression of these fission machinery components have been seen to change with age, but studies have shown that retarding fission both increases longevity in yeast but promotes mitochondrial dysfunction and insulin insensitivity in mice (Jheng et al., 2012; Scheckhuber et al., 2007). Fusion is carried out by machinery which first joins the outer mitochondrial membrane such as MFN1/2, and then the inner mitochondrial membrane using OPA1 (Tezze et al., 2017). The same machinery which
mediates fusion and fission also facilities intracellular trafficking through associations with MIRO1/2 and Milton1/2 which link mitochondrion to the actin-myosin cytoskeleton for transport (Mishra and Chan, 2014). In articulated cellular bodies such as neurons with axons spanning millimeters, mitochondria have been observed to attach to the cytoskeleton and engage in long distance anterograde and retrograde transport as a part of their turnover process (Course and Wang, 2016). During cellular division, mitochondrial networks undergo fission resulting in a larger more granular pool of mitochondria for segregation to daughter cells. At the individual organelle level, mitochondrion damaged through oxidative stress can be fused other mitochondrion as a form of functional complementation, resulting in a single mitochondrion with improved metabolic efficiency. Thus, fusion and fission, commonly termed mitochondrial dynamics, are critical processes in the homeostatic maintenance of intracellular mitochondrial populations and add a layer of complex regulation over biogenesis.

Peroxisomes are also important and entwined with discussion of mitochondria as they function in concert with mitochondria to facilitate fatty acid oxidation through lipid catabolism (Huang et al., 2017). The so-called master regulator of mitochondrial biogenesis, Pgc1a, also induces peroxisome proliferation, an indication of their co-dependency. Regulators of mitochondrial biogenesis such as Sirtuins, AMPK, and mTOR also simultaneously alter peroxisome biogenesis (Sharma et al., 2019). Alterations to peroxisome positioning during cellular division skews keratinocyte cell fate, as does knockdown of genes encoding structural components of peroxisomes such as pex11b (Asare et al., 2017).

Taken all together, a picture emerges whereby mitochondria play a pivotal role in determining epidermal stem cell fate and cell division modality, which in turn effectively dictates the biology of the skin epidermis. Mitochondrial dysfunction due to defective ROS handling in
epidermal Sod2 knockout mice reveal profound thinning of the skin reminiscent of the aging phenotype, as well as delayed wound healing (Velarde et al., 2012, 2015). A mouse with defective DNA polymerase gamma known as the POLG mutator mouse suffers a high rate of mitochondrial mutations due to deficient DNA repair develops an extreme progeroid phenotype (Trifunovic et al., 2004). Working in the opposite direction in a similar vein to the rescue of inactive atrophied muscle tissue by forced Pgc1a expression, interventions which bolster Pgc1a expression in skin also similarly rescue it from functional decline caused by age. Endurance exercise promoting the secretion of the cytokine IL-15 has been directly shown to prevent thinning of the skin in aged mice and humans while increasing expression of Pgc1a (Crane et al., 2015), and subsequent work presented in a later chapter here has extended that rescue to wound healing and improved proliferation of aged tissue \textit{in vitro} (Wong et al., 2019). Exercise also partially rescues the progeroid phenotype of the POLG mutator mouse (Safdar et al., 2011), potentially due to IL-15 signaling.

1.7 \textbf{The skin and its dynamic responses to the environment}

Adapting to environmental stress is necessary for survival. The skin epidermis forms the exterior protective barrier of animals and is the first responder to external stresses. It possesses remarkable capacity to preserve internal homeostasis. It can regulate temperature by sweating to shed heat, or by raising hairs to trap air and keep warm. In response to touch and pressure detected by sensory cells within the skin, it can undergo regional thickening of the skin to produce calluses. A hydrophobic anti-microbial mixture of oils and peptides are secreted to the skin surface as to augment the cornified envelope against moisture loss and infection. This hydrophobic milieu not only helps limit formation of ROS due to solar radiation but also contains antimicrobial peptides
and maintains the acid mantle, hydrophobic film of pH 4.5-6 which aids in neutralizing surface pathogens and together with lipids secreted by keratinocytes, aids in keeping the skin waterproof (Lovászi et al., 2017). Upon exposure to solar radiation, melanin is produced and distributed within the epidermis by melanocytes, which acts in concert with the cornified envelope to attenuate the genotoxic effects of sunlight. Sunburn occurs when high energy ultraviolet rays cause sufficient DNA damage to the epidermal cells of the skin to trigger apoptosis cell cycle arrest coupled with the DNA damage response (Ponten et al., 2001), and has been classically defined as the dose which causes erythema of the skin, which is symptomatic of inflammation and skin capillary vasodilation resulting in a grossly visible reddening of the skin surface (Blum and Terus, 1946). Apoptotic sunburned cells trigger an inflammatory response and epidermal hyperplasia peaking at 48 hours after the exposure, and pain signaling through the TRPV4 ion channel in mouse skin (El-Abaseri et al., 2006; Moore et al., 2013; Ouhtit et al., 2000). If the exposure results in sunburn, the outer layers of the cornified envelope are shed and form a fragile but light scattering protective layer over the damaged area while cells in lower layers undergo DNA repair and rapid proliferation to thicken and strengthen the skin barrier where damaged. As touched on above, hair pigmentation responds to psychological stresses as well and may act as a type of evolved biological signaling cue among social animals as signal of experience. Proliferation and differentiation within the skin is coordinated by circadian rhythms generated by the suprachiasmatic nuclei such that these delicate processes primarily occur in dark, out of sunlight.

Compromise of the barrier by wounding triggers a robust healing response. This healing response is well studied and occurs in a stereotyped manner with many similarities between mouse and man. The wound is first stabilized by clotting, which stops blood loss and further contamination of the wound. An inflammatory response begins in parallel with the recruitment of
neutrophils and macrophages to the wound to fight contamination and activate dermal and epidermal healing processes (Ellis et al., 2018). One of the main early steps of wound healing is the formation of early granulation tissue in the wound bed consisting of unorganized collagen and newly proliferated fibroblasts, but this can only occur after the inflammatory phase subsides. Together with formation of the early granulation tissue, keratinocytes become activated at the epidermal wound edges and begin to proliferate and migrate to seal the wound surface in a process known as re-epithelialization (Braiman-Wiksman et al., 2007). In mice, an interim structure (the proliferative hub) at the wound edges composed of proliferative thickened basal epidermis sustains re-epithelialization by providing keratinocytes (Fig. 1) (Aragona et al., 2017). These steps address the immediate outcome of wounding and unfold in the span of approximately 10 days in normal young mice. Wounds continue to heal for much longer, with gradual restructuring of the underlying dermal ECM, paring back of vasculature, as well as other processes. Inappropriate regulation of the inflammatory phase of wound healing is often present in chronic and poorly healing wounds (Ellis et al., 2018).

One major difference between mouse and human skin is the presence of the panniculus carnosus muscle (PC) over the majority of the mouse body, in particular over the dorsal trunk, which is a common site for wounding (Fig. 1). Contraction of the PC greatly accelerates wound healing by reducing the total wound area by over half, thus reducing the amount of proliferation and migration needed by both the dermis and epidermis (Davidson et al., 2013). While PC mediated wound contraction is evolutionarily advantageous for prey animals, it confounds efforts to model human wound healing, which exhibits very little wound contraction and heals primarily by re-epithelialization (Reinke and Sorg, 2012). Thus, strategies to mitigate wound contraction are necessary to accurately model human wound healing using the skin of rodents. Alternative self-
splinted epithelial sites exist like rodent ear, tail, and paw skin (Aragona et al., 2017; Gawriluk et al., 2016), but these sites are unsuitable for wound healing studies as they are readily accessible by the mouse and prone to interference. In addition to the those sites being susceptible to normal self-grooming behavior, they are also limited in surface area for wounding, and thus do not provide adequate resolution in tracking wound closure with daily imaging (Davidson et al., 2013; Wong et al., 2011). Another approach is to place full thickness wounds immediately behind the head of mice, where they are inaccessible. Contraction of the PC is prevented by installation of a metal or silicone ring which is sutured and glued in place and splints the wound open (Davidson et al., 2013; Park et al., 2014a). Mechanical splinting of wounds in rodent models has been demonstrated to produce wound healing kinetics and biology more akin to human wound healing, and is the method used in the work described herein to investigate the wound healing response. These splinted dorsal wound healing models in mice exhibit predictable and consistent wound closure kinetics and can expose slight wound healing impairments like those seen between young and old skin (Wong et al., 2019).

1.8 The skin and its functional decline with age

Lifelong maintenance of this barrier is critical to quality of life. However, aging decreases the structural integrity and physiologic resiliency of the skin leading to poor tissue repair and complications from unhealed wounds (Fisher et al., 2002; Giacomoni and Rein, 2014). Why skin undergoes deterioration with age not yet fully understood, but aged skin exhibits hallmarks of aging such as mitochondrial dysfunction, senescence, exhaustion and depletion of stem cell reserves, and altered cell signaling (López-Otín et al., 2013). Compromised skin barrier function, whether by aging or disease, is caused by imbalances in the regulation of cellular divisions of the
epidermis, resulting in either too much differentiation or proliferation (Asare et al., 2017). Compromise of the skin barrier in frail elderly individuals > 65 years old with other pre-existing conditions and disabilities contributes to the morbidity and mortality of wounds like diabetic skin ulcers, pressure sores, and surgical wounds and contributes to over-utilization of antibiotics (Howell-Jones et al., 2005). Dysregulation of proliferation and differentiation in the skin epidermis can also cause increased epidermal stem cell activation which accelerates wound healing but is also a causative factor in epithelial cancers (Naik et al., 2017). Despite the significant burden of impaired wound repair and skin cancer in the aged population, existing pharmacologic treatments are inadequate in many cases due to a reliance on pathways that are already dysregulated due to either age or disease (Park et al., 2014b).

Few effective treatments for poorly healing wounds in aged skin exist because the underlying reasons for why skin deteriorates with aging are poorly understood. Initial theories that epidermal stem cell counts decline with age (Giangreco et al., 2008) have since given way to more nuanced explanations based on recent findings delineating heterogeneous stem cell populations (Changarathil et al., 2019) and the contribution of resident immune cells (Doles et al., 2012; Keyes et al., 2016). A fine balance between self-renewal and lineage commitment of epidermal stem cells is necessary for proper skin function, which is impaired with organismal chronological aging (Changarathil et al., 2019; Velarde et al., 2015). One potential cause of aged skin and poorly healing wounds may be due to the effects of mitochondrial dysfunction and inflammation on epidermal stem cell fate, the so-called mitochondrial theory of aging (Stout and Birch-Machin, 2019). This theory posits that mitochondrial genomes accrue damage at a more rapid rate and are relatively inferior in DNA repair compared to the nuclear genome, and as this damage accrues over an organism’s lifetime, mitochondrial function gradually declines in an uncompensated manner.
Through the loss of mitochondrial function, tissues are less able to maintain and renew themselves at the cellular level, and as a whole these events may serve to explain how aging occurs. Given the crucial role of mitochondria and attendant peroxisomes during homeostatic cell division in the skin (reviewed earlier), their role in aging warrants further discussion.

In response to damage, the mitochondria execute apoptotic programming to induce cell death. Energy status is also integrated within mitochondria, as low energy signaling via AMPK ultimately result in activation of mitochondrial gene expression. As the multitude of environmental stresses imposed on life are distilled into the language of energy and damage at the cellular level, mitochondria are thus poised to respond comprehensively to the external world. This is illustrated elegantly through studies in *C. elegans* demonstrating that mitochondria exert not only cell autonomous but long-distance organism-wide changes in response to sensed stress. Specifically, stress imposed on cells resulting in impaired mitochondrial biogenesis trigger a cell autonomous mitochondrial unfolded protein response, and this event is communicated organism wide if it occurs in certain classes of cells such as neurons (Tatar and Sedivy, 2016). As this signal propagates throughout the organism, epigenetic changes are initiated even in distal cells removed from the actual stress event. The outcome of this long-distance transmission is priming of the organism at a cellular level to anticipate the stress event. The results of this type of adaptive response is palpable in both worms and mice, explaining the two-fold lifespan extensions attained with knockout of genes (*jmdj*-1.2, *jmdj*-1.3, and *lin*-65) within this signaling axis (Merkwirth et al., 2016). Thus, mitochondria integrate important external and internal signals in both cell autonomous and organism wide contexts to optimize for host survival under stress conditions, with important implications on longevity and healthspan.
1.9 Conclusion

Hopefully this brief overview of skin epidermal biology in vertebrates has impressed upon the reader the importance and ideal suitability of the skin as a model to study stem cell dynamics. Much is known. Much remains to be understood. The skin is uniquely poised for study of stem cell behavior as it is an evolved stratified organ with stereotypical structure and more importantly, evolutionarily refined responses to a variety of environmental stresses which can be experimentally controlled. Findings unearthed from studies within the skin can serve as the gateway to deeper understanding of the stem cell biology of other epithelialized tissues such as the eyes, lungs, kidneys, and gastrointestinal tract. Mechanical biology and the role of differential extracellular matrix compositions on the regulation and definition of stem cell niches remains a critically underexplored area, in particular in vivo. The role and responses of stem cells in tightly packed compartments to bioenergetic signaling cues represents another gap in our understanding, in particular with the view of how age-related changes in these parameters associate with impaired tissue function. The dermal-epidermal junction and how hair follicles and other epidermal appendages are patterned has been studied in the context of development in mice and humans but remains underexplored under homeostatic adult conditions. The spiny mouse exhibits complete and total epidermal regeneration after sustaining wounds of all sizes as well as resistance to hallmarks of aging and may represent a crucial model to catalyze the next round of biological insights into the skin epidermis, in particular hair follicle regeneration.

Aside from basic biology, many pertinent biomedical questions can be answered in the skin. Skin cancers such as melanomas and squamous cell carcinomas remains the most prevalent
form of cancer in humans, and occur primarily in the IFE as opposed the HF (Bedogni and Paus, 2020). The immune privilege of hair follicles and rarity with which they become the site of cancer initiation remains poorly understood but are full of potential. Mechanical forces exerted on the nucleus and how they may contribute to diseases, as well as normal cell biology can be conveniently studied in the context of the skin and inform about the ways by which highly mobile immune cells protect their nucleus. The means by which anucleate cells lose their nucleus still remains relatively poorly understood, but the epidermis is a tractable model in which to further study how it occurs, with relevance elsewhere like in the blood and eye where anucleate cells also feature prominently. Psoriatic lesions and non-healing wounds due to chronic cryptic inflammation remain significant dermatological issues which greatly detract from quality of life in afflicted individuals. Acutely, accidents resulting in extensive injuries place enormous demand on supplies of skin for grafting. For example, for extensive burn injuries, donor site skin for autologous grafts are scarce, and cultured epithelial autografts (CEA) are used (Shpichka et al., 2019). However, CEAs take weeks to prepare due to poor colony forming rates, particularly in cultures from aged individuals (Atiyeh and Costagliola, 2007). Cadaver skin can be grafted, but only as a temporary wound dressing as it is rejected by the host after 2-3 weeks (Alsbjörn and Sørensen, 1985) and supplies can be depleted by acute demand (Rogers et al., 2013). Advances in identifying discrete stem cell populations and the regulatory role of topology and biomechanical signaling hold enormous promise in addressing these biomedical challenges.
Figure 1. The skin in development, homeostasis, and wound healing. (1) The panniculus carnosus is a striated layer of muscle responsible for twitching responses and assists during wound closure. (2) Subdermal adipose tissue is involved in hair cycling and skin remodeling. (3) Reticular dermis is a dense network of fibroblasts and extracellular matrix (ECM). (4) Papillary dermis is a loose network of fibroblasts and ECM. (5) The basement membrane dividing the epidermis from the dermis. (6) Basal epidermis consisting of epidermal stem cells (ESCs). (7) Spinous epidermis produced by differentiating ESCs. (8) Granular epidermis produced by continued differentiation and stratification of the spinous layer. (9) Cornified envelope formed by terminally differentiating keratinocytes from the granular layer. (10) The proliferative hub consisting of activated ESCs formed in response to injury. (11) Granulation tissue (healing wound). (12) Eschar, coagulated and dried pus and cell debris (healing wound). (13) Epithelial tongue migrating and proliferating from the proliferative hub to seal the wound. (14) Dermal fibroblasts maintain the dermis and respond to wounding by becoming contractile myofibroblasts within the granulation tissue. (15) Newly formed wound beds are host to coagulated blood, immune cells, and activated fibroblasts. (16) A layer of dried pus and cellular debris seals the wound. (17) Sweat glands that empty to the interfollicular epidermis are important for thermal regulation. (18) Sebaceous glands are attached to hair follicles (HFs) and their secretions are important to epidermal homeostasis by maintaining an acidic pH and waterproofness. (19) Normal anagen HF with a club hair and a newly synthesizing hair shaft. The bulge is immediately below the basal layer and hosts HF and melanocyte stem cells. The outer root sheath in orange encapsulates a companion layer divider and inner root sheath in pink. Transit amplifying matrix cells in green derive from HFSCs and produce the hair shaft. (20) The arrector pili muscle is responsible for standing hairs up and goosebumps. (21) Quiescent telogen hair follicle. (22) First anagen is developmentally encoded and occurs postnatally after HF development. (23) Developing hair peg interact with dermal signals and pattern based on gradient diffusion. (24) Developing hair germs invaginate into the dermis. (25) Dermal signals nucleate the formation of the dermal placode. (26) The dermal papilla in development recruits epidermal downgrowths which will become HFs. (27) During telogen, the DP is external to the HF. (28) During anagen, the DP is incorporated closely into the bulb of the HF as it extends and proliferates to support hair shaft synthesis.
1.11 References


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Chapter 2: The exercise cytokine interleukin 15 rescues slow wound healing in aged mice
2.1 Abstract

Impaired wound healing in elderly individuals increases infection risk and prolongs surgical recovery, but current treatment options are limited. Low doses of interleukin 15 (IL-15) that mimic exercise responses in the circulation improve skin structure and increase mitochondria in uninjured aged skin, suggesting that IL-15 is an essential mitochondrial signal for healing that is lost during aging. Here we used gene microarray analysis of old and young murine epidermal stem cells and demonstrate that aging results in a gene signature characteristic of bioenergetic dysfunction. Intravenous IL-15 treatment rescued chronological aging-induced healing defects and restored youthful wound closure in old, sedentary mice. Additionally, exercise-mediated improvements in the healing of aged skin depend upon circulating IL-15. We show that IL-15 induces signal transducer and activator of transcription 3 (STAT3) signaling characteristic of young animals, reduces markers of growth arrest, and increases keratinocyte and fibroblast growth. Moreover, exercise or exercise-mimicking IL-15 treatment rescued the age-associated decrease in epidermal mitochondrial complex IV activity. Overall, these results indicate that IL-15 or its analogs represent promising therapies for treating impaired wound healing in elderly patients.

2.2 Introduction

The integrity of the skin barrier is critical for blocking pathogens and other environmental hazards and the proper maintenance of skin structure requires timely, coordinated tissue repair. Skin healing is complex and requires the coordination of clotting, the recruitment of multiple types of immune cells and significant amounts of tissue re-growth (Guo and Dipietro, 2010). Skin trauma is repaired relatively quickly in young skin, however in the elderly wounds heal more slowly (Ashcroft et al., 2002) and this older population is particularly susceptible to non-healing, chronic wounds (Margolis et al., 2002b, 2002a). Delays in skin healing dramatically increase the risk of infection, prolong recovery from surgery and increase morbidity and mortality in older adults.
(Gould et al., 2015). As a result, the care and management of non-healing ulcers or poorly healing wounds costs approximately $10-25 billion per year in the U.S. alone (Sen et al., 2009). Overall, improving skin healing in the elderly is a major challenge for medicine and new treatment strategies are needed.

Aging is broadly associated with various forms of cellular dysfunction such as genomic instability, inflammation and stem cell dysfunction (Birch-Machin and Bowman, 2016; López-Otín et al., 2013). While the mechanisms responsible for poor healing remain unclear, there is evidence that aging delays epidermal stem cell activation (Giangreco et al., 2008; Keyes et al., 2016), shifts immune cell populations (Swift et al., 2001) and alters vascular repair after injury (Swift et al., 1999). In association with these changes aged skin exhibits epidermal and dermal thinning, has lower rates of post-injury keratinocyte proliferation, and experiences slowed wound re-epithelialization. Past work has employed expression profiling of aged keratinocytes to better understand the mechanisms of epidermal dysfunction and these data suggest that changes in the cell cycle, disrupted cytokine signaling and altered growth cues may prevent rapid healing (Keyes et al., 2016). As a result of this work, keratinocyte STAT3 was identified as a promising target for reversing age-induced healing defects as it is required for re-epithelialization after skin injury (Sano et al., 1999) and dysfunctional STAT3 signaling at the wound edge is thought to impede keratinocyte proliferation in old mice (Keyes et al., 2016). However, we do not understand what triggers age-related changes in keratinocyte STAT3 signaling over the lifespan and we currently lack treatments that can restore this pathway and augment healing in aged skin.

Several therapeutic approaches have been identified that improve skin healing and reduce scarring in young, healthy skin, such as negative pressure therapy, cellular scaffolds or supplemental growth factors, however none of these therapies has proven effective at reversing
healing defects in aged skin (Han and Ceilley, 2017). There are several roadblocks to developing effective healing treatments for the elderly: 1) few studies specifically target intrinsic, aging-induced cellular dysfunction; 2) aged skin cells have impaired signal transduction responses (Beer et al., 1997), which may reduce the effectiveness of growth factors or other mitogenic treatments in aged skin; 3) excessive exposure to exogenous growth factors may cause the malignant transformation of cells or promote tumor growth in aged, cancer-prone cells. Thus, it may be more prudent to first focus on treating the pathophysiology of skin aging in order to curtail healing defects. In a similar vein, we reasoned that therapies known to successfully reverse skin aging would also improve poor skin healing.

Aerobic exercise is a robust lifestyle intervention that produces widespread health benefits over the lifespan including reduced mortality and a lower incidence of neurologic, cardiovascular and cancer-related disease (Chakravarty et al., 2008). Notably, pre- and post-operative exercise is associated with improved recovery from various types of surgery in elderly adults (Carli and Zavorsky, 2005). Exercise is also one of the few interventions shown to enhance wound healing in the aged skin of both mice (Keylock et al., 2008) and humans (Emery et al., 2005), however the mechanism underlying these benefits is not known. Since exercise has such a well-established safety profile to protect against systemic disease, mimicking exercise signaling is an ideal way to treat age-related disorders, particularly in the frail elderly that are unable to exercise. We previously discovered that regular exercise reverses some of the structural effects of epidermal and dermal aging in the skin and increases the abundance of skin mitochondria in humans and mice (Crane et al., 2015). By screening the blood of athletes, we identified exercise-induced IL-15 as the circulating signal that increases skin mitochondria. Additionally, we found that a low, exercise-mimicking dose of recombinant mouse IL-15 (rmIL-15; 25 ng/kg) provided daily for 5 weeks to
old mice was sufficient to improve skin structure, restore tissue mitochondrial abundance and reduce circulating inflammatory cytokines akin to exercise (Crane et al., 2015). This was of interest because IL-15 is considered pro-inflammatory and induces T-cell proliferation (Steel et al., 2012). Conversely, regular exercise is broadly anti-inflammatory (Petersen, 2005), but stimulates far lower levels of IL-15 in circulation than the concentrations associated with innate immune responses (Crane et al., 2015). Overall, these data suggested that increasing mitochondrial abundance in the skin is a potential way to treat skin aging and that the low pulses of IL-15 stimulated by exercise are a physiologic regulator of skin mitochondria. As circulating levels of IL-15 are reduced in aged mice (Quinn et al., 2010) and disrupted IL-15 signaling is observed in non-healing human wounds (Jones et al., 2016), we reasoned that our low-dose rmIL-15 therapy may restore youthful signaling and improve healing in aged animals.

We show that daily, low-dose injections of rmIL-15 restore youthful wound closure, increase mitochondrial activity, reduce cellular senescence and normalize STAT3 signaling in aged, sedentary mice. Additionally, we find that the improvement in wound re-epithelialization observed in old, exercise-trained mice is dependent on IL-15 in circulation, demonstrating that IL-15 is an important anti-aging exercise signal.

2.3 Results

_Epidermal aging is associated with mitochondrial dysfunction_

While the structural deterioration and slow healing evident in aged skin have been known for some time, we are only just beginning to understand the mechanisms that drive chronological skin aging. Wound closure is the primary endpoint used to assess healing therapy efficacy (Flanagan, 2003), so identifying age-related factors that regulate keratinocyte maintenance or re-epithelialization is a logical starting point to understand the basis of healing defects. Previous
research implicates senescent cells (Giangreco et al., 2008; Herbig et al., 2006; Keyes et al., 2016; Sorrentino et al., 2014), inflammation (Swift et al., 2001), and dendritic T-cells (Giangreco et al., 2008; Keyes et al., 2016) in skin aging and the resulting impairment in re-epithelialization. To gain additional insight into the underlying mechanisms of aging within the epidermis, we re-analyzed a publicly available gene expression dataset comparing FACS sorted young and old murine epidermal stem cells (ESCs, CD49f<sup>high</sup>/CD34<sup>−</sup>) collected from uninjured dorsal skin that originally examined age-related changes in circadian rhythms (GSE84511) (Solanas et al., 2017). We analyzed differentially expressed genes in young versus old ESCs irrespective of the time of day (n = 24 per age group), giving us substantial power to detect aging effects, and then employed two established differential gene expression analysis tools, GAGE (generally applicable gene set enrichment for pathway analysis) (Luo et al., 2009) and GSEA (gene set enrichment analysis) (Sergushichev, 2016). GAGE evaluates significantly differentially expressed genes without an arbitrary fold-change cut-off to identify sets of coordinately perturbed genes (up and/or down) and their related pathways, increasing the sensitivity and reproducibility of pathway analysis (Luo et al., 2009). Our GAGE analysis identified several significantly altered pathways previously associated with ESC aging such as JAK/STAT signaling and senescence, as shown by simultaneous aging associated elevations in canonical STAT signaling with pro-survival (Akt), cell cycle arrest (Cyclin D1), and anti-apoptotic signaling (Bcl-X<sub>L</sub>) (Fig. 1A, S1A, S1B). Additionally, GAGE highlighted pathways that have never been connected to ESC aging, including the cellular energy sensor AMP-activated protein kinase (AMPK) and metabolic peroxisome proliferator-activated receptor (PPAR) signaling (Fig. 1A). Pathview is a graphical display package which overlays gene expression metrics on Kyoto Encyclopedia of Genes and Genome (KEGG) pathway diagrams. Detailed networks of the JAK/STAT pathway
and AMPK signaling alterations using Pathview show comprehensive downstream pathway dysregulation (up and down) in response to aging (Fig. S1A-B). Subsequently, GSEA analysis was used to identify cellular functions consistently up-regulated or consistently down-regulated in response to aging. We found that metabolic pathways related to glucose and carbohydrate metabolism were upregulated and that mitochondrial synthesis and ATP production were downregulated in aged ESCs (Fig. 1B), suggesting a shift away from oxidative phosphorylation-derived cellular energy. A selection of differentially expressed genes from the identified pathways highlights these changes (Fig. 1C). Age-induced mitochondrial deterioration is well-described in other stem cells such as muscle and neural progenitors (Zhang et al., 2018), but these data provide the first evidence that the epidermal stem cell compartment experiences broad metabolic dysfunction during aging. Together, these data suggest that mitochondrial defects are mirrored across multiple cell types in aging skin and may drive its aging process.

**Administration of IL-15 to sedentary mice rescues defective would closure**

We have previously shown that IL-15 reverses skin aging in intact skin, but the impact of low dose rmIL-15 treatment on skin healing has not been established. Interestingly, IL-15 receptor α subunit expression (IL-15Rα) was identified through our pathway analysis to be increased in old versus young epidermal stem cells (Fig. 1C), which is analogous to greater IL-15Rα expression levels found in non-healing versus healing wounds in humans (Jones et al., 2016). Moreover, Keyes et al. identified IL-15 as one of a handful of downregulated genes in old versus young mouse epidermal keratinocytes isolated from the leading wound edge (Keyes et al., 2016) and circulating IL-15 levels are reduced in old mice (Quinn et al., 2010). In combination this suggested that IL-15 treatment may improve wound healing in aged skin. To assess this, we used our previously
described regimen of IL-15 therapy in which aged mice were pre-treated for 33 days with daily intravenous rmIL-15 (old rmIL-15, 25 ng/kg) or PBS vehicle injections (old controls) as well as a young, PBS-injected control group (Crane et al., 2015). After this pre-treatment phase, excisional wound surgery was performed and the closure of wounds was tracked over 9 days, which is the approximate time to full wound closure in young mice. Splinted, full-thickness wounds were utilized to prevent confounding wound contraction prevalent in murine wounds and to promote greater re-epithelialization akin to human wound healing as described by others (Davidson et al., 2013). Due to the long wound healing period after pre-treatment and the rapid turnover rate of the murine epidermis (Koster, 2009), we continued daily injection treatments of rmIL-15 or vehicle control until 5 days post-surgery (Fig. 2A). The young control group was largely fully healed after 9 days (> 95% original wound area healed), whereas old control mice had significantly less advanced wound closure at 9 days post-wounding (Fig. 2B-D). Remarkably, we found that rmIL-15 treatment in old mice resulted in significantly smaller wounds at 9 days post-wounding compared to old controls and their degree of healing was indistinguishable from young control mice (Fig. 2B-D). We also analyzed the rate of wound closure over time and this showed a significantly decreased wound closure rate in the old PBS group relative to young PBS, but a restoration of healing rate in rmIL-15 treated old mice (Fig. 2E). Additionally, when we extrapolated these healing rates to time until full wound closure, we found that old control mice were projected to heal approximately 2 days slower than both young control and old rmIL-15 treated groups (Fig. 2F).

It has been recently shown that a transiently thickened region of Keratin 14 positive epidermal progenitors forms inward from the original wound margin, termed the proliferative hub (Aragona et al., 2017). This thickened hub sustains the extending epithelium as the wound closes
but resolves and thins when re-epithelialization is completed. This phenomenon has not yet been investigated in aged mouse skin, so we sought to use it to better understand the impact of our therapy on the extent of re-epithelialization. Thus, we stained day 9 post-wounding skin cross-sections for Keratin 14 and 5-ethynyl-2’-deoxyuridine (EdU) incorporation and assessed the average thickness of the Keratin 14 layer 300 microns toward the center of the wound from the original wound margin guided in part by EdU staining. We found that old control mice had a hyper-thickened proliferative hub at day 9 post-wounding compared to young controls, suggesting a less advanced stage of re-epithelialization, however, the old rmIL-15 treated group had a reduced proliferative hub thickness compared to old controls and this thickness was indistinguishable from young mice (Fig. 2G-H). Thus, short-term IL-15 therapy is able to restore a youthful rate of skin healing in aged mice.

**Recombinant IL-15 therapy rescues age-associated changes in STAT3 and cellular senescence in vivo**

Since STAT3 signaling is implicated in keratinocyte aging (Keyes et al., 2016), we sought to clarify how it was impacted by aging and IL-15 therapy in vivo. Using the intact skin excised during wound surgery, we found that staining for nuclear STAT3 phosphorylated at tyrosine 705 (pSTAT3Y705) was higher in interfollicular epidermal cells and dermal fibroblasts (vimentin+ dermal cells) of old control mice compared to young controls, however, rmIL-15 treatment largely abrogated this elevation in both cell types (Fig. 3A-B). Conversely, STAT3 phosphorylation at its S727 site (pSTAT3S727) was reduced in old versus young control mice yet restored in old rmIL-15 treated mice (Fig. 3A, C). Thus, the two STAT3 regulatory sites are altered in opposite directions by both aging and rmIL-15 treatment within the epidermis and dermal fibroblasts. Since we have
previously shown our rmIL-15 regimen increases mitochondrial activity in whole mouse skin and within cultured human fibroblasts (Crane et al., 2015), we sought to assess whether these changes occurred in isolated epidermis. Thus, we measured complex IV activity in whole epidermal protein lysates from each group. We found that complex IV activity tended to be reduced in young versus old control mice ($p = 0.097$, Fig. S2A). Additionally, old mice treated with rmIL-15 had significantly greater complex IV activity than old control mice (Fig. S2A). These data show that rmIL-15 treatment augments epidermal mitochondrial function.

Cellular senescence has also been implicated as a driver of aging in numerous tissues (Childs et al., 2015) and biomarkers of senescence such as the loss of nuclear proteins HMGB1 or Lamin B1 occur more frequently in aged skin (Davalos et al., 2013; Freund et al., 2012; Wang et al., 2017). Thus, an increased burden of growth arrested cells is a logical explanation for at least some of the age-associated delays in cell growth during wound healing. Given the improved cellular re-growth after injury found in old rmIL-15-treated mice, we reasoned that this treatment would be associated with a lower burden of cellular senescence. To assess senescence, we quantified the presence or absence of nuclear HMGB1 and nuclear Lamin B1 levels via immunofluorescence in intact skin samples collected during wound surgery. We found that nuclear HMGB1 staining was reduced in epidermal cells and dermal fibroblasts of old control mice relative to young control, but that this loss was not observed in the old rmIL-15 treated mice (Fig. S2B-C). However, the aging-associated loss of nuclear Lamin B1 observed in old control mice compared to young controls was only partially rescued by rmIL-15 treatment in the epidermis and was not impacted by rmIL-15 therapy in dermal fibroblasts (Fig. S2B, D). The difference in response between senescence biomarkers is likely due to HMGB1 being more related to transient growth
arrest (p53-related), whereas Lamin B1 is more reflective of terminal growth arrest (Rb/p16\textsuperscript{ink4a}).

Taken together, IL-15 therapy reduces the proportion of senescent cells in aged skin.

IL-15’s most well-established physiologic function is in innate immunity where it activates and expands T-cells and NK cells and this function has been leveraged as an immunotherapy to reduce the growth of some types of cancers (Steel et al., 2012). However, IL-15’s effects on T-cell proliferation and its anti-tumor activity typically require at least 3 orders of magnitude higher dosing than the dose we utilized in our experiments (Zhang et al., 2009). Nevertheless, because T-cells play a prominent role in wound healing (Keyes et al., 2016), we wanted to assess the impact of our recombinant IL-15 treatment on this immune population. First, we assessed spleen weights across all of our treatment groups to ascertain any broad changes in immune system activation. Old mice had greater spleen weights (as a percent of body mass) compared to young mice, but this was similar between old control and old rmIL-15 treated mice (Fig. S2E). We then assessed T-cells in the epidermis by staining for the T-cell marker CD3 in day 33 intact skin collected during wound surgery. However, we found no differences between our treatment groups in the proportion of CD3\textsuperscript{+} cells within the epidermis (Fig. S2F). Finally, we quantified circulating, peripheral blood cytotoxic T cells (CD3\textsuperscript{+}/CD8\textsuperscript{+}) by flow cytometry in our experimental cohort after 31 days of pre-treatment and just prior to wound surgery. Similar to epidermal T-cells, we found no changes in the abundance of circulating cytotoxic T-cells across our treatment groups (Fig. S2G). Thus, our dose of IL-15 does not appear to expand circulating or resident epidermal T-cells or overtly activate the immune system in aged mice.

To gain further insight into how IL-15 was working, we next sought to examine whether this benefit was specific to aged skin. We repeated our experiment of 33 daily injections of rmIL-15 or PBS vehicle prior to wound surgery in young mice. We presumed that the rate of re-
epithelialization and healing was somewhat maximal in young skin. In agreement with this hypothesis, we found that rmIL-15 treatment in young mice did not impact day 9 wound closure or the rate of wound healing compared to young control mice (Fig. S3A-D). However, there was a modest trend for a reduction in projected time until closure (< 1 day, \( p = 0.0502 \), Fig. S3E). This suggests that young skin is able to “catch up” at later stages of healing which somewhat negates the benefits of IL-15 therapy. Since IL-15 treatment impacted pSTAT3 signaling in aged skin, we again measured the proportion of skin cells with strong nuclear pSTAT3\(^{Y705}\) and cellular pSTAT3\(^{S727}\) in tissue cross sections. We found that rmIL-15 treatment in young mice only produced significant changes in interfollicular epidermal cells, but not dermal fibroblasts, reducing nuclear pSTAT3\(^{Y705}\) positive cells and increasing pSTAT3\(^{S727}\) positive cells compared to young vehicle controls (Fig. S3F-I). Thus, recombinant IL-15 treatment does not appreciably impact healing in young mice and modestly alters STAT3 signaling in the epidermis.

**IL-15 directly increases the cell growth of mouse fibroblasts and keratinocytes**

Our data clearly show that low-dose IL-15 improves the pathophysiology of aged skin and its healing, but it was still unclear whether this was a direct, cell autonomous effect. We have previously shown that post-exercise levels of recombinant human IL-15 (10 pg/mL) stimulate human dermal fibroblast proliferation (Crane et al., 2015), so we mirrored these conditions using post-exercise levels of IL-15 found in mice (100 pg/mL). To examine whether low-dose IL-15 acts directly on mouse keratinocytes, we employed an explant culture method that recapitulates age-related growth defects in vitro and exposed young and old skin explants to vehicle or 100 pg/mL of rmIL-15 immediately after plating. After 48 hours of treatment, young mouse keratinocytes had a larger average area of outgrowth than old keratinocytes and rmIL-15 treatment was able to rescue
growth defects in old explants to that of young mice (Fig. 3D-E). To more specifically characterize the cell composition of rmIL-15 stimulated cellular growth, we stained outgrowth for the epidermal stem cell marker Keratin 14, keratinocyte differentiation markers Keratin 10 and Loricrin, and the fibroblast marker Vimentin. We observed that virtually all of the rmIL-15 stimulated outgrowth from aged skin explants after 48 hours of culture was positive for Keratin 14, with occasional Loricrin positive cells and no visible Keratin 10 or Vimentin staining (Fig. 3F-G). This demonstrates that rmIL-15 treatment rescues growth defects in aged skin explants primarily by augmenting epidermal stem cell growth. Finally, since we observe many parallel effects on both keratinocytes and fibroblasts in vivo, we sought to test the effect of 100 pg/mL IL-15 on mouse fibroblast growth. Primary mouse dermal fibroblasts showed a significant increase in proliferation when exposed to rmIL-15 for 72 hours and this effect is blocked when the cells are treated with the JAK inhibitors ruxolitinib or tofacitinib prior to rmIL-15 (Fig. 3H-I). Thus, low dose IL-15 acts cell autonomously on mouse epidermal stem cells and fibroblasts to stimulate cell growth in a JAK-dependent manner. In a separate experiment, we measured the acute response of pSTAT3Y705 and pSTAT3S727 relative to total STAT3 in fibroblasts exposed rmIL-15 via western blotting. Since the cytokine IL-6 is well known to signal via STAT3 Y705 phosphorylation, we used this as a reference for comparison. IL-15 treatment only increased pSTAT3S727, whereas IL-6 only induced pSTAT3Y705 in comparison to untreated cells (Fig. 3J-L). Therefore, unlike the classical STAT3 activator IL-6, low-dose IL-15 increases pSTAT3S727 and not pSTAT3Y705.

Exercise improves wound healing in aged mice via IL-15 signaling

Exercise is one of the only interventions that has been shown to improve wound healing in both aged mice (Keylock et al., 2008) and humans (Emery et al., 2005), however the underlying
mechanism of this effect is not known. Since our rmIL-15 therapeutic regimen mimics physiologic elevations of post-exercise IL-15 in circulation, we sought to test whether this signaling was essential for the benefits of exercise on wound healing in aged skin. Thus, we subjected 2 groups of old mice to daily treadmill exercise for 33 days, with one group receiving IL-15 neutralizing antibody treatment (Old EX-nAb) and the other receiving control IgG antibody (Old EX) prior to each exercise session. Additionally, we included young and old sedentary control groups (Young SED, Old SED) that also received daily IgG control antibody injections. After 33 days of pre-treatment, we again performed splinted wound surgery and monitored healing over 9 days. To sustain our therapeutic window and in order to be congruent with the prior work showing that exercise improves wound healing in old mice (Keylock et al., 2008), we continued the exercise and injection treatments for 5 days post-surgery (Fig. 4A). As might be expected, the continuation of exercise treatment after wounding surgery produced significantly more variability in day 1 to 5 of healing than in sedentary mice. Despite this, we found that aging resulted in impaired healing by day 9 compared to young sedentary controls, yet exercise treatment reversed this effect in old exercised mice versus old sedentary mice (Fig. 4B-D). Remarkably, old exercised mice receiving IL-15 neutralizing antibody had similar day 9 wound closure as the sedentary old mice, demonstrating that IL-15 signaling is essential for the effects of exercise to speed wound closure in aged skin (Fig. 4B-D). There was no effect on the rate of wound healing across the 4 groups (Fig. 4E). Analyzing the projected time until full wound closure revealed a significant 1.5 day difference between young, sedentary control and old sedentary mice groups, but projected healing times for old exercised and old exercised with neutralizing antibody groups were not significantly different compared to the young group (Fig. 4F). We then measured the Keratin-14 positive epidermal proliferative hub using day 9 post-wounding skin tissue cross-sections. We again found
that old sedentary mice had a significantly thickened proliferative hub compared to young sedentary mice, however this was reduced to the thickness of young mice in exercised old mice (Fig. 4G-H). Importantly, old exercised mice that received IL-15 neutralizing antibody had greater proliferative hub thickness than old exercised and young sedentary mice (Fig. 4G-H), further supporting the necessity of IL-15 for the beneficial effects of exercise on healing. These findings corroborate the work of others showing that exercise improves wound closure and re-epithelialization in old mice and we extend these results by showing that it is dependent on IL-15 in circulation.

Since recombinant rmIL-15 therapy in sedentary mice corrected age-induced changes in STAT3 signaling, we sought to examine this and additional aspects of mitochondrial and inflammatory signaling within our exercise experiment. Using intact skin excised during wound surgery, we again stained and quantified nuclear pSTAT3Y705- and pSTAT3S727-positive epidermal and dermal fibroblast cells. We found that old sedentary mice had more pSTAT3Y705-positive cells than young mice but that this was abrogated in old exercised mice (Fig. 5A-B). Additionally, old exercised mice receiving IL-15 neutralizing antibody had more pSTAT3Y705-positive cells than young sedentary mice or old exercised mice (Fig. 5A-B). Consistent with the inverse relationship of pSTAT3S727-positive cells to those with pSTAT3Y705, we found fewer pSTAT3S727-positive cells in old sedentary versus young sedentary mice (Fig. 5A, C). In addition, old exercised mice had more pSTAT3S727-positive cells than old sedentary animals, and this effect was lost in old exercised mice receiving IL-15 neutralizing antibody (Fig. 5A, C). Thus, exercise reverses aging-induced changes in STAT3 signaling in skin and this is dependent upon circulating IL-15.

Given the dual role of the Y705 and S727 phosphorylation sites in regulating inflammatory and mitochondrial signaling, respectively, we sought to characterize downstream epidermal gene
expression of these processes in response to aging and exercise. To capture in vivo gene expression changes in response to our treatment, we used whole epidermis separated from intact skin using dispase enzymatic digestion. Consistent with the downregulation of mitochondrial signaling observed in aged ESCs (Fig. 1), we found lower mRNA expression of the mitochondrial complex I subunit Ndufs2 in old sedentary mice compared to young sedentary mice (Fig. 5D). Interestingly, epidermal Ndufs2 mRNA expression in old exercised mice was indistinguishable from young sedentary mice, but remained lower in old exercised mice receiving IL-15 neutralizing antibody (Fig. 5D). Additionally, while Cox7B mRNA was not altered in young sedentary versus old sedentary mice, it was significantly elevated in old exercised mice compared to young sedentary mice (Fig. 5D). However, old exercised mice treated with IL-15 neutralizing antibody exhibited no difference in Cox7B mRNA compared to young or old sedentary mice. There were no significant changes in the complex III subunit Uqcr10 or the mitochondrial regulator Pgc1a between treatment groups (Fig. 5D). To corroborate these mitochondrial changes, we again measured Complex IV activity in epidermal protein lysates. We found that old sedentary mice, but not old exercised mice, had significantly lower complex IV activity than young sedentary mice (Fig. 5E). Additionally, old exercised mice that received IL-15 neutralizing antibody had significantly reduced complex IV activity compared to young sedentary mice and old exercised mice (Fig. 5E).

When assessing epidermal inflammatory genes, we found that old exercised mice exhibited significantly higher Cxcl1 mRNA than young control or old control mice, but old exercised mice receiving IL-15 neutralizing antibody had comparable expression to young and old control mice (Fig. 5F). Similarly, old exercised mice, but not exercised mice receiving IL-15 neutralizing antibody, had greater epidermal Cxcl2 expression than old control mice (Fig. 5F). We also assessed
the mRNA expression of the pro-inflammatory cytokine IL-6 (*Il6*), a classical activator of pSTAT3\(^{705}\) signaling, where we expected an age-induced elevation in *Il6* expression as seen in ESCs (Fig. 1C). Unexpectedly, we found that epidermal *Il6* was reduced in old versus young control mice, but was restored in old exercised mice to that of young control mice (Fig. 5F). Moreover, old exercising mice given neutralizing antibody had significantly lower *Il6* than young controls (Fig. 5F). Finally, we measured *Il1b* mRNA, but found no differences between treatment groups (Fig. 5F). Collectively, these data demonstrate that exercise depends upon circulating IL-15 to reverse aging-induced changes in epidermal mitochondrial and cytokine signaling. Finally, since we have shown that exercise and IL-15 therapy each independently reverse age-induced epidermal thinning, we sought to test if circulating IL-15 was essential for this effect of exercise in old mice. As we have previously observed, old control mice had reduced epidermal thickness versus young controls, but this was restored in old, exercised mice (Fig. 5G-H). However, old exercising mice receiving IL-15 neutralizing antibody had significantly lower epidermal thickness that old exercised mice (Fig. 5G-H), showing that exercise-induced IL-15 signaling is essential for the restoration of youthful epidermal structure.

**Exercise-mediated changes in skin cell senescence partially depend upon circulating IL-15**

Since recombinant IL-15 therapy was able to reverse the pattern of epidermal and dermal senescence in aged skin, we reasoned that exercise training in old mice would produce similar effects. Thus, we again assessed indicators of skin cell senescence (i.e. loss of nuclear HMGB1 or Lamin B1) in our exercise cohort. We found that the number of cells with nuclear HMGB1 in epidermal cells and dermal fibroblasts was again reduced in old versus young sedentary mice (Fig. S4A-B). This loss of nuclear HMGB1 seen with aging was rescued in old exercised mice to that
of young sedentary mice, however the rescue was completely abrogated by IL-15 neutralizing antibody treatment (Fig. S4A-B). Additionally, the proportion of epidermal cells and dermal fibroblasts with positive staining for nuclear Lamin B1 levels was reduced in old versus young sedentary mice but was restored to young levels in old exercised animals (Fig. S4A, C). Old exercised mice receiving IL-15 neutralizing antibody had fewer dermal fibroblasts with nuclear Lamin B1 compared to old exercising mice, yet the proportion of epidermal cells with nuclear Lamin B1 was similar in old exercised mice treated with or without neutralizing antibody (Fig. S4A, C). Additionally, there were no significant changes in the epidermal mRNA expression of the cell cycle regulators p21 or p16\textsuperscript{ink4a} in response to aging or exercise (Fig. S4D). Therefore some, but not all, of the benefits of exercise to mitigate skin growth arrest are derived from IL-15 signaling.

### 2.4 Discussion

The poor healing ability of aged skin is a major medical issue (Ashcroft et al., 2002), but we lack therapies for this debilitating problem. Wound closure defects in the elderly are especially challenging to manage because this population frequently undergoes surgery, has a greater risk of open skin lesions (i.e. bedsores), and often suffers from disorders that impede tissue repair such as vascular disease or diabetes. The identification of new therapies to treat poorly healing wounds has stalled in recent decades and only 3 healing therapies have been approved for clinical use in the last 24 years (Eaglstein et al., 2012). One reason for this lack of progress is that the underlying cellular defects responsible for aging are not well understood and potential therapies must overcome impairments in signal transduction in aged cells (Gould et al., 2015). To begin addressing these problems we have used a highly powered bioinformatics approach to associate several known pathways of keratinocyte aging, such as JAK/STAT signaling and cellular
senescence, to an area not previously connected to healing defects: bioenergetic dysfunction. We establish that daily pulses of IL-15 via injection or exercise, which boost mitochondrial abundance (Crane et al., 2015), restores youthful keratinocyte STAT3 signaling, reduces markers of growth arrest and improves healing in aged skin. We observe that aging impedes wound healing by about 2 days, which is in line with the delay in healing observed in old versus young humans (Holt et al., 1992). Moreover, our finding that IL-15 can speed wound closure by ~2 days is similar to the improvement seen following becaplermin (PDGF) treatment in ischemic and hyperglycemic mouse models (Uhl et al., 2003). However, in more severe clinical healing disorders, such as lower extremity ulcers, becaplermin treatment is able to reduce wound healing time by multiple weeks (Smiell et al., 1999). Thus, IL-15 may have more substantial healing benefits if it is used to treat severe skin ulcers.

We opted to use intravenous tail vein injections to mirror the post-exercise secretions of IL-15 into the blood based around our prior work (Crane et al., 2015). We understand that a more practical delivery method is therapeutically desirable, however, drug delivery to the wound bed directly has its own confounding issues. First, healing wounds have a very proteolytic environment, so the bioavailability of rmIL-15 could be severely impacted and potentially in a different manner in young and old wounds. Second, most carrier formulations (i.e. ointments) make it difficult to visualize the leading edge of the wound for closure measurements. It is likely that a water-soluble small molecule would get around these issues, but this requires further investigation. Nevertheless, our data demonstrate that intravenous IL-15 administration or exercise reverse fundamental aspects of skin aging and suggest that targeting these downstream molecular pathways can guide future efforts to improve poor skin healing.
Tissues directly involved in exercise such as skeletal muscle or the heart positively adapt over time to improve their physiological function. This adaptation occurs in response to a wide variety of cellular cues, including mechanical stress, calcium fluctuations and other complex signals that would be difficult to recapitulate with drug injections (Egan and Zierath, 2013). Thus, it is notable that a single factor such as IL-15 can enhance wound closure in aged skin and is the responsible exercise signaling factor for healing. We believe that IL-15 is effective for several reasons. First, healing is complex and treatments likely need to impact multiple facets of tissue remodeling and regrowth to be effective. We find that the actions of IL-15 are mirrored in keratinocytes and dermal fibroblasts in vitro and in vivo, suggesting direct, multi-potent benefits of IL-15 treatment. Second, we find that IL-15 is able to reverse basic aspects of cellular aging, which likely permits enhanced tissue re-growth after injury. IL-15 restored epidermal mitochondrial (Ndufs2, Cox7b) and cytokine mRNA (Il6, Cxcl1, Cxcl2) as well as mitochondrial enzyme activity in aged epidermis. However, low dose IL-15 does not increase nuclear pSTAT3Y705, suggesting a re-sensitization of keratinocyte recruitment or activation that became lost during aging. This idea fits well with prior research showing that circulating IL-15 is reduced in aged mice (Quinn et al., 2010) and IL-15 mRNA expression is lower in old vs. young keratinocytes at the leading edge of a healing wound (Keyes et al., 2016). However, whether the loss of IL-15 signaling itself or other factors initially trigger skin aging and defects in tissue healing remains unknown.

Our understanding of how mitochondrial signaling and energy generation impact wound healing in the skin is limited, particularly in the context of intrinsic, chronological aging. However, there is some evidence for the involvement of mitochondrial deterioration in the other form of skin aging, UV-radiation induced photoaging. This type of aging is primarily due to exposure to UV-
A and UV-B radiation in sunlight, which causes both nuclear and mitochondrial DNA damage and also increases the release of mitochondrial free radicals (Birch-Machin and Bowman, 2016). Human photoaged skin exhibits a high incidence of large-scale mtDNA deletions including two characteristic 3895-base pair and 4977-base pair common deletions which are significantly elevated compared to non-photoaged skin from the same subject (Birch-Machin and Swalwell, 2010). These mtDNA deletions target portions of Complex I and IV, which impairs mitochondrial ATP generation (Naidoo et al., 2018).

The role of mitochondrial function in chronological aging-associated disease has been extensively investigated in other organs, however its impact in the skin is relatively understudied. The most direct example of mitochondrial dysfunction in driving tissue aging is the progeroid aging phenotype of polymerase gamma mutator mice (PolG mice) that have error prone replication of their mitochondrial DNA (mtDNA) (Trifunovic et al., 2004). These animals systemically accumulate excessive mtDNA mutations, have reduced mitochondrial energy generation, and experience accelerated aging throughout the body including the skin. Notably, treatments that boost cellular metabolism such as endurance exercise (Safdar et al., 2011) or the drug bezafibrate (Dillon et al., 2012), rescues the epidermal, dermal and sub-dermal adipose layer thinning of the skin observed in PolG mice. The striking reversal of progeroid aging in PolG mice by exercise training (Safdar et al., 2011) was the initial catalyst for our previous investigation regarding exercise-induced circulating factors that impact skin aging (Crane et al., 2015). Based upon these initial observations in uninjured skin we now show that circulating IL-15 is part of a critical signaling circuit that restores aged skin repair through increased re-epithelialization and an enhancement in epidermal stem cell growth. Our findings add to the growing body of literature detailing how physical activity improves health and combats disease such as studies showing that
exercise augments the post-injury repair of cardiac muscle (Vujic et al., 2018) and peripheral nerves (Park and Höke, 2014).

Unlike other stem cell populations, aging does not reduce the total number of epidermal stem cells, but does appear to stall their growth potential in vitro as well as their proliferation after wounding (Giangreco et al., 2008; Keyes et al., 2016). While slow healing in aged skin has been associated with altered keratinocyte STAT3 signaling (Keyes et al., 2016), there is little known about the role of cellular senescence. The delay in keratinocyte proliferation after wounding in healthy, aged mouse skin implies transient pausing of the cell cycle rather than extensive permanent growth arrest. Our data corroborate this idea and show that aging alters the alarmin and transient senescence marker HMGB1 but does not impact permanent senescence markers such as p16\textsuperscript{ink4a}. Additionally, we show that both HMGB1 and STAT3 dynamically respond to aging, exercise and IL-15 treatment in the skin. This is not entirely unexpected as both factors activate pro-inflammatory processes after stress (Davalos et al., 2013; Horiuchi et al., 2017; Yu et al., 2009). Notably, a similar loss of HMGB1 has been associated with poor wound healing in diabetic skin and exogenous HMGB1 treatment restored diabetic healing defects (Straino et al., 2008), however this prior study did not examine skin STAT3 signaling. Thus, changes in STAT3 may drive the loss of HMGB1 from the nucleus or vice versa to stall the cell cycle and delay re-epithelialization, but more work is needed regarding the interaction of these pathways in the context of wound re-epithelialization.

While the biology of STAT3 has been extensively studied in disease, until now, all of our knowledge regarding STAT3 in aging skin has focused on its Y705 phosphorylation site, which is well-established to increase in the aged epidermis (Doles et al., 2012; Keyes et al., 2016). However, no studies have previously characterized aging-induced changes to the STAT3 S727
site. Classical pro-inflammatory cytokines such as IL-6 or GM-CSF increase STAT3\(^{Y705}\) phosphorylation as well as induce senescence (Kojima et al., 2013), and this class of cytokines is increased in the blood during aging as a consequence of low-grade inflammation (Franceschi and Campisi, 2014). However, it has not been clear how one might physiologically reverse these cellular changes. Mechanistically, increasing pSTAT3\(^{S727}\) is one of the only known ways to promote dephosphorylation of pSTAT3\(^{Y705}\) via upregulation of the phosphatase TC45 (Wakahara et al., 2012). At the same time pSTAT3\(^{S727}\) also directs the mitochondrial localization of STAT3 and the resulting enhancements in bioenergetic function (Wegrzyn et al., 2009). Since we show increased cellular pSTAT3\(^{S727}\) after IL-15 treatment and exercise, this seems the most likely explanation for the corresponding reduction in pSTAT3\(^{Y705}\). Thus, there appears to be a counterbalancing role for pSTAT3\(^{S727}\) in both cellular metabolism and cell growth during wound repair through moderation of pSTAT3\(^{Y705}\)-mediated signaling.

These studies expand our understanding of the health benefits of exercise in reducing the risk of age-related disease. Our findings suggest that other skin or epithelial disorders may be improved by regular exercise or conversely worsened by a sedentary lifestyle. Some indications of this connection have been established as vigorous physical activity adherence is associated with a lower risk of the inflammatory skin disorder psoriasis (Frankel et al., 2012). Additionally, our findings have high translational potential as they are rooted in the physiologic exercise signaling observed in mice and humans and exercise is already known to improve healing in elderly subjects. Finally, higher doses of recombinant human IL-15 are already FDA approved for cancer immunotherapy, potentially reducing regulatory hurdles in applying a lower dose as a treatment for poorly healing wounds. Overall, these studies demonstrate that IL-15 therapy is a safe and efficacious means of reversing wound healing defects in aging skin.
2.5 Experimental procedures

Cell Culture

Primary dermal fibroblasts were isolated by liberase (Sigma) enzymatic digestion of the underarm skin from adult female C57Bl/6 mice as described (Seluanov et al., 2010). Cells were cultured at 37°C in DMEM (VWR) supplemented with 15% FBS (Peak Serum), 1% glutamine (Gibco), 1% penicillin (Gibco), and 1% streptomycin (Gibco) under a 5% CO₂ atmosphere and experiments were performed on cells at passage 3-6. For proliferation, fibroblasts at approximate 70% confluence were first pre-treated with 0.4 µg/mL ruxolitinib (Cayman), 0.4 µg/mL tofacitinib (AdipoGen), or vehicle control (DMSO), and then 3 hours later were treated with 100 pg/mL rmIL-15 or vehicle (PBS). After 72 hours, cells were counted using Trypan Blue dye exclusion on a hemocytometer. For STAT3 western blotting, fibroblasts at approximately 70% confluence were treated with either vehicle control (PBS), 100 pg/mL rmIL-15 (R&D Systems #447-ML/CF), or 100 ng/mL rmIL-6 (R&D Systems #406-ML/CF) for 30 minutes/ Cells were then collected in ice cold RIPA lysis buffer (Boston BioProducts, 50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM DTT, Pierce Complete Protease and Phosphatase inhibitor (Thermo Scientific)) and frozen in liquid nitrogen for later western blotting.

Keratinocyte explant culture was performed by shaving and depilating telogen dorsal back skin from C57Bl/6 mice (3-5 months old for young and 23-24 months old for old) using clippers and NAIR before skin excision. The excised tissue was then incubated for 3 minutes in a solution of povidone-iodine, then twice in a solution of 70% ethanol for a total of 3 minutes, and finally rinsed in sterile PBS. The skin was then pinned epidermis side down and scraped with a beveled scalpel blade until all subdermal adipose had been removed and the dermis was abraded and translucent. A 4 mm biopsy punch was used to excise individual explants, which were plated
dermis side down into a 24-well tissue culture plate (BioLite, Thermo) with a 1 hour drying period to promote explant adhesion. Following the drying period, 400 µL of media (DMEM supplemented with 15% FBS, 1% glutamine, 1% penicillin, and 1% streptomycin) was added in order to submerge the explant. Explants were cultured at 37°C with 5% CO₂ and were cultured for 48 hours after treatments before imaging cell outgrowth.

All fibroblast and keratinocyte explant experiments were performed in triplicate technical replicates to generate data for a single biological replicate (i.e. $n = 1$ for each unique mouse donor). Primary mouse fibroblast identity was determined by morphology. Primary mouse keratinocyte identity was confirmed by staining for Keratin 14, Keratin 10, or Loricrin as well as negative staining for Vimentin.

_Tissue Lysis and Western Blotting_

Freshly dissected epidermis was separated from dermis using dispase (Roche) enzymatic digestion for approximately 1.5-2 hours at 37°C. Isolated epidermis was then homogenized in lysis buffer (50 mM HEPES, 150 mM NaCl, 100 mM NaF, 10 mM Na pyrophosphate, 5 mM EDTA, 250 mM sucrose, 1 mM DTT, 1% Triton-X, and Pierce Protease and Phosphatase Inhibitor solution (Thermo Scientific)). Primary dermal fibroblast and epidermal lysates were homogenized by freeze thaw 3 times in liquid nitrogen followed by passage through a 27-gauge needle to ensure complete lysis. The protein concentration of epidermal lysates was assessed using a BCA kit (Pierce). For the fibroblast analysis of STAT3, 20 µg of protein per sample was prepared for SDS-PAGE by boiling in reducing 4x SDS-sample buffer (Boston BioProducts) for 5 minutes at 98°C. Samples were loaded on a Mini-PROTEAN 4-15% TGX Stain-Free Tris/tricine pre-cast gel (Bio-Rad) and run with Laemmli SDS-Page running buffer (Bio-Rad) for approximately 90 minutes at
100 V. Gels were semi-dry transferred onto PVDF membranes using the Trans-Blot Turbo system (Bio-Rad) and all imaging was done with a ChemiDoc MP (Bio-Rad). Membranes were blocked and antibodies were diluted in Tris-buffered saline with 0.1% Tween-20 and 5% bovine serum albumin fraction V (Bio-Rad). Primary antibodies and their usage were as follows: β-actin (rabbit, Cell Signaling #4967, 1:1000) total STAT3 (rabbit, Cell Signaling #30835S, 1:1000), pSTAT3<sup>Y705</sup> (rabbit, Cell Signaling #9145, 1:1000), pSTAT3<sup>S727</sup> (rabbit, Abcam #AB30647, 1:1000). Membranes were stripped and re-probed using Restore Western Blot Stripping Buffer (Thermo) between pSTAT3<sup>S727</sup>, pSTAT3<sup>Y705</sup>, and total STAT3. Secondary antibody used was anti-rabbit-IgG-HRP (H+L) (goat, Bio-Rad, 1:1000) with Clarity Western ECL Substrate (Bio-Rad) solution. Band densitometry was measured in Image Lab (Bio-Rad) to quantify relative phosphorylation normalized to β-actin. Western blotting was performed in triplicate with three biologically unique primary fibroblast cultures.

**Animals**

All animals used for the chronological aging experiments were young, adult (3-5 months old) or old (23-24 months old) female C57BL/6 mice. Female mice were used because the thickness of the skin and the rate of wound healing differs between male and female mice, and because female mice are less prone to fighting and barbering when group housed. Mice were randomly allocated to treatment groups prior to the interventions. During the study, mice were group housed except after wounding surgery when they were individually housed to minimize disturbance to the wound site. All mice were maintained in an AAALAC approved animal facility and all procedures were approved by the Northeastern University IACUC according to NIH standards.
In vivo Treatments

Old exercising mice that received IL-15 neutralizing antibody or IgG control antibody were exercised daily on a 15-degree incline using a 6-lane rodent treadmill (Columbus Instruments) at 16 m/min pace after initial 1 week ramp up from 10 m/min. Exercising mice underwent training for 33 days plus 5 days post-wound surgery but did not exercise on the day of surgery. Post-surgery the exercising mice ran at 8 m/min to minimize wound aggravation. Recombinant mouse IL-15 (rmIL-15) (R&D Systems #447-ML/CF) was administered at 25 ng/kg body weight by tail vein injection. IL-15 neutralizing antibody (R&D Systems #AF447) or normal goat IgG control antibody (R&D Systems #AB-108-C) was provided at 8,750 ng/kg body weight, via tail vein injection daily prior to exercise. Compounds were administered between 2-4 hours prior to treadmill exercise sessions, once daily for 38 consecutive days.

Wounding Surgery

Prior to surgery, all mice were anesthetized with isoflurane, provided subcutaneous carprofen for pain relief (5 mg/kg) (TCL) and hair was shaved with clippers. The skin was then swabbed with povidone-iodine (APLICARE) and ethanol. Thereafter, two 6 mm diameter dorsal full-thickness wounds were excised using a dermal biopsy punch (Medline) and this sample was used for intact skin histology and mRNA analysis. Wounds were made in telogen skin in all experiments. Wounds were splinted using a 10 mm inner diameter stainless steel ring (Grainger) secured with 4-6 monofilament nylon 5-0 sutures (Oasis) and cyanoacrylate adhesive (Krazy Glue) as described (Davidson et al., 2013). A clean Tegaderm (3M) wound dressing was loosely applied around the circumference of the mice over the wounds to cover the splinted wounds. Carprofen (5
mg/kg) was administered subcutaneously for the following 3 post-operative days. Starting on the
day of surgery, wounds were photographed daily using a DMC-FZ70 camera (Panasonic) when
the Tegaderm dressing was changed until 9 days post-surgery. Wound site photography lighting
conditions and imaging parameters on the camera were kept identical between days. Proliferating
cells were labeled using 100 mg/kg BrdU (Sigma) that was administered intraperitoneally 24 hours
before wounding or 100 mg/kg EdU (Cayman Chemical) that was administered intraperitoneally
24 hours before sacrifice. Wound surgery occurred on day 33.

**Histology and Immunofluorescence**

Tissues were fixed overnight in 10% neutral buffered formalin, stored in 70% ethanol at
4°C, processed by automated tissue processor (Thermo HistoStar), and embedded in paraffin. A
microtome (Leica) was used to cut 4 µm skin cross-sections, which were allowed to dry overnight
before de-waxing and further processing. Heat induced epitope retrieval was performed on de-
waxed slides with either citrate or Tris-EDTA buffer prior to immunofluorescence staining. Skin
cross-sections were blocked for 30 minutes at room temperature in 5% normal goat serum PBS,
which was also the primary antibody diluent, unless the target was a phospho-protein in which
case 5% normal goat serum TBS was used. Primary antibodies and their usage were as follows:
pSTAT3S727 (rabbit, Abcam #AB30647, 1:200), pSTAT3Y705 (rabbit, 1:100, Cell Signaling
#9145), Keratin 14 (chicken, BioLegend #906004, 1:500), Vimentin (chicken, ThermoFisher
#PA1-10003, 1:2000), HMGB1 (rabbit, Abcam #AB79823, 1:250), Lamin B1 (rabbit, Abcam
#AB16048, 1:1000), Keratin 10 (rabbit, BioLegend #905404, 1:500), Loricrin (rabbit, BioLegend
#905104, 1:500). Primary antibody incubations were carried out overnight at 4°C except Keratin
14, Keratin 10, Loricrin, and Vimentin, which were 2 hours at room temperature. Following either
PBS or Tris-buffered saline (TBS) wash, secondary detection antibodies conjugated with either AlexaFluor-647 or AlexaFluor-488 (Invitrogen) were diluted in either PBS or TBS at 1:200 were applied for 30 minutes at room temperature. Mounting media with DAPI used was either ProLong Gold (Invitrogen) or Vectashield Vibrance (Vector Labs), which were used interchangeably and allowed to cure prior to imaging. EdU staining was done using the Click-iT EdU AlexaFluor Imaging Kit (Invitrogen) according to the manufacturer’s instructions following primary antibody staining but prior to secondary antibody incubations.

For immunofluorescence staining of skin explants, samples were grown on cover glass and surrounded by a sterile 0.5 mm press-to-seal silicone gasket (Invitrogen) to reduce staining volume. After 48 hours of skin explant outgrowth, cells were fixed for 1 hour at room temperature in 10% neutral buffered formalin followed by 10 minutes of cell permeabilization using 0.1% Triton X-100 in PBS. Cells were then blocked for 30 minutes in 5% normal goat serum in PBS followed by a 2 hour room temperature incubation with primary antibody and 30 minute room temperature incubation with secondary antibody as described above. Hard set mounting media with DAPI was applied and cured prior to imaging.

Hematoxylin and Eosin staining was performed on 4 µm de-waxed sections using standard histology protocols using Gill’s Hematoxylin No.1 and Eosin Y (Sigma). Colorimetric stained slides were mounted with Permount (Fisher). All brightfield imaging was done using an inverted EVOS XL (Life Technologies) microscope. All fluorescence imaging was performed on an Revolve R4 (Echo Labs) microscope equipped with Olympus UPlanFL 10x/0.30, and UPlanFL 20x/0.50 air objectives using DAPI, FITC, and Cy5 fluorescence channels.

*Image Analysis*
All image processing and analysis was conducted using ImageJ FIJI (version 2.0.0-rc-69/1.52i, NIH), Photoshop CC 2019 (version 20.0.4, Adobe), and Illustrator CC 2019 (version 23.0.3, Adobe). Daily images of the wound site were used to quantify wound closure over time. Images were processed to normalize camera distance and angle using the size of the metal splint as a standardization factor. Wound measurements were obtained by tracing the area of the visible wound margin on each day relative to the splint area. Wound closure over time was normalized as a percentage of the original day 0 wound. The rate of wound healing was analyzed by fitting wound closure on days 0-9 on a linear best fit line.

Immunofluorescence images first underwent thresholding over entire image set of all samples from the experiment followed by manual counting of positive cells for at least 3 unique fields from each sample. All epidermal analyses only counted interfollicular epidermal cells and excluded hair follicles. Dermal fibroblasts were identified by vimentin staining. pSTAT3\(^{Y705}\) positive cells were those that had overlap with their DAPI nuclear signal. pSTAT3\(^{S727}\) positive cells were those that had DAPI-associated cytoplasmic signal. HMGB1 positive cells were those that had overlapping signal with their nuclear DAPI signal. Lamin B1 positive cells were those that exhibited perinuclear signal relative to their DAPI signal. Vimentin positive cells were those that had DAPI-associated periplasmic signal. Keratin 14, Keratin 10, and Loricrin positive cells were those that had DAPI-associated cytoplasmic signal.

Average thickness of the epidermal proliferative hub was measured over 300-micron span from wound margin, guided by EdU staining along with reference to the subdermal adipose layer and serially sectioned H&E samples (Aragona et al., 2017). Composite images for thickness analysis were tiled by manually registering images of 10x fields with approximately 20%
overlapping content using Photoshop (Adobe), and individual thickness measurements were taken using ImageJ FIJI (NIH).

Epidermal thickness measurements were performed on H&E stained non-wounded skin cross-sections as previously described (Crane et al., 2015) by measuring the orthogonal distance from the basement membrane to the end of the spinous layer several times over the interfollicular epidermis.

Keratinocyte outgrowth was imaged in a circular overlapping clockwise pattern from 12 o’clock 24 hours after plating and areas of outgrowth were imaged at 48 hours after plating. Outgrowth at 48 hours post-explanting were measured, and the maximal area outgrowth field for each discrete outgrowth on each explant was recorded by tracing its outline and calculating the enclosed area using Illustrator (Adobe). The average of the 3 highest of these maximal outgrowth area fields was taken and used as representative growth from the explant. For each data point, this process was done 2 times on 2 different pieces of explant skin from the same mouse.

Gene Expression

The epidermis was separated from dermis using dispase (Roche) enzymatic digestion for 2 hours at 37 °C. After digestion, the epidermis was scraped using a scalpel blade and immediately frozen at –80 °C until later analysis. RNA was extracted by homogenizing separated epidermis in TRIzol (Invitrogen) reagent using a bead mill apparatus (MPBio 5G) followed by column purification (Zymo Direct-zol RNA mini prep), on column DNase treatment and subsequent elution. 300 ng of total RNA was then reversed transcribed to cDNA (ABI HC cDNA synthesis kit), diluted 1:30 in ultrapure water and mRNA expression was assessed using qPCR with TaqMan or SYBR chemistry.
**Flow Cytometry**

Whole blood was collected on day 31 of the rmIL-15 or exercise experiments from the submandibular vein. Blood was collected into K2-EDTA MiniCollectTubes (Greiner Bio-One) and RBCs were lysed using PharmLyse (BD Biosciences). Lymphocytes were then pelleted and incubated with FITC-CD3 (BD Pharminigen #553061) and APC-CD8 (BD Pharminigen #561093) antibodies and analyzed in conjunction with unlabeled and compensation controls on a BD FACSARia instrument. Data analysis was done using FlowJo software for MacOSX.

**Microarray Analysis**

Microarray data from GSE84511 (Solanas et al., 2017) was obtained from GEO using the Bioconductor suite of tools in R (version 3.5.1) on MacOSX. Differential gene expression between young and old epidermal stem cells were obtained using the LIMMA package (version 3.36.2) to contrast all aged animal GSM entries against all young animal GSM entries using group-means parameterization, empirical Bayes statistics for differential expression, and Benjamini-Hochberg false discovery rate adjustment (Ritchie et al., 2015). Gene set enrichment analysis using the FGSEA package (version 1.6.0) (default pathways, \( p. \ adj < 0.05 \), 10000 permutations) and bi-directional KEGG pathway analysis using the GAGE package (version 2.30.0) was performed on differentially expressed genes using mouse (mmu) gene sets for bioprocesses, molecular factors, and cellular compartments with wiring diagrams generated using the Pathview package (version 1.20.0) for GAGE (Luo et al., 2009; Sergushichev, 2016).

**Cytochrome C Oxidase Activity**
Cytochrome C oxidase activity was measured as the decrease of absorbance due to oxidation of cytochrome $c$ in epidermal lysates as described previously (Crane et al., 2015). Briefly, sodium dithionite (Fisher, S310-100) was used to reduce Cytochrome $C$ (Sigma, C-2506) in assay buffer (50 mM potassium phosphate buffer with 1 mM EDTA, pH 7.4). Reduced cytochrome $c$ was added to 20 µL of sample lysate per well in a 96-well plate and the absorbance at 550 nm was monitored over 90 seconds as a fitted linear slope ($R^2 > 0.95$). Samples were measured in duplicate and absolute enzyme activity was normalized to protein concentration:

$$\frac{(Slope_{average}/18.5)}{([Protein] \times Volumesample)/1000}$$

**Statistical Analysis**

Graphpad Prism (version 7.0d) was used to calculate statistics. Data were log transformed or square root transformed prior to statistical analysis. Significance was set as $p < 0.05$. Comparisons of two groups were performed using a student’s $t$ test. Comparisons of more than 2 treatment groups were analyzed using a one-way or two-way analysis of variance (ANOVA) as appropriate for the number of treatment factors. If statistical significance was achieved via ANOVA, specific differences between groups were identified using Holm-Sidak or Tukey’s post-hoc analysis.

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**Conflict of interest:** JC is a co-holder of a patent involving the use of IL-15 as a therapy for skin disorders.

Figure 1. Metabolic dysfunction is a hallmark of aged murine epidermis. (A) Bi-directional KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway perturbation analysis using GAGE (Generally Applicable Gene Set Expression) depicting significant defined biological pathways (KEGG Pathways) with genes significantly up and/or down with age.

(B) Uni-directional Gene Set Enrichment analysis (GSEA) showing sets of genes either up or down with age associated with specific gene ontology terms. Gene ontologies are ordered by their normalized enrichment score that signifies how uniform the gene expression change is across all member genes of a set (Gene Set Rank).

(C) Candidate differentially expressed genes in young versus old mouse epidermal stem cells. All analysis in A-C was generated using raw data from GSE84511. Significance was determined by a Benjamini-Hochberg False Discovery Rate adjusted \( p < 0.05 \).
**Figure 2. Exogenous IL-15 therapy rescues wound healing in aged, sedentary mice.** (A) Experimental design of intravenous pre-treatments and wound surgery with key experimental days denoted on a timeline. (B) Representative photographs of wound healing progress in young control (Young PBS), old control (Old PBS) and old IL-15 (Old rmIL-15) treated mice on the indicated days post-wounding. Wound margins are delineated by the dotted white line. Scale bar = 5 mm. (C) Quantification of wound areas from (B). (D) Final wound closure at time of sacrifice on day 9 expressed as a proportion of the original wound area. (E) Wound closure rate per day determined by average slope of best fit lines. (F) Projected time for full wound closure based on wound closure rate calculated in (E). *Significantly different (p < 0.05) relative to Young PBS mice. #Significantly different (p < 0.05) relative to Old PBS mice.

(D) Final wound closure at time of sacrifice on day 9 expressed as a proportion of the original wound area. (E) Wound closure rate per day determined by average slope of best fit lines. (F) Projected time for full wound closure based on wound closure rate calculated in (E). *Significantly different (p < 0.05) relative to Young PBS mice. #Significantly different (p < 0.05) relative to Old PBS mice.

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Figure 3. Exogenous IL-15 therapy rescues STAT3 phospho-signaling and cell growth in aged, sedentary mice. (A) Representative images of pSTAT3\textsuperscript{Y705} and pSTAT3\textsuperscript{S727} immunofluorescence staining in intact skin collected during wound surgery (day 33 of treatment). The epidermal basement membrane is indicated by the white dashed line. Scale bar = 50 μm. (B and C) Quantification of staining shown in (A) in epidermal cells and dermal fibroblasts. \( n = 4-8 \) mice per group. (D) Brightfield images and magnified insets of young and old skin explants after 48 hours of treatment with or without 100 pg/mL of rmIL-15. Scale bar = 100 μm. Inset scale bar = 25 μm. Dashed lines indicate the borders of keratinocyte outgrowth and (E) quantification of outgrowth area. \( n = 3 \) per treatment. (F) Representative images of Keratin 14 and Keratin 10 as well as (G) Loricrin and Vimentin immunofluorescent staining from young (3 months) and old (25 months) skin explants after 48 hours of growth. Scale bar = 100 μm. (H-I) Relative counts of primary dermal fibroblasts after 72 hours of treatment of 100 pg/mL rmIL-15 following 3 hours of pre-treatment with either 0.4 μg/mL ruxolitinib (H) or 0.4 μg/mL tofacitinib (I). (J) Representative western blots with molecular weight (MW) indicators and quantification of (K) STAT3 Y705 and (L) S727 phosphorylation in primary dermal fibroblasts treated with PBS, 100 pg/mL rmIL-15, or 100 ng/mL rmIL-6 for 30 minutes. \( n = 5 \) per treatment. Data are mean±SEM. *Significantly different (\( p < 0.05 \)) relative to Young PBS mice. #Significantly different (\( p < 0.05 \)) relative to Old PBS mice †Significantly different (\( p < 0.05 \)) relative to PBS control ‡Significantly different (\( p < 0.05 \)) relative to rmIL-15.
Figure 4. Exercise-induced improvements in wound healing depend on IL-15 signaling in aged mice. (A) Experimental design of intravenous pre-treatments, the exercise regimen and wound surgery with key experimental days denoted on a timeline. (B) Representative images of wound healing progress in young sedentary (Young SED), old sedentary (Old SED), old exercised (Old EX) and old exercised mice receiving IL-15 neutralizing antibody (Old EX-nAb) on the indicated days post-wounding. Wound margins are delineated by the dotted white line. Scale bar = 5 mm. (C) Quantification of wound areas from (B). (D) Final wound closure at time of sacrifice on day 9 expressed as a proportion of the original wound area. (E) Wound closure rate per day determined by average slope of best fit lines. (F) Projected time for full wound closure based on wound closure rate calculated in (E). n = 4-8 mice per group in A-E. (G) Quantification of the proliferative hub thickness in each treatment group. n = 3-5 mice per group. (H) Stitched panoramic immunofluorescence images of Keratin 14 stained stem cells and EdU labeling in day 9 post-wounded skin showing the wound bed (WB) and proliferative hub (PH) region of each side. The space between the white arrow (original wound edge) and the yellow arrow (300 microns inwards) indicates the proliferative hub region. Scale bar = 100 μm. Data are mean±SEM. * Significantly different (p < 0.05) relative to Young SED mice. †Significantly different (p < 0.05) relative to Old SED mice. ‡Significantly different (p < 0.05) relative to Old EX mice.
Figure 5. Neutralization of IL-15 in exercising mice prevents the rescue of STAT3 phospho-signaling, mitochondrial and inflammatory signaling, and epidermal structure. (A) Representative immunofluorescence images of pSTAT3Y705 and pSTAT3S727 staining in intact skin collected after 33 days of treatment in young sedentary (Young SED), old sedentary (Old SED), old exercised (Old EX) and old exercised mice receiving IL-15 neutralizing antibody (Old EX-nAb). The epidermal basement membrane is indicated by the dashed white line. Scale bar = 50 \( \mu \text{m} \). n = 3-6 mice per group. (B and C) Quantification of epidermal cells and dermal fibroblasts for (B) pSTAT3Y705 positive cells as well as (C) pSTAT3S727 positive cells. n = 3-6 mice per group. (D) Mitochondrial epidermal mRNA expression in each treatment group relative to young control mice as measured by qPCR. n = 5-7 mice per group. (E) Mitochondrial cytochrome c oxidase activity measured on Day 42 intact skin epidermal lysates. n = 3-5 mice per group. (F) Inflammation associated epidermal mRNA expression in each treatment group relative to young control mice as measured by qPCR. n = 5-7 mice per group. (G) Brightfield microscopy images of hematoxylin and eosin stained skin from each treatment group. Scale bar = 100 \( \mu \text{m} \). (H) Quantification of epidermal thickness. n = 3-6 mice per group. Data are mean±SEM. *Significantly different (p < 0.05) relative to Young SED mice. **Significantly different (p < 0.05) relative to Old SED mice. *Significantly different (p < 0.05) relative to Old EX mice. NS, non-significant (p > 0.05).
Figure S1. JAK/STAT and AMPK signaling pathways in ESCs change extensively in response to aging. (A) JAK/STAT and (B) AMPK pathway gene expression perturbations in young vs aged mouse epidermal stem cells from dataset GSE84511. Each node represents a set of genes and the color coding reflects gene expression changes within the node. Wiring diagrams generated using the Pathview and GAGE bioconductor package with 5% FDR (BH).
Figure S2. Recombinant mouse IL-15 treatment reduces hallmarks of senescence in skin without impacting T-cells. (A) Cytochrome c oxidase activity measured on Day 42 intact skin epidermal lysates. n = 4-6 mice per group. (B) Representative immunofluorescent images of epidermal and dermal HMGB1 and Lamin B1 staining of young control (Young PBS), old control (Old PBS) and old rmIL-15 (Old rmIL-15) treated intact skin taken during wound surgery and (C, D) quantification of images from (A). n = 4-8 mice per group. (E) Spleen weights of mice from each treatment group normalized to individual body weights. n = 4-6 mice per group. (F) Representative micrographs of epidermal CD3 staining of young control, old control, and old rmIL-15 intact skin taken during wound surgery after 33 days of treatment and quantification. n = 4-6 mice per group. (G) Flow cytometry analysis of whole blood CD3+/CD8+ cells acquired after 31 days of treatment in uninjured mice. n = 4-6 mice per group. Dashed line denotes epidermis. Data are mean±SEM. *Significantly different (p < 0.05) compared to Young PBS. #Significantly different (p < 0.05) compared to Old PBS. NS, non-significant (p > 0.05).
Figure S3. Recombinant IL-15 therapy in young mice augments wound healing and alters STAT3 signaling. (A) Representative images of wound healing progress and (B) quantification of wound closure over time in control (Young PBS) and rmIL-15 treated (Young rmIL-15) young mice. Dashed white line indicates wound margin. Scale bar = 5 mm. (C) Final wound closure at time of sacrifice on day 9 expressed as a proportion of the original wound area. (D) Wound closure rate per day determined by the average slope of best fit lines. (E) Projected time for full wound closure based on wound closure rate calculated in (D). n = 8-11 mice per group for A-E data. (F) Representative images of pSTAT3<sup>Y705</sup> immunofluorescent staining in intact skin collected during wound surgery and (G) quantification in the indicated treatment groups. (H) Representative immunofluorescent images of pSTAT3<sup>S727</sup> staining of intact skin collected during wound surgery and (I) quantification. n = 3 mice per group. Dashed line denotes epidermis. Data are mean±SEM. *Significantly different (p < 0.05) relative to Young PBS.
Figure S4. Neutralization of IL-15 in exercising mice prevents the rescue of cellular senescence in aged skin. (A) Representative images of epidermal and dermal HMGB1 and Lamin B1 immunofluorescent staining of young control (Young SED), old control (Old SED) and old rmIL-15 (Old rmIL-15) treated intact skin after 33 days of treatment. (B) Quantification of HMGB1 and (C) Lamin B1 images from (A). $n = 3$–6 mice per group. (D) Epidermal mRNA expression of the senescence markers p21 (Cdkn1a) and p16ink4a in each treatment group as a fold of young control mice. $n = 5$–7 mice per group. Dashed line denotes epidermis. Data are mean±SEM; *Significantly different ($p < 0.05$) versus Young SED mice. #Significantly different ($p < 0.05$) relative to Old SED mice. φSignificantly different ($p < 0.05$) compared to Old EX mice. NS, non-significant ($p > 0.05$).
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Chapter 3: Depletion of PGC1α in the skin epidermis basal cell niche models chronological aging.

(Manuscript currently under preparation)
3.1 Abstract

Skin is the first line of defense against the environment including solar radiation, dehydration, lacerations, temperature changes, and infections. The outer skin epidermis constantly renews itself from the expansion and differentiation of resident stem cells, shedding dead cells off of the surface. Imbalanced epidermal differentiation can result in defective skin, which is structurally altered and responds poorly to stress such as wounding and solar radiation. Metabolic signaling is understood to control the fate of differentiating cells in hair follicles, muscle, and neurons, but not yet in interfollicular epidermis. To investigate its role in the maintenance of the epidermis, we engineered the epiPgc1a mouse which allows for inducible deletion of the mitochondrial transcriptional co-activator, Pgc1a, in the basal layer of the IFE. We quantified morphology, protein, and gene expression of Pgc1a deficient skin under basal and stressed (TPA, UVB, and wounding) states. Basal deleted skin was macroscopically normal and comparable to control skin but exhibited a NAD+ metabolism perturbation gene expression signature. Upon applying proliferative stresses, deleted epidermis revealed a tissue repair defect resulting in slow wound healing, and skewed differentiation of keratinocytes in response to TPA and UVB.

3.2 Introduction

Skin covers the body and prominently responds to environmental challenges. It protects the body from injury, infection, dehydration, temperature, and the cancer-causing effects of solar radiation. It achieves all this by, through continual renewal of the epidermis by proliferation and differentiation of keratinocytes. Keratinocytes proliferate and differentiate as they migrate to the skin surface and reconfigure their keratin expression to give the skin its toughness and
waterproofness (Fuchs, 1995). To balance the constant loss of cells from the basal layer, epidermal stem cells occasionally produce two stem cells, or one stem cell and one differentiated cell, or two differentiated cells, processes known as asymmetric and symmetric divisions (Clayton et al., 2007; Williams et al., 2011). Maintenance of the epidermis depends on appropriate regulation of cellular division; skewing of these processes change the structure and stress responses of the skin. Psoriasis, eczema, tumors, poor tissue repair, and thin fragile skin associated with aging, may all be due to skewed differentiation of keratinocytes in the skin (Lopez-Pajares et al., 2013). Mitochondria are central to energy metabolism and play a major role in determining cell fate in neurons (Zheng et al., 2016), muscle (Bernard et al., 2015), and hair follicles (Hamanaka et al., 2013), but little is known about their role in the interfollicular epidermis. The coenzyme nicotinamide adenine dinucleotide (NAD+) is a redox carrier in mitochondria and is also critical to many enzymatic reactions such as DNA repair and histone deacetylation as an ADP-ribose source (Johnson and Imai, 2018). It is replenished within cells by salvage and de novo synthesis. Salvage is controlled by the circadian clock circuit and supplies the majority intracellular NAD+ in eukaryotic cells (Foteinou et al., 2018; Sporty et al., 2009; Tran et al., 2016). Both reduced cellular NAD+ levels and mitochondrial dysfunction have been linked to aging phenotypes in various tissues, and NAD+ supplementation has been demonstrated to restore impaired tissue function and confer longevity in mice (Zhang et al., 2016b). The skin epidermis is a proliferative and dynamic tissue which must respond to solar radiation induced DNA damage while coordinating repair and proliferation to circadian cycles, but the role of NAD+ and it’s impact on the skin epidermis is unknown. The gene PPARGC1a encodes a coactivator protein, Pgc1a, which stimulates the expression of many mitochondrial and energy metabolism genes, including the NAD+ salvage pathway via the circadian circuit (Liu et al., 2007; Wu et al., 1999). The importance
of Pgc1a expression for maintenance of the skin epidermis is also currently unknown. To investigate the role of Pgc1a expression on cellular mitochondria and NAD+ metabolism epidermis, we deleted its expression in the adult skin epidermis. We find that loss of epidermal Pgc1a primarily impacts regulation of intracellular NAD+ levels with minimal perturbation to mitochondrial activity or quantity in the epidermis. These biochemical changes in the epidermis alter its stress responses as evidenced by skewed keratinocyte differentiation causing an impaired response to wounding.

3.3 Materials and Methods

Animal handling and care

PPARGC1α<sup>fl/fl</sup> mice were obtained from Jackson Labs (Jackson Labs #009666) and mated with Keratin14<sup>Cre-ERT/+</sup> mice (Jackson Labs #005107) to generate Keratin14<sup>Cre-ERT/+</sup>;PPARGC1α<sup>fl/fl</sup> animals. All mice in experiments expressing the Cre transgene were hemizygous. To induce Cre recombination, tamoxifen was dissolved in 70% ethanol and applied topically to shaved dorsal skin daily for 7 days as 5 mg/day for two days, followed by a two-day break (typically for the weekend), and then continued for 5 more days. Mice were 8-16 weeks old at the time of tamoxifen administration and were not used for experiments until in telogen and at least 21 days after the last day of tamoxifen application. All mice were housed in independently ventilated cages maintained under controlled environmental conditions (12h/12h) light/dark cycle, 23°C) in an AAALAC approved animal facility. Mice received chow food and water <i>ad libitum</i> and sacrifice was performed by cervical dislocation. All animal procedures were conducted under the approval of the Northeastern University IACUC. TPA (12-O-tetradecanoylphorbol 13-acetate, LC labs) dissolved in acetone was applied as a single daily dose for 7 days. For the single dose, we applied
100 µL of 81 µM TPA or acetone vehicle to each half of the shaved dorsal skin in mice under isoflurane anesthesia and obtained skin samples 24 hours after the last dose. Mice were given EdU (50 mg/kg) via intraperitoneal injection 3 hours (acute TPA) or 24 hours (acute UVB) prior to sacrifice. For the acute UVB exposure, mice received ketamine/xylazine anesthesia (50 mg/kg ketamine; 5 mg/kg xylazine) i.p. and were exposed to a single dose of 200 mJ/cm² of UVB radiation using a dosimeter calibrated UV instrument (Tyler Research). After 48 hours, mice were sacrificed, and skin samples collected.

Genotyping

Ear punches were collected from mice at weaning (21 days of age) for genotyping of Ppargc1a flox or Keratin14Cre-ERT. All qPCR, including HRM, was performed on a QuantStudio 3 instrument (ThermoFisher). Genomic DNA was extracted from ear tissue by adding 15 uL of QuickExtract DNA extraction solution (EpiCentre) and heating for 65°C for 6 minutes followed by 98°C for 2 minutes in a heat block. After a brief centrifugation, 10 µL of sample was diluted 1:25 in ultrapure water for PCR analysis. Ppargc1a lox p sites were genotyped by high resolution melt (HRM) PCR using primer sequences provided by Jackson Labs. Primers – M-Pgc1a flox Forward TCCAGTAGGCAGAGATTTATGAC; M-Pgc1a flox Reverse TGTCTGGTTTGACAATCTGCTAGGTC. HRM PCR conditions per reaction were as follows: 5 µL of Melt Doctor HRM Master Mix (ABI), 0.3 µL forward primer, 0.3 µL reverse primer, 2.4 µL ultrapure water and 2 µL of genomic DNA. The primer stocks used for qPCR were 20 µM for Pgc1a flox. HRM was analyzed using HRM Melt v3.1 software (ThermoFisher) to identify wild-type, heterozygous and homozygous animals for each flox site. The presence or absence of the Keratin14CreERT transgene was genotyped by SYBR qPCR using primer sequences provided by
Jackson Labs – Keratin14CreERT forward: CGCATCCCTTTCCAATTTAC; Keratin14CreERT reverse: GGGTCCATGGTGATACAAGG. qPCR conditions per reaction were as follows: 5 μL of PowerUP SYBR Master Mix (ABI), 0.3 μL forward primer, 0.3 μL reverse primer, 2.4 μL ultrapure water and 2 μL of genomic DNA. The working primer stocks used for Keratin14CreERT qPCR were 20 μM.

**Wound surgery**

Prior to surgery, all mice were anesthetized using isoflurane anesthesia, provided subcutaneous carprofen analgesia (5 mg/kg) (TCL) and the dorsal hair was shaved with clippers. The skin was then disinfected using povidone-iodine (APLICARE) and ethanol followed by the surgical excision of two 6 mm diameter dorsal full-thickness wounds using a sterile skin biopsy punch (Medline). Wounds were made in telogen skin in all experiments. Wounds were splinted using a sterile 10 mm inner diameter stainless steel ring (Grainger) that was secured by 4-6 monofilament nylon 5-0 sutures (Oasis) and cyanoacrylate adhesive (Krazy Glue) as previously described (Wong et al., 2019). After sutures were tied, a clean Tegaderm (3M) wound dressing was loosely applied around the circumference of the mice to cover the splinted wounds. Carprofen (5 mg/kg) analgesia was administered subcutaneously for the first 3 post-operative days. Depending on the endpoint, wounds were photographed daily using a DMC-FZ70 camera (Panasonic) and the Tegaderm dressing was changed daily until either 9 days post-surgery or until full closure. Wound site photography lighting conditions and imaging parameters on the camera were kept identical between days. At sacrifice, wound skin was collected and applied to cardstock backing to maintain structural integrity, and later processed for histology.
**Keratinocyte explants**

To prepare skin for keratinocyte culture, the dorsal skin of mice in the telogen phase was shaved using clippers and depilated with NAIR prior to skin collection. Skin was then excised, incubated for 2 minutes in a solution of povidone-iodine, twice in a solution of 70% ethanol (total of 2 minutes), and rinsed in sterile PBS. Skin was then placed epidermis side down and scraped with a beveled scalpel blade to remove all subdermal adipose tissue and part of the dermal tissue. A 4 mm biopsy punch was used to excise individual tissue explants, which were then plated dermis side down into 24-well tissue culture plates (Thermo BioLite) followed by a 1 hour drying period for explant adhesion to the surface. After the drying period, 400 µL of media (DMEM supplemented with 15% FBS, 1% glutamine, 1% penicillin, and 1% streptomycin) was added to submerge the explant. Explants from both genotypes were treated with and without 1 mM nicotinamide (NAM), 1 mM nicotinamide riboside (NR), and 1 mM nicotinamide mononucleotide (NMN) and placed in a cell culture incubator at 37°C with a 5% CO₂ atmosphere. Explant outgrowth was assessed at 72 hours after treatment. Explants were prepared for imaging first by fixation with 10% neutral buffered formalin followed by staining with 0.1% crystal violet in a 30% methanol solution. The explants were then washed with water until the run-off was clear. Multiple overlapping brightfield images of each explant and outgrowth were then acquired at low magnification.

**Histology**

Tissues were fixed overnight in 10% neutral buffered formalin and thereafter transferred to 70% ethanol at 4°C until processing. Tissues were processed using an automated tissue
processor (Thermo HistoStar, Kalamazoo, Michigan, USA) and embedded in paraffin wax. A microtome (Leica Biosystems, Buffalo Grove, Illinois, USA) was used to cut 4 μm skin cross-sections, which were allowed to dry overnight before de-waxing and further processing. Heat induced epitope retrieval was performed on de-waxed slides with either citrate or Tris-EDTA buffer prior to immunofluorescence staining. Skin cross-sections were blocked for 30 minutes at room temperature in 5% normal goat serum PBS, which was also the primary antibody diluent, unless the target was a phospho-protein in which case 5% normal goat serum TBS was used. Primary antibodies and their usage were as follows: Keratin 14 (chicken, BioLegend #906004, 1:500, San Diego, California, USA), HMGB1 (rabbit, Abcam #AB79823, 1:250, Cambridge, Massachusetts, USA), Keratin 10 (rabbit, BioLegend #905404, 1:500), Loricrin (rabbit, BioLegend #905104, 1:500), BrdU (rat, Abcam #AB6326, 1:200). Primary antibody incubations were carried out overnight at 4°C except Keratin 14, Keratin 10, and Loricrin, which were 2 hours at room temperature. Following either PBS or Tris-buffered saline (TBS) wash, secondary detection antibodies conjugated with either AlexaFluor-647 or AlexaFluor-488 (Invitrogen, Carlsbad, California, USA) were diluted in either PBS or TBS at 1:200 were applied for 30 minutes at room temperature. Mounting media with DAPI (4’,6-diamidino-2-phenylindole) used was either ProLong Gold (Invitrogen) or ProLong Diamond (Invitrogen), which were used interchangeably and allowed to cure prior to imaging. For thymine dimer staining, a mouse-on-mouse blocking kit (Vector Labs #BMK-2202, Burlingame, California, USA) was used per the manufacturer’s instructions.

Hematoxylin and Eosin staining was performed on 4 um de-waxed sections using standard histology protocols using Gill’s Hematoxylin No.1 and Eosin Y (Sigma Aldrich). Colorimetric stained slides were mounted with Permount (Fisher Scientific, Pittsburgh, Philadelphia, USA). All
brightfield imaging was done using an inverted EVOS XL (Life Technologies, Carlsbad, California) microscope. All fluorescence imaging was performed on either an Revolve R4 (Echo Labs, San Diego, California, USA) microscope equipped with Olympus UPlanFL 10x/0.30, and UPlanFL 20x/0.50 air objectives using DAPI, FITC, and Cy5 fluorescence channels or an Axio Observer Z1 (Zeiss, White Plains, New York, USA), equipped with Plan Apochromat 5x/0.16, 10x/0.45, and 20x/0.8 air objectives and 40x/1.3 and 63x/1.4 oil objectives using DAPI, GFP, DsRed, and Cy5 fluorescence channels.

**Image analysis**

All image processing and analysis was conducted using ImageJ FIJI (version 2.0.0-rc-69/1.52i, NIH), Photoshop CC 2019 (version 20.0.4, Adobe), and Illustrator CC 2019 (version 23.0.3, Adobe, San Jose, California, USA). Immunofluorescence images first underwent thresholding over entire image set of all samples from the experiment followed by manual counting of positive cells for at least 5 unique fields from each sample. All epidermal analyses only counted interfollicular epidermal cells and excluded hair follicles. Nuclear HMGB1 positive cells were those that had overlapping signal with their nuclear DAPI signal. Keratin 14, Keratin 10, and Loricrin positive cells were those that had DAPI-associated cytoplasmic signal. Epidermal thickness measurements were performed on H&E stained intact skin cross-sections as previously described (Crane et al., 2015) by measuring the orthogonal distance from the basement membrane to the outer edge of the cellular epidermis. Explants images were obtained by imaging the fixed and stained explants using a 4x objective under brightfield conditions on the Revolve R4 (Echo Labs, San Diego, California, USA) in the inverted configuration. For each explant, at least four image fields were captured to allow for panoramic stitching. Outgrowth areas were quantified on
stitched images by using the pencil tool in ImageJ/FIJI and normalized to the similarly traced area of the original explant tissue.

**Statistical Analysis**

All data were analyzed in Prism 8.0 (GraphPad, San Diego, California, USA) using a two-way analysis of variance (ANOVA) on either log or square root transformed data with Tukey’s HSD post-hoc testing for differences between treatments and species. Statistical significance was set as p<0.05.

3.4 Results

*Pgc1a deleted epidermis maintains normal morphology but signs of NAD+ pathway perturbances*

We crossed the K14-cre inducible driver mouse with the lox-flanked Pgc1a mouse; the resulting epiPgc1a mouse allows targeted deletion of Pgc1a in basal epidermal cells upon treatment with tamoxifen. We then compared the skin of these deleted mice with littermate controls under basal and stress states using both histological, surgical, molecular, and cell culture to determine if loss of Pgc1a in the basal epidermis results in a skin defect owing to mitochondrial or NAD+ disruptions. We treated the shaved dorsal skin of age and sex matched adult K14-cre+ and K14-cre- epiPgc1a mice with topical tamoxifen to induce cre-mediated deletion of the loxP flanked region covering exons 3-5 of Pgc1a coding sequence (Lin et al., 2004; Vasioukhin et al., 1999), and allowed their skin to recover from the ethanol treatment vehicle for at least 4 weeks before use in experiments. Residual Pgc1a expression in epidermal digests from deleted mice was determined by RT-qPCR to be approximately 20% of control, without concomitant reduction of the related
mitochondrial genes *Coxb*, *Ndufs2*, and *Uqcr10* (Fig. 1A). We then measured epidermal protein expression of mitochondrial electron transport chain components (VDAC, SDHA, and TOM20) by western blot, and found that control expression levels were comparable to levels in deleted epidermis (Fig. 1B). Mitochondrial protein levels corroborated by the equivocal activity levels of epidermal cytochrome c oxidase (Fig. 1C) and mitochondrial genome copy number between genotypes (Fig 1D). Gene expression levels of the cell cycle regulators *CDKN2A* (p16^ink4a^) and *CDKN1A* (p21), as well as *SERPINE1*, which are p53 target genes, were also similar in control and deleted epidermal digests (Fig. 1E). In contrast, expression levels of *Bax*, which promotes mitochondrial apoptosis processes, were nearly double in deleted epidermis compared with control epidermis (Fig. 1E). Since Pgc1a is also a regulator of NAD+ salvage (Tran et al., 2016), we measured epidermal expression levels of the related pathway genes and found significantly elevated *Nampt*, and *Parp1*, while *Nmnat1* trended up (*p=0.067*) in deleted skin (Fig. 1F). Unlike the readily observed epidermal thinning seen in aged vs young skin (Fig. S1A), gross observation of morphological features using hematoxylin and eosin staining showed epidermis with normal morphology, and average epidermal thickness was similar between control and deleted mice (Fig. 1G). Immunofluorescence staining of basal skin also revealed similar expression levels, patterns, and thicknesses of the basal layer marker keratin-14 and the spinous layer marker keratin-10 between control and deleted mice (Fig. 1H). However, further analysis of baseline differences between control and deleted epidermis revealed significantly decreased levels of nuclear HMGB1, the loss of which is associated with senescence and inflammation, similar to deficits seen in old vs young epidermis (Fig. 1I).
Deleted skin epidermis responds differently when stressed by wounding, topical TPA treatment, and acute UVB irradiation

To better understand the effects of Pgc1a loss in the epidermis, we sought to compare the epidermal response to different types of stress. In a wound healing experiment where bilateral 6 mm circular wounds were made through the full thickness of shaved dorsal skin and splinted open to prevent contraction by installing a splint, full wound closure of deleted skin lagged behind control skin by two days (Fig. 2A-B), a delay similar to what is seen geriatric skin (24 months and older) compared to young skin (Wong et al., 2019). Stressing deleted skin with topical TPA, a proliferation inducer, applied to half of the shaved dorsum with acetone vehicle applied to the other half resulted in robust hyperproliferation and hyperplasia of the basal layer specific to the TPA treated epidermis. However, the average amount of proliferation and epidermal thickness increase due to TPA treatment was similar between control and deleted skin (Fig. S2A-B). However, measurement of keratin-10 thickening indicated this epidermal layer became significantly thicker in deleted skin compared with control skin in response to TPA (Fig. 2C-D). Follow up analysis of the average thickness of the keratin-14 and loricrin expressing basal and cornified envelope epidermal layers (Fig. 2E-F), and the number of nucleated loricrin+ (Fig. 2L) cells, did not expose further differences between control and deleted skin due to TPA. Acute irradiation with UVB causes cell cycle arrest coupled with DNA repair a subsequent increase in epidermal proliferation (El-Abaseri et al., 2006). Irradiated skin thickened as expected per the stereotyped sunburn response (Ouhtit et al., 2000), but the resulting change in epidermal thickness was similar between control and deleted skin (Fig. S2C-D). Comparable to TPA treatment, the keratin-10 expressing spinous layer was thickened in epiPgc1a skin but not WT control (Fig. 2G-H), while the keratin-14 and loricrin layers were unchanged from control (Fig. 2I-J). Unlike what
was seen after TPA treatment, UVB the number of loricrin+ cornified cells in irradiated skin was maintained in the deleted group but decreased in the control group (Fig. 2K-M). Finally, we observed enlarged round basal layer nuclei in both control and deleted mice following TPA and UVB treatments, which are characteristic keratin-14 basal layer cells ascending to the first suprabasal layer (Lee et al., 2012), but nuclear size measurements revealed no significant differences in average size between genotypes in either treatment group (Fig. S2E-H).

**Addition of NAD+ to epiPgc1a deleted and aged skin explants rescues outgrowth**

We used split thickness skin explants to further investigate the tissue repair defect seen *in vivo* after epidermal deletion of Pgc1a. Baseline observations of cell proliferation characteristics were in good agreement with the wound healing results obtained *in vivo*: explants from deleted skin grew poorly compared to those taken from control skin (Fig. 3A-C). The magnitude of the differences in relative explant outgrowth area seen in control vs deleted explants approximated that of young vs old explants (Fig S3A-B). Supplementation of NAD+ using salvage pathway precursors (nicotinamide/NAM, nicotinamide riboside/NR, and nicotinamide mononucleotide/NMN) into the growth media of deleted explants greatly improved their outgrowth, while having minimal effect on control explants (Fig. 3A-C), with similar results seen in old compared to young explants (Fig. S3A-B).

**3.5 Discussion**

Skin is conspicuous protective and sensory organ which responds to environmental stresses. Understanding its underlying stress response mechanisms can improve the quality of life of individuals suffering from skin disorders and diseases such as fragile and thin skin, poor wound
healing, inflammatory conditions like psoriasis and eczema, aberrant pigmentation, hair loss, and cancer. However, these conditions are exacerbated or provoked with age, and have resisted efforts to decipher their etiology because the fundamental factors underpinning aging-related functional decline of the skin remain poorly understood. The skin epidermis as a model for studying cellular behavior has the advantages of well understood morphology, accessibility, and adapted responses to a multitude of stresses, which provides ample learning opportunities. Perturbances to mitochondria at the gene, protein expression, and activity levels have been consistently shown to be associated with aging related tissue function deficits (Birch-Machin and Bowman, 2016; Gonzalez-Freire et al., 2015; Kim et al., 2018). Our previously published data looked specifically at aged mouse skin and showed that thinned epidermis due to age could be rescued with epidermal Pgc1a expression boosting interventions (Crane et al., 2015). We later further extended these findings to wound healing (Wong et al., 2019), establishing a jumping off point to further study the role of Pgc1a bioenergetic regulation of epidermal maintenance. Genetic mouse models with defective epidermal mitochondrial antioxidant ability (Velarde et al., 2012) or whole body mtDNA repair (Kujoth et al., 2005) also have progeroid epidermal thinning and concomitant tissue maintenance defects, but are exaggerated in their phenotype severity compared with naturally aged mice. These data motivated us to develop a mouse model to more specifically investigate the contribution of Pgc1a itself on maintenance of the skin epidermis.

Here, we present data describing characteristics of an inducible mouse model of Pgc1a deletion in the basal epidermis. We sought to investigate the effects of deleting this central regulator of energy, mitochondrial, and NAD+ metabolism on the maintenance of the skin epidermis. Pgc1a is an important regulator of mitochondrial biogenesis, positioned downstream of energy sensing by AMPK and is capable of promoting the transcription of a variety of
mitochondrial genes through CREB and NRF pathways (Fernandez-Marcos and Auwerx, 2011). Aside from its canonical role, it is also crucial in maintaining NAD+ levels within the cell by regulating BMAL and CLOCK of the circadian circuit, which in turn regulate NAMPT and NMNAT1 of the salvage pathway (Foteinou et al., 2018; Liu et al., 2007; Tran et al., 2016). Both mitochondrial function and cellular NAD+ levels have been independently studied as causes and determinants of aging and longevity. Several theories exist using these concepts to explain the phenomenon of why organismal aging occurs (Imai and Guarente, 2014; Sharma et al., 2019).

We achieved on average an 80% reduction of Pgc1a gene expression in the epidermis after deleting with topical tamoxifen. However, this significant reduction of Pgc1a expression did not alter mitochondrial gene expression, protein levels, activity, or number in epidermal digests. Changes were detected in the gene expression levels of BAX, which is responsible for executing mitochondria mediated apoptosis (Smaili et al., 2000), as well as NAD+ salvage pathways (NAMPT, NMNAT1), and the NAD+ dependent DNA repair enzyme Parp1. To our surprise, deleted skin did not exhibit epidermal thinning, and expression of keratins and epidermal intermediate filaments was normal when we assessed keratin-14, keratin-10, and loricrin expression in intact skin. In contrast, aged skin exhibits both decreased levels of Pgc1a and marked epidermal thinning (Crane et al., 2015). Since there were elevated levels of epidermal senescent cells reported in the K14-SOD2 mouse (Velarde et al., 2012), we measured the gene expression levels of the stable cell cycle arrest and senescence protein p16, and the transient cell cycle arrest protein p21, and found no change owing to Pgc1a deletion, which agrees with the overall normal morphology seen in deleted epidermis. A significant amount of nuclear HMGB1 was lost in basal epidermal cells following deletion, which phenocopies the decline measured in aged epidermis. This, together with the gene expression signature described above, characteristic of an NAD+
metabolism perturbance, suggested that stressing the epidermis might reveal more differences. Overall, the loss of Pgc1a in the epidermis does not appear to impact mitochondria or alter cell cycling, and overall the result is deleted intact and unstressed skin is largely normal. This contrasts with findings reported in the vasculature, where Pgc1a loss was associated with mitochondrial abnormalities, reduced Sirt1 expression, and a vascular senescence phenotype (Xiong et al., 2013).

We next sought to expose underlying differences that would arise upon applying proliferative and also genotoxic stress on the deleted skin. Tissue repair is a complex process which demands a coordinated and orderly response in the form of clotting, immune recruitment, and the migration and proliferation of dermal and epidermal cells to fill in the wound bed with mesenchymal granulation tissue and to seal off the surface of the wound via re-epithelialization of keratinocytes (Braiman-Wiksman et al., 2007). The splinted full thickness wound is an effective way to approximate human wound healing in murine skin by limiting the ability of the panniculus carnosus muscle to contract and close the wound (Davidson et al., 2013). Employing this model, we found approximately a two day delay in wound closure time in deleted mice, which is in good agreement with wound healing delays in both aged human skin (Bonifant and Holloway, 2019), and aged mouse skin (Wong et al., 2019). To reconcile the incongruous findings that Pgc1 deletion yields macroscopically normal skin at baseline but also impaired wound healing, we turned to non-wounding stressing. The phorbol ester TPA is a known skin irritant which induces epidermal hyperplasia by activation of the MAPK pathway in keratinocytes without causing damage (Schönwasser et al., 1998), whereas acute UVB irradiation is known induce genotoxic DNA adducts which trigger DNA damage repair pathways with subsequent hyperproliferation (Ouhtit et al., 2000). There was a marked relative increase in the thickness of the keratin-10 spinous layer with both TPA and UVB treatments in deleted mice, which may reflect a skewing of keratinocytes
toward differentiation instead of basal cell proliferation. At the same time, the constant thickness of the keratin-14 basal and loricrin cornified envelope layers indicate that renewal of the basal stem cell pool is not compromised, and that further differentiation of spinous keratinocytes into corneocytes is not accelerated. Whether the thicker spinous layer in deleted mice induced to hyperproliferate occurs after basal cell hyperplasia or instead of it can’t be determined directly from the current data. However, the wound healing defect together with the keratin-10 phenotype could be explained by a skewing of basal keratinocytes toward precocious differentiation. With basal cell proliferation rates being equal between genotypes and conditions, altered rates of symmetric and asymmetric divisions could be required to maintain the epidermis. Although conversion of spinous keratinocytes into cornified envelope corneocytes was not altered to the point of changing the average thickness, we did count significantly more loricrin+ nucleus retaining corneocytes after TPA treatment in deleted skin. In UVB irradiated deleted skin, we saw the opposite pattern with maintained numbers of loricrin+ nucleated corneocytes while control skin underwent a marked decrease. Nucleated corneocytes in the cornified envelope are characteristic of parakeratotic epidermis, which derives from different basal epidermal stem cell populations compared with epidermis with anucleate corneocytes (Gomez et al., 2013; Sada et al., 2016). Taken together, these data could be explained by a skewing of keratinocyte cell fate toward early differentiation. This might reflect altered heterogeneity of the epidermal basal stem cell compartment (Changarathil et al., 2019).

Pgc1a coordinates both mitochondrial gene expression and also, indirectly, NAD+ salvage. Historically, dietary insufficiencies lead to the disease known as pellagra, caused by a lack of the NAD+ precursor Vitamin B3. Dermatitis is a pellagra associated disease phenotype, demonstrating the necessity of NAD+ metabolism for skin homeostasis. NAD+ metabolism is needed as a redox
carrier for mitochondrial metabolism, and also an ADP-ribose source for enzymatic reactions relating to genome stability and remodeling. Through these mechanisms, NAD+ metabolism has been connected to aging and aging related disorders, a field now under active research.

The explant outgrowth assay is a model of early wound healing by re-epithelialization as it generates migrating and proliferating keratin-14 expressing keratinocytes which exit the tissue to fill the surrounding space. It is a controlled environment enabling precise and consistent exposure of the tissue to test compound, unlike the in vivo scenario. We found that NAD+ repletion rescued impaired outgrowth of explants prepared from deleted Pgc1a deleted skin. At baseline, epidermal loss of Pgc1a yielded a noticeable growth defect of skin explants grown in vitro, phenocopying the defect observed comparing the poor growth of aged vs young explants. In both deleted as well as aged explants, supplementation of the growth media with NAD+ replenishing compounds completely rescued their growth potential and resulted in explants with outgrowths indistinguishable from their controls. NAD+ supplementation has also been shown in vitro to modulate stratification and senescence within stratified organotypic skin cultures (Tan et al., 2019), is in agreement with our results here. Taken together, these results indicate that our observed slow wound healing in vivo may be ascribed in part to epidermal NAD+ deficiencies caused by Pgc1a deletion.

Altogether these data indicate that, in Pgc1 deleted skin, NAD+ levels are decreased without overt mitochondrial defects and levels can be restored by a responsive salvage pathway. Intracellular NAD+ levels are likely sufficient to support basal maintenance of the epidermis, but additional stresses exceed the reserve capacity present in those keratinocytes. These findings are supported and explained by our NAD+ pathway gene expression analysis in deleted epidermis showing commensurate upregulation of Nampt, Nmnat1, and Parp1 expression. They are also
consistent with existing literature reports that NAD+ repletion improves tissue function of muscle (Ryu et al., 2016), kidney, and liver (Katsyuba et al., 2018). PARP1 is a major consumer of NAD+; its regulated both by genomic instability cues but also by NAD+ availability (Bian et al., 2019; Palazzo and Ahel, 2018). The $K_m$ of NAD+ dependent enzymes like SIRT1 and PARP1 have been experimentally determined to be optimal for normal cellular levels of NAD+ (Cantó and Auwerx, 2012). Thus, increased gene expression of PARP1 suggests either increased genomic instability or insufficient NAD+ levels for PARP1 function. This is supported by the observed significantly increased expression of NAMPT, which critically aids in recycling of NAM to NMN and then NAD+. NAMPT expression is energy stress responsive and increases due to exercise or fasting (Audrito et al., 2020).

The differences seen in deleted epidermis pushed to undergo proliferation with wounding, TPA, and UVB may also be explained by other roles fulfilled by PARP1. It also promotes pluripotency by protecting key genes such as Sox2 and Nanog from epigenetic repression through NAD+ dependent histone modifications (Roper et al., 2014). Reduced PARylation activity in murine embryonic stem cells was shown to significantly alter cell fate commitment (Liu and Kraus, 2017). These are developmental observations, but cutaneous healing relies on many aspects of its developmental biology (Bielefeld et al., 2013; Chigurupati et al., 2007). Cockayne syndrome (CS) results from NAD+ depletion by PARP1 because of genomic instability caused by defects in CS genes (Cordisco et al., 2019; Crochemore et al., 2019). Depletion of NAD+ produces general metabolic dysfunction, senescence, UV sensitivity, and progeria. Notably, skin defects are prominent in CS. Normal cellular function is restored either by blocking PARP activity, NAD+ repletion with NR, or expression of normal CS proteins. One more role for PARP is in the regulation of mitosis through transfer of poly-ADP-ribose (PARylation) to regulate centrosome
function and spindle assembly (Slade, 2019). Thus, proliferative stresses such as the ones we apply here appear to expose deficiencies rooted in PARP activity and NAD+ supply. Of the two nonwounding stresses, TPA and UVB, UVB tended to produce more severe phenotypes, potentially related to its additional genotoxic burden. Our observation of skewed differentiation may be explained by the tendency of the epidermis to expel defective keratinocytes via stratification and shedding as an innate response (Lopez et al., 2009; McMullan et al., 2003; Ren et al., 1997; Ying et al., 2018). Fate committed terminally differentiated expressing keratinocytes such as those expressing loricrin do not activate in response to wound healing (Morasso and Tomic-Canic, 2005), which accounts for our observed healing and outgrowth results.

The precise involvement of bioenergetic signal integration on epidermal stem cells and skin homeostasis in vivo during aging is still far from understood. Specific and conditional transgenic models coupled with topical delivery, like the one presented here, are needed and can more specifically answer questions thus far left ambiguous for lack of better mouse models. We explored the effects of conditional epidermal deletion of a key regulator of bioenergetics which is decreased with age, Pgc1a. We revealed a milder than expected mitochondrial phenotype and macroscopically normal skin in deleted mice. Proliferative and genotoxic stresses revealed a deficiency in intracellular NAD+ levels, impaired wound healing, and skewing of keratinocyte cell fate toward differentiation. The canonical role of Pgc1a in mitochondrial biogenesis and data from more severe mitochondrial perturbations hinted at a more dominant role for mitochondria in the skin than we see here. Instead, we found our deletion phenotypes to be explained by the effects of Pgc1a on NAD+ metabolism and PARP1 activity on the genome. Aging results in impairments to epidermal function which manifest as diminished epidermal architecture and poor wound healing and brings with it as a hallmark reduced levels of Pgc1a. Intracellular NAD+ levels also oscillate
to the circadian rhythm (Nakahata et al., 2009). Circadian rhythms guide the tempo of epidermal renewal to minimize exposure of sensitive DNA replication to sunlight (Brown, 1991), and circadian arrhythmia was shown to induce premature differentiation of human epidermal stem cells in vitro (Janich et al., 2013). The data presented here should be taken as a jumping off point for further investigations into the role of Pgc1a in the skin epidermis, its interactions with circadian biology and NAD+ metabolism, and connections to the etiology of aging.
Figure 1. epiPgc1a KO mice have morphologically normal epidermis with a gene expression signature of perturbed NAD+ metabolism. (A) Relative gene expression of mitochondrial biogenesis genes (Ppargc1a, Cox7b, Ndufs2, Uqcr10). (B) Western blotting for mitochondrial proteins (VDAC, SDHA, TOM20) with the housekeeping protein β-actin. (C) Enzyme activity of mitochondrial cytochrome c oxidase. (D) Relative quantification of mitochondrial DNA to nuclear DNA. (E) Relative gene expression of p53 target genes (p21, Bax, Serpine1, p16ink4a). (F) Relative gene expression of genes involved in NAD+ metabolism (Nampt, Nmnat1, Parp1, Sirt1). (G) Hematoxylin and eosin stained epidermis and epidermal thickness measurement. Scale bar is 100 μm. (H) Immunofluorescence of epidermal stratification markers keratin 14 (K14) and loricrin (Lor) with DAPI nuclear counterstaining. Scale bar is 50 μm. (I) Quantification and representative immunofluorescence images of epidermal nuclear HMGB1. Scale bar is 100 μm. Epidermal isolates obtained by enzymatic digestion were analyzed for A-F. n = 3-7 mice. Data are mean±SEM for A, E, and F. *Significantly different (p < 0.05) to control.
(Figure legend on following page)
Figure 2. Pgc1a deleted epidermis exhibits impaired tissue repair and skewed differentiation.
(A) Quantification of wound areas and representative photographs of wound healing progress in wildtype control and epidermal Pgc1a deleted mice over 9 days. $n = 7$-$8$ mice. Scale bar = 5 mm. (B) Time until full wound closure from a separate wound healing cohort. $n = 3$ mice. (C) Quantification and representative images of keratin 10 layer thickness increase of deleted mice treated with TPA relative to wildtype control. Scale bar = 50 μm. (D) Absolute thickness differences of keratin 10 thickness of (C). (E-F) Quantification of keratin 14 and loricrin expressing layer thicknesses of wildtype control and Pgc1a deleted mice after TPA treatment. (G) Quantification and representative images of keratin 10 layer thickness increase of deleted mice irradiated with UVB relative to wildtype control. Scale bar = 50 μm. (H) Absolute thickness differences of keratin 10 thickness of (G). (I-J) Quantification of keratin 14 and loricrin expressing layer thicknesses of wildtype control and Pgc1a deleted mice after UVB irradiation. (K-M) Absolute and relative quantification of loricrin expressing and nucleus retaining corneocytes of wildtype control and Pgc1a deleted mice from both TPA and UVB experiments. $n = 4$-$8$ mice for C-M. *Significantly different ($p < 0.05$) to control.
Figure 3. Pgc1a deleted skin yields poor explant outgrowth and is rescued with NAD+ precursors. (A-B) Average absolute and relative quantification of normalized skin explant outgrowth proportions from wildtype control and Pgc1a deleted mice treated either with PBS vehicle or 1 mM of NAD+ salvage pathway precursors nicotinamide (NAD), nicotinamide riboside (NR), and nicotinamide mononucleotide (NMN). (C) Representative panoramic stitched images of explants with their outgrowths stained purple with crystal violet for measurement. Scale bar is 2 mm. \( n = 3-4 \) mice. *Significantly different (\( p < 0.05 \)) to control.
Figure S1. Normal aging associated thinning of the skin epidermis. (A) Quantification of the cellular epidermis thickness of young (3-5 months old) and old skin (23-25 months old) from C57Bl/6 strain mice. Scale bar is 100 μm. n = 6-9 mice. *Significantly different (p < 0.05) to control.
Figure S2. Epidermal thickness, proliferation, and nuclear size of Pgc1a deleted skin. (A-D) Average absolute and relative quantification of cellular epidermal thickness from hematoxylin and eosin stained skin cross sections after TPA or UVB exposure. (E-H) Average absolute and relative area measurements of basal epidermal cell nuclear size from DAPI staining. (I-L) Average absolute and relative counts of EdU positive basal epidermal cells. (n = 5-9). *Significantly different (p < 0.05) to control.
Figure S3. Normal aged skin explant outgrowth potential is restored with NAD+ supplementation. (A-B) Average absolute and relative outgrowth area proportions of young (3-5 months old) and old (23-25 months old) skin explants from C57Bl/6 strain mice treated with either PBS vehicle or 1 mM NAD+ salvage pathway precursors nicotinamide (NAM), nicotinamide riboside (NR), and nicotinamide mononucleotide (NMN). n = 3 mice. *Significantly different (p < 0.05) to control.
3.7 References


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Chapter 4: *Acomys cahirinus* exhibit attenuated hallmarks of aging and rapid cell turnover after UV exposure in the skin epidermis

4.1 Abstract

The study of long-lived and regenerative animal models has revealed diverse protective responses to stressors such as aging and tissue injury. Spiny mice (Acomys) are a unique mammalian model of skin regeneration, but their response to other types of physiological skin damage have not been investigated. In this study, we examine how spiny mice skin responds to acute UVB damage or chronological aging compared to non-regenerative C57Bl/6 mice (M. musculus). We find that, compared to M. musculus, the skin epidermis in A. cahirinus experiences a similar UVB-induced increase in basal cell proliferation but exhibits increased epidermal turnover. Notably, A. cahirinus uniquely form a suprabasal layer co-expressing Keratin 14 and Keratin 10 after UVB exposure concomitant with reduced epidermal inflammatory signaling and reduced markers of DNA damage. In the context of aging, old M. musculus animals exhibit typical hallmarks including epidermal thinning, increased inflammatory signaling and senescence. However, these age-related changes are absent in old A. cahirinus skin. Overall, we find that A. cahirinus have evolved novel responses to skin damage that reveals new aspects of its regenerative phenotype.

4.2 Introduction

Maintenance and repair of the skin barrier are essential to prevent infection and protect the body from external hazards. However, normal stress and damage during an organism’s lifespan deteriorates skin structure and repair through undefined mechanisms. The skin epidermis is composed primarily of keratinocytes derived from basal stem cells that continually renew the differentiated spinous, granular, and cornified suprabasal layers (Fuchs, 2008). Together, these cells protect against environmental insults such as radiation, physical injury, dehydration, and
pathogens. A major environmental hazard to the skin is ultraviolet (UV) solar radiation, which induces potentially mutagenic DNA damage if left unrepaired. UV-damaged cells undergo either apoptosis or cell cycle arrest coupled with DNA repair before re-entry into the cell cycle which results in thickening of the epidermis (El-Abaseri et al., 2006). Aging of the skin induces cellular senescence, thinning of the epidermis and reduced basal stem cell renewal and proliferation (Liu et al., 2019). While we are beginning to understand some of the mechanisms that underlie the slow repair of aged skin (Keyes et al., 2016; Wong et al., 2019), further research is needed.

Long-lived or stress-resistant model organisms permit a better understanding of adaptive mechanisms of tissue homeostasis and repair. For example, the Snell dwarf mouse attains a long lifespan through impaired pituitary hormone signaling and its study has informed our understanding of how organismal physiology and longevity are balanced (Brown-Borg, 2011; Flurkey et al., 2002). The naked mole rat *Heteroecephalus glaber* (*H. glaber*) has evolved distinctive mechanisms of cancer resistance which may be mediated by microenvironment and immune system (Hadi et al., 2018; Seluanov et al., 2009; Tian et al., 2015). In a similar vein, models of vertebrate regeneration such as salamanders and newts have been used extensively to understand the optimal healing of nervous, muscle and connective tissue (Joven et al., 2019), but a critical barrier to deriving clinical therapies from this work has been a lack of mammalian models of tissue repair. The recent discovery that spiny mice (*Acomys* spp., hereafter referred to as *Acomys*) can fully regenerate skin and hair following wounding or burn injury (Maden, 2018; Seifert et al., 2012), a feature not observed in typical strains of mice (*Mus musculus*; *Mus*), lends well to uncovering novel regenerative mechanisms in skin tissue. However, it is not known how *Acomys* responds to other types of skin stress, including UV-damage and chronological aging.
Here, we examine epidermal responses to acute UVB exposure and chronological aging in the conventional C57Bl/6 *M. musculus* strain compared to *A. cahirinus*. We find that *A. cahirinus* has attenuated pathological hallmarks of UVB exposure and aging in conjunction with distinctive differences in epidermal turnover, senescence and inflammation. These processes may underlie the unique skin regeneration phenotype of *A. cahirinus*, and such mechanisms can inform therapeutic strategies to manage skin diseases such as skin cancer and aging.

### 4.3 Materials and Methods

**Mice**

For all studies, C57Bl/6 (*M. musculus*) mice were acquired from Jackson Labs (#000664). For the UVB experiments, 5-6 month old male *A. cahirinus* animals were shipped from the University of Florida and compared to 3-4 month old male C57Bl/6 mice. *M. musculus* were housed 4-5 to a cage in mouse cages while *A. cahirinus* were housed 2-3 to a cage in rat cages with a 12h/12h light cycle. Animals were housed at Northeastern University for at least 2 weeks prior to UVB or sham treatments to acclimate. For the aging studies, young, female animals of each species at 3-4 months of age were used and old, female animals (*M. musculus*: 2 years of age; *A. cahirinus*: 4 years of age) were analyzed. The young and old *M. musculus* mice were housed at Northeastern University while the young and old *A. cahirinus* animals were housed at the University of Florida and University of Kentucky. For the UVB studies, all animals were sacrificed by cervical dislocation. For the aging studies, animals were euthanized either by cervical dislocation (*M. musculus*) or isoflurane overdose (*A. cahirinus*). All procedures were performed according to IACUC approved procedures from each institution.
**Ultraviolet Irradiation**

Animals were sedated with intraperitoneal ketamine/xylazine anesthesia (50 mg/kg ketamine; 5 mg/kg xylazine) and shaved with clippers prior to UVB exposure. Animals were exposed to a UVB dose of 200 mJ/cm\(^2\) using a dosimeter calibrated UV instrument (310 nm peak output) (Tyler Research, Alberta, Canada). Full-thickness dorsal skin samples in telogen phase were collected and fixed at 24- and 48-hours after irradiation in addition to sham controls and paraffin embedded cross-sections were prepared for histologic analysis. BrdU (5-Bromo-2’-deoxyuridine, Sigma Aldrich, St. Louis, Missouri, USA) was injected intraperitoneally at 100 mg/kg in sterile PBS 24 hours prior to sacrifice. Animals were sacrificed by cervical dislocation.

**Histology**

Tissues were fixed overnight in 10% neutral buffered formalin and thereafter transferred to 70% ethanol at 4°C until processing. Tissues were processed using an automated tissue processor (Thermo HistoStar, Kalamazoo, Michigan, USA) and embedded in paraffin wax. A microtome (Leica Biosystems, Buffalo Grove, Illinois, USA) was used to cut 4 μm skin cross-sections, which were allowed to dry overnight before de-waxing and further processing. Heat induced epitope retrieval was performed on de-waxed slides with either citrate or Tris-EDTA buffer prior to immunofluorescence staining. Skin cross-sections were blocked for 30 minutes at room temperature in 5% normal goat serum PBS, which was also the primary antibody diluent, unless the target was a phospho-protein in which case 5% normal goat serum TBS was used. Primary antibodies and their usage were as follows: Keratin 14 (chicken, BioLegend #906004,
1:500, San Diego, California, USA), HMGB1 (rabbit, Abcam #AB79823, 1:250, Cambridge, Massachusetts, USA), Lamin B1 (rabbit, Abcam #AB16048, 1:1000), Keratin 10 (rabbit, BioLegend #905404, 1:500), Loricrin (rabbit, BioLegend #905104, 1:500), BrdU (rat, Abcam #AB6326, 1:200), thymine dimer mouse, Novus #NB600-1141, 1:400, Centennial, Colorado, USA), cleaved caspase-3 (rabbit, Cell Signaling #9661, 1:100, Danvers, Massachusetts, USA), γH2AX (rabbit, Cell Signaling #9718, 1:40). Primary antibody incubations were carried out overnight at 4°C except Keratin 14, Keratin 10, and Loricrin, which were 2 hours at room temperature. Following either PBS or Tris-buffered saline (TBS) wash, secondary detection antibodies conjugated with either AlexaFluor-647 or AlexaFluor-488 (Invitrogen, Carlsbad, California, USA) were diluted in either PBS or TBS at 1:200 were applied for 30 minutes at room temperature. Mounting media with DAPI (4′,6-diamidino-2-phenylindole) used was either ProLong Gold (Invitrogen) or ProLong Diamond (Invitrogen), which were used interchangeably and allowed to cure prior to imaging. For thymine dimer staining, a mouse-on-mouse blocking kit (Vector Labs #BMK-2202, Burlingame, California, USA) was used per the manufacturer’s instructions.

Hematoxylin and Eosin staining was performed on 4 μm de-waxed sections using standard histology protocols using Gill’s Hematoxylin No.1 and Eosin Y (Sigma Aldrich). Colorimetric stained slides were mounted with Permount (Fisher Scientific, Pittsburgh, Philadelphia, USA). All brightfield imaging was done using an inverted EVOS XL (Life Technologies, Carlsbad, California) microscope. All fluorescence imaging was performed on either an Revolve R4 (Echo Labs, San Diego, California, USA) microscope equipped with Olympus UPlanFL 10x/0.30, and UPlanFL 20x/0.50 air objectives using DAPI, FITC, and Cy5 fluorescence channels or an Axio Observer Z1 (Zeiss, White Plains, New York, USA), equipped with Plan Apochromat 5x/0.16,
10x/0.45, and 20x/0.8 air objectives and 40x/1.3 and 63x/1.4 oil objectives using DAPI, GFP, DsRed, and Cy5 fluorescence channels.

**Image analysis**

All image processing and analysis was conducted using ImageJ FIJI (version 2.0.0-rc-69/1.52i, NIH), Photoshop CC 2019 (version 20.0.4, Adobe), and Illustrator CC 2019 (version 23.0.3, Adobe, San Jose, California, USA). Immunofluorescence images first underwent thresholding over entire image set of all samples from the experiment followed by manual counting of positive cells for at least 3 unique fields from each sample. All epidermal analyses only counted interfollicular epidermal cells and excluded hair follicles. Nuclear HMGB1 positive cells were those that had overlapping signal with their nuclear DAPI signal. Lamin B1 positive cells were those that exhibited perinuclear signal relative to their DAPI signal. Keratin 14, Keratin 10, and Loricrin positive cells were those that had DAPI-associated cytoplasmic signal. Epidermal thickness measurements were performed on H&E stained intact skin cross-sections as previously described (Crane et al., 2015) by measuring the orthogonal distance from the basement membrane to the outer edge of the cellular epidermis.

**Gene Expression**

RNA was extracted by homogenizing whole skin in TRIzol (Invitrogen) reagent using a bead mill apparatus (MPBio 5G, Irvine, California, USA) followed by column purification (Zymo Direct-zol RNA mini prep, Irvine, California, USA) with on column DNase treatment and subsequent elution. 600 ng of total RNA was then reversed transcribed to cDNA (ABI HC cDNA synthesis kit, Waltham, Massachusetts, USA), diluted 1:30 in ultrapure water and mRNA
expression was assessed using qPCR with SYBR chemistry. CDK-interacting protein 1 (Cdkn1a) was used as a housekeeping gene and its expression did not significantly differ between species or with UVB treatment. Data was analyzed by the delta Ct (ΔCt) method.

Primers are as follows:

\[
\begin{align*}
M.\ musculus\ Cxcl1-Forward: & \text{ ACTCAAGAATGTCGCAGAGCGG; M. musculus Cxcl1-} \\
\text{Reverse:} & \text{ GTGCCATCAGAGCAGTCTGT.} \\
A.\ cahirinus\ Cxcl1-Forward: & \\
\text{Reverse:} & \text{ CCCATGGTTCGGAAGTGTGT;} \\
\text{GTTGTCAGACGCGACAGACC.} & \text{ M. musculus Il1b-Forward:} \\
\text{TGCCACCTTTTGCAGTGATG;} & \text{ M. musculus Il1b-Reverse:} \\
\text{TGATGTGCTGCTGCGAGATT.} & \text{ A. cahirinus Il1b-Forward: CTGGGCTCCAGAGACACCAAG;} \\
\text{A. cahirinus Il1b-Reverse:} & \text{ GAACCCCTGCATCAAATCTCA.} \\
\text{M. musculus Cdkn1a-Forward:} & \\
\text{CAGTCGCGCCTAGAGTGAT.} & \text{ A. cahirinus Cdkn1a-Forward:} \\
\text{Reverse:} & \text{ CGGTGCAGAGTCTAGGGA;} \\
\text{AGGATTGGACATGGTGCTG.} & \text{ M. musculus Cdkn1a-Reverse:} \\
\text{TGCACTCTGGTATCTCAGCG;} & \text{ A. cahirinus Cdkn1a-Reverse:} \\
\text{CAGTCGCGCCTAGAGTGAT.} & \text{ A. cahirinus Cdkn1a-Reverse:}
\end{align*}
\]

**Statistical Analysis**

All data were analyzed in Prism 8.0 (GraphPad, San Diego, California, USA) using a two-way analysis of variance (ANOVA) on either log or square root transformed data with Tukey’s HSD post-hoc testing for differences between treatments and species. Statistical significance was set as \( p<0.05 \).
4.4 Results

UV-irradiation induced changes to skin epidermal morphology and epidermal stratification of *M. musculus* and *A. cahirinus*.

While *A. cahirinus* can fully regenerate skin wounds and burns without scarring (Maden, 2018; Seifert et al., 2012), it has not been established how this regenerative ability translates to other types of skin damage. Since UVB radiation is a common form of epidermal damage (Brash et al., 1991), we sought to compare the morphological changes between *A. cahirinus* and *M. musculus* epidermis following an acute dose of UVB known to induce epidermal proliferation and DNA damage in *M. musculus* (Trevithick et al., 1992). We first measured the total thickness of the cellular epidermis using hematoxylin and eosin staining. As expected, *M. musculus* had a significantly thicker epidermis at 48 hours post-UVB compared to sham or 24 hours (Fig. 1A). In contrast, there was no appreciable difference in epidermal thickness in response to UVB in *A. cahirinus* at 24 hours (*p=0.21*) or 48 hours (*p=0.80*) compared to sham (Fig. 1B). To understand the processes underlying this differential response, we examined rates of cell division by analyzing post-UVB epidermal incorporation of the synthetic nucleoside 5-bromo-2’-deoxyuridine (BrdU). During imaging, we noted species specific patterns of BrdU labeling in basal and suprabasal epidermal compartments (Fig. 1C). To distinguish these changes, we analyzed patterns of label retention in the two compartments separately. In the basal epidermis, BrdU incorporation was significantly lower in *M. musculus* basal epidermis 24 hours after UVB-exposure, and significantly higher at 48 hours following UVB compared to both sham and 24 hours (Fig 1D). In contrast, *A.
*cahirinus* epidermis did not exhibit changes in BrdU incorporation at 24 hours post-exposure but was significantly elevated at 48 hours compared to both the sham and 24 hour groups (Fig. 1D). In the suprabasal epidermis, both *M. musculus* and *A. cahirinus* exhibited greater BrdU incorporation at 48 hours compared to sham, however this UV-induced proliferation was greater in *M. musculus* (Fig. 1E).

Since stem cell fate decisions control the balance of cell division and upward transport through the suprabasal layers (Williams et al., 2011), we next stained for keratin 14 (K14) to mark the basal stem/progenitor layer, keratin 10 (K10) to mark the spinous layer, and for the Loricrin (Lor) expressing cornified envelope after UVB in each species. During imaging, we noticed species specific patterns of UVB-induced epidermal differentiation, most notably the unique double-positive K14+/K10+ suprabasal layer in UVB-exposed *A. cahirinus* (Fig. 1F). We then measured the thicknesses of each of the labeled basal (K14+/K10−), double-positive (K14+/K10+), spinous (K14−/K10+), and cornified (Lor+) epidermal layers. Exposure to acute UVB resulted in a significantly thicker K14+ layer in *M. musculus* by 48 hours but not in *A. cahirinus*. However, a double-positive K14+/K10+ suprabasal epidermal layer was uniquely evident only in *A. cahirinus* at 24 and 48 hours post-UVB (Fig. 1G). The thickness of the Lor+ layer between species was similar at all time points and was not statistically different (*p=0.0986*) in *A. cahirinus* at 48 hours (Fig. 1G and Fig. S1A). Overall, *A. cahirinus* skin exhibits a unique program of epidermal stratification without hyperproliferation. This distinctive pattern of differentiation consists of a transient intermediate layer with both basal and spinous characteristics in response to UVB exposure.
Differences in UV-induced damage and epidermal cell death between *M. musculus* and *A. cahirinus*

In *M. musculus* and humans, differentiation and eventual shedding of skin cells via upward transport through the epithelium serves as a method of eliminating damaged and compromised cells (Freije et al., 2014). Since we observed an altered differentiation pattern in *A. cahirinus* in response to UVB, we reasoned that this could impact the removal of damaged cells. We first assessed this by measuring the UVB-induced DNA photoproduct thymine dimer (T-T dimer) in the epidermis (Fig. 2A). As expected from this dose of UVB, we found significantly higher thymine dimer positive cells in *M. musculus* basal epidermis by 24 hours with a near return to sham levels by 48 hours (Fig. 2B), yet in the suprabasal layer levels rose significantly by 24 hours and remained elevated at 48 hours (Fig. 2C). In *A. cahirinus*, while we found an induction of thymine dimer positive cells 24 hours following UVB, this UV-response was reduced compared to *M. musculus* animals in both the basal and suprabasal epidermis (Fig. 2B-C). To extend our thymine dimer results, we next assessed the DNA repair marker γH2AX (Fig. 2D). *M. musculus* had greater basal and suprabasal epidermal γH2AX labeling at 24 hours and 48 hours after UVB (Fig. 2E and 2F). *A. cahirinus* also had more γH2AX-positive cells versus sham at 24 hours in the basal layer, with a similar pattern in the suprabasal layers at 24 and 48 hours (Fig. 2E and 2F). However, similar to thymine dimers, there were more γH2AX-positive cells in *M. musculus* compared to *A. cahirinus* at 24 and 48 hours post UVB exposure (Fig. 2E and 2F).

To understand the contribution of apoptosis to the post-UVB epidermal response, we measured the abundance of cleaved caspase-3 (CC3) positive cells (Fig. 2G). In the basal layer, there was a similar UVB-induced response between species, with an increase in cleaved caspase-3 expressing cells at 24 hours followed by a return to sham levels 48 hours later (Fig. 2H). This
was in contrast to the suprabasal epidermis, where at 24 hours *A. cahirinus* exhibited a significantly greater number of cleaved caspase-3 expressing cells compared to *M. musculus*. However, there was a similar abundance of cleaved caspase-3 expressing cells between species at 48 hours (*M. musculus* significantly elevated vs baseline; \( p=0.0913 \) for *A. cahirinus* vs baseline) (Fig. 2I). Thus, *A. cahirinus* epidermis have modest changes in cell death responses following UVB exposure compared to *M. musculus*.

**Attenuated skin epidermal inflammatory response following UV-irradiation in *A. cahirinus***

Inflammation is a major component of epidermal remodeling following UVB exposure (Barker et al., 1991). To gain further insight into the differential UVB responses between *M. musculus* and *A. cahirinus*, we analyzed the epidermal abundance of the damage associated molecular pattern, HMGB1 (Fig. 3A). Loss of nuclear HMGB1 is a biomarker of cell stress, senescence, inflammation, and autophagic responses particularly in UVB-exposed basal epidermis (Bald et al., 2014; Davalos et al., 2013). Quantification of HMGB1 labeling revealed that *M. musculus* experiences a significant loss of basal nuclear HMGB1 by 48 hours after UVB (Fig. 3B). However, we found that post-UVB *A. cahirinus* basal epidermis retained nuclear HMGB1 labeling similar to sham levels (Fig. 3B). Suprabasal levels of HMGB1 were similar between species and treatments (Fig. 3C), suggesting that this effect is restricted to basal progenitors. A milieu of pro-inflammatory signaling within the skin also occurs concomitantly to the epidermal hyperplastic response (Ouhtit et al., 2000). To better understand the UV-induced inflammatory response in each species, we measured mRNA levels of the inflammation associated genes *Il1b*, *Cxcl1*, *Tgfb1*, and *Mmp9* in whole skin using species specific primers and qPCR. Increased epidermal expression of *Il1b* and *Cxcl1* mediate inflammatory responses after UV-exposure (Qiang et al., 2017), and
knockout mouse studies have shown loss of Tgfb1 and Mmp9 are associated with increased and prolonged inflammation in the skin epidermis (Wang et al., 1999; Yoshinaga et al., 2008). Acute UVB-irradiation resulted in significant increase in Cxcl1 in M. musculus skin at 48 hours, but levels in A. cahirinus were not significantly altered by UVB exposure (Fig. 3D). In contrast, Il1b expression was not significantly changed by acute UVB-irradiation in either species but was consistently lower in A. cahirinus vs. M. musculus (Fig. 3E). Transcript levels of Tgf1b in M. musculus skin significantly decreased at 24 hours but A. cahirinus levels were unchanged (Fig. 3F). Similarly, levels of Mmp9 in M. musculus skin significantly decreased at 24 and 48 hours but A. cahirinus levels were not significantly altered by UVB (Fig. 3G). Thus, compared to M. musculus, A. cahirinus epidermis resists UVB-driven inflammatory gene expression changes, consistent with their anti-inflammatory tissue damage response seen after wounding and burn injury (Brant et al., 2015; Gawriluk et al., 2019; Maden, 2018).

**Aging associated epidermal thinning, inflammatory signaling, and senescence are absent in A. cahirinus**

Total organismal aging is the sum of chronological aging and environmental exposure. We sought to further explore the differences in cellular stress response in these two species by examining their epidermal responses to chronological aging. A. cahirinus have a maximal lifespan of 5.9 years, versus 3.5 years in M. musculus (C57Bl/6) (Edrey et al., 2012). Thus, to study aging in these species, we used young animals from each species at 3-4 months old as well as aged 2-year old M. musculus and 4-year old A. cahirinus animals. At these ages the species are roughly matched as a fraction of their respective maximum lifespans: approximately 9.4% for young M. musculus, 8.4% for young A. cahirinus, 56% for old M. musculus, and 68% for old A. cahirinus.
Using formalin fixed skin cross-sections for both species, we used hematoxylin and eosin staining of the skin to reveal morphological changes due to age (Fig. 4A). This revealed an expected decrease in the thickness of the cellular epidermis of old versus young *M. musculus* (Fig. 4B), however, the cellular epidermal thickness of *A. cahirinus* remained unchanged with age (Fig. 4B). To better understand the cellular epidermal thickness differences, we measured the nuclear envelope protein and senescence associated biomarker Lamin B1 (Fig. 4C). Similar to previous research by ourselves (Wong et al., 2019) and others (Freund et al., 2012; Wang et al., 2017), we found a decrease in basal epidermal Lamin B1 with age in *M. musculus* (Fig. 4D). However, *A. cahirinus* basal epidermal Lamin B1 labeling was similar to young *M. musculus* and was not different in old *A. cahirinus* versus young (Fig. 4D). To corroborate these findings regarding senescence, we also evaluated the proportion of epidermal cells with nuclear HMGB1 staining (Fig. 4E). As previously reported by ourselves (Wong et al., 2019) and others (Davalos et al., 2013), we observed a decrease in epidermal nuclear HMGB1+ cells with age in *M. musculus* (Fig. 4F), yet HMGB1 in old *A. cahirinus* remained unchanged from young animals (Fig. 4F). Collectively, these data show that *A. cahirinus* have reduced hallmarks of epidermal aging.

4.5 Discussion

The comparative study of exceptionally long-lived, stress-resistant, or regenerative organisms can be used to provide insight into evolved mechanisms of cellular repair. In this study, we found substantial differences in how the regenerative rodent *A. cahirinus* responds to UVB skin damage compared to *M. musculus*. This included altered patterns of keratinocyte proliferation and differentiation, an earlier induction of UVB-induced cell death, and an attenuated inflammatory response. The acanthosis commonly observed in *M. musculus* after UVB was not
evident in *A. cahirinus* despite similar rates of basal cell proliferation, suggesting an alternative fate of newly formed keratinocytes. Notably, the *A. cahirinus* epidermis forms a unique middle suprabasal layer of keratinocytes with both basal and spinous characteristics (K14+/K10+) which may facilitate increased keratinocyte upward transport and removal. This was supported by the observations of fewer retained damaged cells (thymine dimers, γH2AX) as well as more apoptotic cells transiting the suprabasal epidermis at intermediate stages of repair (24 hours) in *A. cahirinus* compared to *M. musculus*. Taken together, these data suggest that *A. cahirinus skin* epidermis is capable of more rapid removal of damaged and dying cells after UVB-exposure through an enhanced rate of differentiation.

The presence of a co-expressed K14+/K10+ suprabasal layer in the epidermis has been reported by others, but usually this is in the context of skin disorders. For example, in psoriatic human skin biopsies, there is aberrant Notch expression and dual K14/K10 expression (Ota et al., 2014). Dysregulated epidermal calcium gradients can also produce dual expression of K14 and K10, such as in the skin blisterning disorders Hailey-Hailey disease and Darier disease (Mikkelsen et al., 2018; Robia and Young, 2018), and in TRPV4 KO mice (Moore et al., 2013) which suffer defective tight junction formation (Sokabe et al., 2010). Interestingly, TRPV4 is also a nociceptor and those mice had altered pain perception and inflammatory responses after sunburn (Moore et al., 2013). Aberrant cell cycle regulation can also cause dual K14/K10 expression as mice lacking epidermal C/EBPβ and C/EBPα exhibit defective cornified envelope formation (Lopez et al., 2009). In contrast to these pathological examples of K14/K10 co-expression, this suprabasal layer formed after UVB in *A. cahirinus* appears to be a physiological skin damage response. Notably, *A. cahirinus* executes this distinctive program of epidermal differentiation without marked changes
in inflammatory signaling factors, which is similar to the blunted inflammatory response reported during wound healing (Brant et al., 2015).

The study of organisms with long lifespans such as the naked mole rat (*H. glaber*) has revealed several unique mechanisms of stress resistance (Pérez et al., 2009; Seluanov et al., 2009). Long-lived model organisms often also exhibit altered inflammatory and cellular damage responses distinct from humans or laboratory mice which provide cancer resistance or augmented tissue repair (Eming et al., 2009; Kowalczyk et al., 2020). In *H. glaber*, cellular senescence is rare, yet cancer rates are very low due to sensitive cellular growth inhibition, modified tumor suppressor pathways, altered extracellular matrix composition, and a stable epigenome (Seluanov et al., 2018). The evolutionary path to these adaptive differences was likely guided by evolutionary adaptation to the hypoxic subterranean environment of *H. glaber* and the fructose biased metabolism resulting from hypoxia (Seluanov et al., 2018). We reasoned that *A. cahirinus* might have also evolved distinct cellular stress mechanisms to enable extensive tissue regeneration, which parallels the unique survival mechanisms recently found in *A. cahirinus* fibroblasts *in vitro* (Saxena et al., 2019). We found that *A. cahirinus* epidermis was remarkably resilient to chronological aging stress with minimal changes in epidermal thickness or biomarkers of aging and senescence. It is interesting to note that the MRL/MpJ healer strains of mice also exhibit reduced inflammatory responses to injury and improved recovery outcomes in the corneal epithelium (Ueno et al., 2005) and ear punch wounds (Kench et al., 1999). Thus, an attenuated inflammatory response may enable the unique growth and repair patterns of *A. cahirinus* epidermis compared to *M. musculus* and may also explain the differences in biological aging between the species.

Our study of the regenerative African spiny mouse, *A. cahirinus*, has revealed a unique skin response to acute UVB-irradiation which consists of rapid differentiation and apoptosis.
without prototypical epidermal hyperplasia or inflammation. However, we do not yet understand the precise cellular mechanisms underlying this response. While proliferation and differentiation may be intrinsically regulated, inflammation and clearance of cells is likely related to the altered immunity and inflammation previously reported during skin repair in *A. cahirinus* (Maden, 2018; Simkin et al., 2017). Moreover, our findings of improved epidermal tissue repair in *A. cahirinus* extend recent work using models of spinal cord injury (Streeter et al., 2019), musculoskeletal regeneration (Gawriluk et al., 2016), and muscle damage (Maden et al., 2018). Future work should clarify the molecular underpinnings of improved tissue repair in *A. cahirinus* in order to advance regenerative medicine.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

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**List of abbreviations**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>K10</td>
<td>Keratin 10</td>
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<td>K14</td>
<td>Keratin 14</td>
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<td>Lor</td>
<td>Loricrin</td>
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<td>PBS</td>
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<td>BrdU</td>
<td>5-Bromo-2’-deoxyuridine</td>
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<td>mJ</td>
<td>Millijoule</td>
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<td>TBS</td>
<td>Tris-buffered saline</td>
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<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>Red fluorescent protein</td>
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Figure 1. UV-irradiation induced changes to skin epidermal morphology and proliferation of *M. musculus* and *A. cahirinus*. A, representative brightfield microscopy images of hematoxylin and eosin-stained skin from control (sham) and UV-irradiated *M. musculus* and *A. cahirinus*, collected 24 and 48 hours after exposure. Scale bar = 100 µm. B, quantification of epidermal thickness. n = 3-6 mice per group. C, representative immunofluorescence images of epidermal BrdU labeling. The epidermal basement membrane is indicated by the yellow dashed line. Scale bar = 25 µm. D and E, quantification BrdU labeling in (D) basal epidermis and (E) suprabasal epidermis. n = 3-6 mice per group. F, representative immunofluorescence images of epidermal differentiation markers keratin 14 (K14), keratin 10 (K10), and loricrin (Lor) labeling with magnified inset of the basal-suprabasal junction. Scale bar = 50 µm. G, quantification of individual differentiation marker layer thickness. n = 3 mice per group. Loricrin images included in supplement. Data are mean ± S.E. *, significantly different (p<0.05).
Figure 2. Differences in removal efficiency of UV-induced damaged and dying epidermal cells through turnover between *M. musculus* and *A. cahirinus*. 

A, representative immunofluorescence images of epidermal thymine dimer (*T-T dimer*) labeling of skin from control (*sham*) and UV-irradiated *M. musculus* and *A. cahirinus*, collected 24 and 48 hours after exposure. 

B and C, quantification of thymine dimer labeling in (B) basal epidermis and (C) suprabasal epidermis. 

D, representative immunofluorescence images of epidermal γH2AX labeling. 

E and F, quantification γH2AX labeling in (D) basal epidermis and (E) suprabasal epidermis. 

G, representative immunofluorescence images of epidermal cleaved caspase-3 (*CC3*) labeling. 

H and I, quantification of cleaved caspase-3 labeling in (H) basal and (I) suprabasal epidermis. Data are mean ± S.E. *, significantly different (*p* < 0.05). The epidermal basement membrane is indicated by the yellow dashed line. All scale bars = 50 µm. All measurements *n* = 3-6 mice per group.
Figure 3. Attenuated skin epidermal inflammatory response following UV-irradiation in *A. cahirinus*. 

*Figure 3. A*, representative immunofluorescence images of epidermal HMGB1 labeling of skin from control (sham) and UV-irradiated *M. musculus* and *A. cahirinus*, collected 24 and 48 hours after exposure. The epidermal basement membrane is indicated by the yellow dashed line. Scale bar = 50 µm. *B and C*, quantification of HMGB1 labeling in (B) basal epidermis and (C) suprabasal epidermis. *n = 3-6 mice per group. D and E*, mRNA expression of (D) *Cxcl1* and (E) *Il1b* in each treatment group as measured by qPCR. *n = 3-7 mice per group*. Data are mean ± S.E. *, significantly different (*p < 0.05*). †, significantly different (*p < 0.05*) to *M. musculus* at the same timepoint.
Figure 4. Aging associated epidermal thinning, inflammation, and senescence are absent in *A. cahirinus*. A, representative brightfield microscopy images of hematoxylin and eosin-stained intact skin from control (Young) and aged (Old) *M. musculus* and *A. cahirinus*. Scale bar = 100 µm. B, quantification of epidermal thickness. C, representative immunofluorescence images of epidermal Lamin B1 labeling of skin from each treatment group. The epidermal basement membrane is indicated by the yellow dashed line. Scale bar = 50 µm. D, quantification of epidermal Lamin B1 labeling. E, representative immunofluorescence images of epidermal HMGB1 labeling of skin from each treatment group. The epidermal basement membrane is indicated by the yellow dashed line. Scale bar = 50 µm. F, quantification of epidermal HMGB1 labeling. All measurements *n* = 5-8 mice per group. Data are mean ± S.E. *, significantly different (*p*<0.05).
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