Impaired Neovascularization in Aging

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Significance: The skin undergoes an inevitable degeneration as an individual ages. As intrinsic and extrinsic factors degrade the structural integrity of the skin, it experiences a critical loss of function and homeostatic stability. Thus, aged skin becomes increasingly susceptible to injury and displays a prolonged healing process.

Recent Advances: Several studies have found significant differences during wound healing between younger and older individuals. The hypoxia-inducible factor 1- α (HIF-1 α) signaling pathway has recently been identified as a major player in wound healing. Hypoxia-inducible factors (HIFs) are pleiotropic key regulators of oxygen homeostasis. HIF-1 α is essential to neovascularization through its regulation of cytokines, such as SDF-1 α (stromal cell-derived factor 1- α) and has been shown to upregulate the expression of genes important for a hypoxic response. Prolyl hydroxylase domain proteins (PHDs) and factor inhibiting HIF effectively block HIF-1 α signaling in normoxia through hydroxylation, preventing the signaling cascade from activating, leading to impaired tissue survival.

Critical Issues: Aged wounds are a major clinical burden, resisting modern treatment and costing millions in health care each year. At the molecular level, aging has been shown to interfere with PHD regulation, which in turn prevents HIF-1 α from activating gene expression, ultimately leading to impaired healing. Other studies have identified loss of function in cells during aging, impeding processes such as angiogenesis.

Future Directions: An improved understanding of the regulation of molecular mediators, such as HIF-1 α and PHD, will allow for manipulation of the various factors underlying delayed wound healing in the aged. The findings highlighted in this may facilitate the development of potential therapeutic approaches involved in the alteration of cellular dynamics and aging.

Keywords: HIF-1 α , PHD, aging, neovascularization, skin

SCOPE AND SIGNIFICANCE

THIS REVIEW FOCUSES on the effects of aging on the hypoxia-inducible factor 1- α (HIF-1 α) signaling pathway and the molecules that regulate it. HIF-1 α is integral to neovascularization and has been shown to upregulate the expression of genes involved in a hypoxic response. Aging engenders impediments to HIF-1 α signaling, slowing

the wound healing process. Developments toward regenerative medicine in wound healing are detailed, with a focus on targeting the age-associated defects in the HIF-1 α pathway.

TRANSLATIONAL RELEVANCE

Multiple treatments are available that attempt to prevent skin from

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aging and accelerate the different phases of healing in aged wounds. With an understanding of the pathophysiology of aging skin, therapies can be developed to specifically target the pathways that are altered with age, leading to the development of new treatments.

CLINICAL RELEVANCE

The aged population is disproportionately affected by chronic wounds. Aged wounds can be highly resistant to treatment, persisting indefinitely. Many patients require multiple rounds of treatment, costing the health care system millions each year. The methods discussed in this review are aimed to give an updated summary of wound healing in aged skin and the signaling pathways affected by aging. With a better understanding of these subjects, new treatments may be developed to improve clinical outcomes.

DISCUSSION OF FINDINGS AND RECENT LITERATURE

Introduction

The skin is the largest organ within the human body, serving several purposes, including the provision of a barrier between the internal organs and the outside environment, homeostatic and temperature regulation, and fluid retention.^{1,2} Under normal conditions, cells within the skin exist as a complex and balanced mosaic with continuous turnover. As humans age, the skin undergoes a visible degeneration, with a reduced ability to perform these functions. The physical structure of skin changes with age, leading to an increased susceptibility to injury and morbidity, with a majority of those over 65 years of age having at least one skin disorder.^{3–5} Such disorders and changes in the skin can exacerbate the process of healing following injury through their influences on the microenvironment. As the elderly population continues to rise, efforts have been made to attain a better understanding of the effects of aging on various processes associated with wound healing.

Overview of aging

Intrinsic and extrinsic aging factors are two commonly accepted categories associated with deleterious changes to the skin and can lead to accelerated aging.⁶ Intrinsic aging consists of the physiological changes over time that are determined by genetics, whereas extrinsic aging is the result of controllable, external factors such as exposure to ultraviolet (UV) radiation, pollution and nicotine, excessive and repetitive use of muscles,

and a lack of proper nutrition and sleep.^{7,8} UV radiation is likely the most important of these factors and is split into two major components: UVA [ultraviolet A (long-wave)], which penetrates deeply into the dermis and generates several reactive oxygen species (ROS) which damage DNA, and UVB [ultraviolet B (shortwave)], which affects the epidermis and is absorbed by DNA, leading to harmful, carcinogenic mutations.⁹ UV generated ROS down-regulate several factors and processes that are essential to maintaining skin integrity, such as transforming growth factor beta (TGF- β) and collagen synthesis.^{10,11} Furthermore, these ROS cause excessive oxidative stress that brings about an inflammatory response and subsequent cascading effects that lead to collagen degradation.¹² UVB can also induce inflammation and various signaling cascades, leading to irregular keratinocyte function and lifespan.^{13–15}

While both intrinsic and extrinsic factors lead to unhealthy alterations in the skin, the exact changes brought about by one or the other often differ.¹⁶ For instance, skin largely protected from harmful UV radiation typically displays a dermal thinning along with a flattening of the dermal–epidermal junction (DEJ) as it ages.^{17–19} Conversely, skin subjected to high amounts of UV radiation displays a dermal thickening, suggesting that differing pathways likely govern the various ways skin can age.²⁰ These changes, while different, yield a loss of function within the skin, leading to a higher susceptibility to disease.²¹

Structure of the skin, how it differs with age

The skin comprises roughly 12–16% of the human body weight and consists of three separate layers: the epidermis, dermis, and hypodermis.^{9,19} The epidermis is the outermost layer and is between 50 to 100 μ m thick in a healthy individual, depending on the location.²² The epidermis primarily consists of keratinocytes that are constantly sloughed off and replaced with younger, healthier cells. This turnover rate is reduced within older individuals, with the time it takes for keratinocytes to migrate from the basal layer to the epidermis increased by up to 50%.²³ The stratum corneum is the most superficial region of the epidermis and provides the skin with its water-resistant properties.²⁴ A complex matrix of hydrophobic lipids and “natural moisturization factors,” including cholesterol, amino acids, and fatty acids, regulate skin moisturization in normal, healthy skin.^{24–26} Aged skin displays significantly lower levels of lipids and amino acids essential to water retention, thereby contributing to higher levels of xerosis (dry

Figure 1. Structure of unwounded, young skin and aged skin. Keratinocytes display a loss of function, proliferating, and migrating at lesser rates than those found in younger, healthy skin. The epidermis in aged skin thus lacks the same cellular upkeep found in young skin. Aged skin displays a significantly thinner dermal-epidermal junction, making aged skin more susceptible to shearing forces and injury. The dermis of aged skin is characterized by lesser organization of ECM components, making it less stable and sturdy than the dermis in young skin. ECM, extracellular matrix. Color images are available online.

skin) and sensitivity to irritants.^{27,28} The stratum granulosum, stratum spinosum, and stratum basale make up the rest of the epidermis, with keratinocytes within the stratum basale proliferating and migrating to the more superficial layers.²⁹

Immediately underlying the epidermis is the DEJ, joining the keratinocyte layers of the epidermis to the infrastructure of the dermis.³⁰ Several studies have observed a notable thinning of the DEJ as a result of intrinsic aging, making aged skin more susceptible to shearing forces, and thus, injury.^{30–32} Furthermore, studies have found that the protein composition of the DEJ changes with age, which could yield lesser adhesion between the epidermis and the dermis in addition to a loss of structural integrity.^{30,33} The deeper dermal layer displays fewer cellular constituents than the epidermis and is primarily composed of extracellular matrix (ECM) components. The ECM provides structure

for an array of nerves and vasculature that support the integrity of the skin. Fewer cells responsible for ECM deposition and tissue remodeling are found within the aged dermis, leading to a reduction in vasculature and collagen.²³ Additionally, collagen and elastin deposition become less organized, resulting in weaker and less elastic skin²³ (Fig. 1).

These age-associated, structural and etiological changes are only some of many that bring about increased susceptibility to injury. This altered microenvironment of aged skin leads to an irregular progression through the stages of regeneration: hemostasis, inflammation, proliferation, and tissue remodeling. Often times, these changes are deleterious and yield slow-healing, chronic wounds (Table 1).

Hemostasis. Immediately following injury to the skin, the regeneration process begins with he-

Table 1. The major characteristics of the phases of wound healing⁵⁹

Inflammatory 2–5 Days	Proliferative 5 Days to 3 Weeks	Remodeling 3 Weeks to 2 Years
Clotting cascade Extensive clotting occurs to attempt to obtain hemostasis	Angiogenesis Provides adequate supply of oxygen, nutrients, and growth factors, and promotes tissue granulation	Scar formation Collagen production and degradation are equalized Collagen remodeled from type III to type I Collagen crosslinked structure forms
Inflammatory response Macrophages release signaling molecules/inflammatory factors (serotonin, histamine, FGF, IL-1) to enhance vasodilation Release of proinflammatory cytokines and growth factors (TGF- β , PDGF, FGF, EGF) causing leukocyte migration Polymorphonuclear neutrophils and macrophages phagocytize debris, bacteria, and breakdown damaged tissue	Granulation Fibroblasts synthesize collagen type III and fibronectin to form a provisional ECM Re-epithelialization Re-epithelialization of the dermis bridges and resurfaces the wound Contraction Wound contraction begins	

ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; IL, interleukin; PDGF, platelet-derived growth factor; TGF- β , transforming growth factor beta.

mostasis. Blood vessels vasoconstrict and platelets adhere to the endothelium and accumulate, forming blood clots that prevent excessive blood loss.³⁴ These platelet clots are responsible for the secretion of various growth factors and signaling molecules, such as platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF- α), and TGF- β .^{23,35,36} Such signals are produced to recruit inflammatory cells to the wound bed and stimulate their activity. Thus, platelets are essential to progression into the inflammatory phase of wound healing. *In vivo* studies have found that aged individuals display an increase in protein aggregation compared with younger ones.^{37,38} Additionally, the release of signaling molecules PDGF, TGF- α , and TGF- β is increased with age as well.³⁸

Inflammation. Following the release of proinflammatory cytokines by platelets and mast cells, circulating neutrophils are recruited to the wound bed. Monocytes are also recruited to the wound, where they subsequently differentiate into phagocytic macrophages. Macrophages and neutrophils work together to debride the wound bed of any harmful biotics and detritus, while also secreting chemokines to recruit other cells to aid with wound resolution. Moreover, ROS are produced by neutrophils to assist with cleaning up the wound. ROS also serve as inflammatory mediators, and are beneficial to wound healing in low, contained concentrations. ROS also have a low specificity, which can become problematic, as an excess of ROS can induce a chronic inflammatory state and degrade a variety of beneficial molecules, including many protein and lipid constituents and even DNA.³⁹ ROS production is higher in the elderly, leading to higher levels of oxidative stress and thus, greater DNA degradation.⁴⁰

Aged individuals display alterations in cell function, cellular adherence, and cell migration, thereby affecting wound healing in a multitude of ways.^{41,42} *In vivo* mouse studies have found that neutrophils are likely essential to regular wound healing in aged mice, as neutrophil depletion significantly decreased the rate of wound closure in mice over 20 months of age.⁴³ This is likely due to a loss of function with age, as *in vitro* studies have found that neutrophils from the elderly display a lesser ability to phagocytose bacteria.⁴⁴ This could potentially lead to wound infection and subsequent chronic inflammation.

In addition to the alterations in neutrophil recruitment, circulating monocytes have been found to be recruited to the wound at a lesser rate in aged individuals. This coincides with a higher percent-

age of mature macrophages that are active in the wound. Such macrophages have been shown to have a loss in function, leading to reductions in angiogenesis, re-epithelialization, and tissue remodeling.²³ This was confirmed by *in vivo* studies, where injection of macrophages from aged mice into younger mice did not accelerate wound healing, whereas injection of macrophages from young mice did.⁴⁵ The exact mechanisms by which these observed changes act to influence wound healing in the aged have yet to be elucidated but provide several implications. Any drastic change in the number of these cell types could affect the rates of phagocytosis, chemokine production, and growth factor production, not only changing the outcome of the inflammatory phase, but also the subsequent wound repair phases as well.

Proliferation. Following a successful immune response, endothelial cells, fibroblasts, and keratinocytes are recruited to the wound bed to stimulate re-epithelialization and angiogenesis. This is typically after 3 days in a healthy individual with acute wounds.⁴⁶ Fibroblasts are recruited to the wound bed by factors secreted by platelets and inflammatory cells, such as PDGF and TGF- β .⁴⁷ Following migration to the wound, fibroblasts proliferate at a high rate and begin to synthesize ECM components, such as fibronectin and collagen, which are deposited into a provisional matrix.⁴² This matrix brings about further cell migration to aid with the healing process, although the ratio of collagen types differs in a repaired dermis from that of the unwounded dermis. Specifically, deposition of collagen I decreases significantly, leading to a higher ratio of collagen III to collagen I.⁴⁸ Furthermore, collagen content in the skin has been found to decrease by roughly 1% per year after reaching adulthood as a result of age-associated, waning fibroblast function.⁴⁹

As they continue proliferating, fibroblasts differentiate into myofibroblasts, characterized by their expression of α -smooth muscle actin (α -SMA).⁵⁰ These actin bundles give myofibroblasts contractile properties that they use to contract the wound, an essential process to wound closure.

Concurrently, local endothelial cells are recruited to aid in angiogenesis. Tissue disruption causes the wound to experience hypoxic conditions, activating several signaling pathways, such as the HIF-1 α pathway, which stimulates endothelial cell proliferation and growth. Studies have examined the expression of endothelial cell adhesion molecule (CAM), taking samples from human wounds. Samples from aged individuals displayed a signif-

icant delay in appearance and peak in both intracellular CAM-1 and vascular CAM-1.⁴¹

Hypoxic conditions also have been shown to impair keratinocyte motility in aged individuals, whereas keratinocyte motility was increased in younger individuals.⁵¹ Matrix metalloproteinase (MMP)-1 and -9 expressions were found to be reduced as well in response to hypoxia in aged, whereas both were upregulated in younger control groups.⁵¹ Both have been implicated to have a significant role in keratinocyte migration.⁵¹ Moreover, vascular endothelial growth factor (VEGF) expression has been positively correlated with hypoxia in younger, healthy individuals, and studies have found that older individuals display lower levels of VEGF expression along with lower HIF-1 α expression.⁵²

Thus, several factors underlie delayed wound regeneration during the proliferation phase, with several of these factors working in concert to exacerbate many of the issues. These alterations in the healing process hold an influence over the final phase of tissue remodeling.

Tissue remodeling. Following re-epithelialization and angiogenesis, cells work to remodel the tissue in a way that restores the skin as closely to its original integrity as possible. The end result of wound healing in nonfetal mammals most often is a scar that is characterized by collagen composition and organization that differ from healthy skin.⁵³ This causes the recovered skin to have a weaker mechanical strength than normal skin.⁵⁴ Interestingly, old subjects display a faster scar maturation rate than younger individuals.⁵⁵ Yet, hypertrophic scars and keloids are rarely found in elderly individuals, possibly due to a reduction of molecules that enhance collagen deposition, such as TGF- β . Studies have found that TGF- β inhibition has been shown to impede excessive scar formation in adults.^{56,57}

With an increase in MMP production and a concordant downregulation of tissue inhibitors of metalloproteinases (TIMPs), aged wounds seem primed for tissue breakdown as well.^{23,54} Animal studies have noted an expedited rate of wound healing following the application of growth factors to wounds in aged subjects.⁵⁸ The exact mechanism by which TGF- β 1 acts to improve wound healing is unknown, although topical delivery was shown to increase fibroblast migration and collagen deposition, decrease MMP-1 production, and increase TIMP-1 expression⁵⁸ (Fig. 2).

Impaired neovascularization in aging

Oxygen is key to many processes of life and is involved in all stages of wound healing in the skin,

with many cells and pathways being reactive to changes in oxygen concentration.⁵⁹ Following injury to the skin, disruption of the vasculature results in a hypoxic environment, which is further exacerbated by high oxygen consumption through the cells present at the edge of the wound and in the granulation tissue.^{60,61} Hypoxia has been found to have myriad effects on cells and their function, such as inducing greater dermal fibroblast proliferation and production of TGF- β 1.^{62,63} Adipose-derived stromal cells (ASCs) are proangiogenic in hypoxic conditions, producing high levels of VEGF.⁶⁴ *In vitro* ASCs display an inverse relationship between oxygen concentration and VEGF production, with significantly higher levels of VEGF being produced in lower levels of oxygen.⁶⁵ Studies revealed that higher levels of HIF-1 α expression are correlated with decreasing oxygen levels. Furthermore, hypoxia has been shown to promote *in vitro* keratinocyte motility⁶⁶ and leads to the secretion of several growth factors. These are but a few of the many roles acute hypoxia plays in the induction of skin healing, and although hypoxia is necessary for regeneration, a return to normoxic conditions is eventually required. Fibroblasts, for example, require sufficient amounts of oxygen to adequately produce the collagen necessary for wound reconstruction. With insufficient oxygen, protocollagen strands are produced instead, which lack the tensile strength that is characteristic of mature collagen.^{59,67} Thus, an understanding of the pathways governed by oxygen is critical to developing effective treatments for wound regeneration.

HIF-1's role in wound healing

With hypoxia being of such importance to regeneration of the skin, the HIF pathways have drawn much attention. Hypoxia-inducible factors (HIFs) are pleiotropic key regulators of oxygen homeostasis. Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor and is found in the nucleated cells of animals.⁵⁹ HIF-1 consists of two subunits: HIF-1 α (or its analogs HIF-2 α and HIF-3 α) and HIF-1 β , which bind to acquire the transcriptional capabilities that promote cell survival during hypoxia. Specifically, HIF-1 regulates the expression of almost 200 genes involved in biological processes such as glycolysis, angiogenesis, apoptosis, adhesion, migration, invasion, and metastasis.^{68–70} Additionally, HIF-1 serves as a crucial modulator in the homeostatic processes during hypoxia by increasing vascularization and regulating anaerobic respiration.^{71,72} The HIF-1 transcription factor activates genes to direct migration

Figure 2. (A) Inflammation. Immediately after injury hemostasis and inflammation occur leading to vasoconstriction. Platelets adhere and form blood clots with a wealth of signaling molecules, recruiting monocytes and neutrophils to the site of the wound. Monocytes then differentiate into mature macrophages at the wound site. Macrophages and neutrophils phagocytize the wound bed and release additional factors to stimulate migration of fibroblasts to the wound site. (B) Proliferation and remodeling. Fibroblasts secrete ECM to form granulation tissue during proliferation. Endothelial cells migrate to the wound and engender neovascularization. Remodeling begins with degradation of collagen and ECM through MMPs. (C) Remodeling. During the remodeling phase, collagen production and degradation equalize. The wound gains its tensile strength by disorganized collagen fibers that are crosslinked and aligned along tension lines. (D) Time course of several wound healing phases.⁵⁹ MMPs, matrix metalloproteinases. Color images are available online.

of mature endothelial cells toward sites of hypoxia through VEGF transcription.^{73–75} These endothelial cells ultimately help to form new vessels, enhancing delivery of oxygenated blood to the hypoxic tissues.⁷⁶

HIF-1 is integral to many homeostatic processes that occur throughout the body, prompting studies to further investigate the intricacies of the HIF pathways. HIF-1 α is an oxygen-sensitive subunit, whereby its expression is dependent on hypoxic conditions.^{77,78} Under hypoxic conditions, HIF-1 α expression is higher and sustained, whereas under normoxic conditions, HIF-1 α is quickly degraded following translation.⁷⁸ This is due to oxygen-dependent hydroxylation of HIF-1 α amino acid

residues by prolyl hydroxylase domain proteins (PHDs), which causes conformational changes in the HIF-1 α protein. These changes make the protein susceptible to ubiquitination through von Hippel–Lindau (VHL) complexes, marking them for proteosomal destruction.⁷⁹ Furthermore, HIF-1 α is regulated by factor inhibiting HIF, which blocks HIF-1 α interaction with its coactivators through oxygen-dependent hydroxylation.⁸⁰ These inhibitory processes prevent excessive accumulation of HIF-1, which through its signaling pathways could cause fibroproliferative healing, characterized by fibrotic scarring.⁵² Under hypoxic conditions, the requisite oxygen for hydroxylation and degradation is unavailable, allowing HIF-1 α

levels to increase.^{71,81} The HIF-1 complex then stabilizes as the HIF-1 α and HIF-1 β subunits bind, allowing it to bind to coactivators, CBP and p300, activating its transcriptional properties (Fig. 3).

The HIF-1 pathway is responsible for transcriptionally upregulating many genes that amplify wound healing. As inflammatory cells initially arrive at a site of injury, there is a dearth of oxygen required for cellular respiration. These hypoxic conditions not only make adenosine triphosphate (ATP) generation more difficult for early responders such as macrophages and neutrophils, but they also drive HIF-1-associated gene expression changes as well. Among these changes is an upregulation of glycolytic genes, which allows inflammatory cells to adapt to the hypoxic microenvironment of a wound and change their metabolic production to that of glycolysis.⁸² Thus, macrophages and neutrophils gain the ability to generate higher amounts of energy even with little oxygen available. This energy is required for virtually any and all functions carried out by these cells, including motility, chemotaxis, phagocytic activity, and aggregation.

Furthermore, HIF-1 has been shown to concurrently repress mitochondrial function, preventing cells from consuming what little oxygen is available for energy production purposes. This is a result of HIF-1 induction of pyruvate dehydrogenase kinase 1 (PDK1), which acts to block a crucial step in the mitochondrial tricarboxylic acid cycle.⁸² Ultimately, this yields a significant drop in mitochondrial oxygen consumption, leading to higher levels of available oxygen found within the wound microenvironment and lesser total cell death.⁸² These changes allow for inflammatory cells to better accumulate in the wound bed and perform their duties. Of note, *in vivo* studies with HIF knockout mice have observed a loss in macrophage motility, highlighting that HIF-1-induced upregulation of glycolysis is crucial to successful resolution of wound healing.⁸³ Moreover, *in vitro* studies observed that HIF-1 was effective in inducing myeloid cell differentiation into macrophages and monocytes.⁸⁴

HIF-1 has also been heavily implicated in the stimulation of angiogenesis, targeting a variety of genes that promote the growth reorganization of

Figure 3. HIF-1 regulation during normoxia, hypoxia. Under normoxic conditions HIF-1 α is inhibited from binding with its coactivators by hydroxylation through PHD-1, -2, or -3 or FIH. PHD hydroxylates select proline residues on HIF-1 α causing a conformational change that allows ubiquitination by a VHL ubiquitin-protein ligase complex followed by proteosomal degradation. FIH hydroxylates asparagine residues within HIF-1 α , preventing interaction between HIF-1 α and its coactivators. The hydroxylation described requires available oxygen. Under reduced oxygen conditions (hypoxia) HIF-1 α is stabilized. With a dearth of available oxygen, hydroxylation of the HIF-1 α subunit cannot occur and it readily binds with its coactivators. The stabilized HIF-1 α subunit binds to HIF-1 β subunit to form an active transcription factor. Active HIF-1 α /HIF-1 β then bind to coactivators CBP and p300 to promote expression of downstream target genes. Aged skin PHD 1–3 levels are unregulated, leading to an increase in their prevalence and subsequent increase in HIF-1 α degradation.⁵⁹ FIH, factor inhibiting HIF; HIF-1 α , hypoxia-inducible factor 1-alpha; PHDs, prolyl hydroxylase domain proteins; VHL, von Hippel–Lindau. Color images are available online.

already extant vessels.^{59,85} Factors such as VEGF, stromal cell-derived factor 1 (SDF-1), and angiopoietin 2 are among those that are upregulated and are necessary for neovascularization.^{52,86} These vessels are responsible for returning surrounding tissue to normoxic conditions, which are required during the later stages of regeneration in the skin, promoting cellular activity and survival. Notably, studies have shown that fibroblasts lose the ability to carry out many of their functions when exposed to chronic ischemia. Thus, proper functioning of the HIF-1 pathway is essential to the growth of vasculature and successful wound healing.

Finally, HIF-1 promotes the upregulation of other genes recognized as integral to proper wound healing, including those that encode molecules, such as integrins, growth factors, and ECM components among others. As such, HIF-1 is clearly among the pathways that are paramount to regeneration of the skin.

While HIF-1 is undoubtedly important for wound healing, excessive expression of HIF-1 can lead to unwanted fibroproliferation in the form of keloids and hypertrophic scarring. Such conditions are characterized by unrestrained angiogenesis, as well as collagen and ECM deposition. Keloids and hypertrophic scars are both difficult to treat in a clinical setting, often requiring surgical correction. Biopsies of said conditions have revealed higher levels of growth factors and their respective receptors, such as TGF- β 1 and VEGF.⁸⁷ Such increases in expression likely drive the development of fibrotic diseases in the skin and are associated with unchecked HIF-1 signaling.

Conversely, deficits in HIF-1 signaling can lead to inadequate wound healing. Chronic wounds are often characterized by a constant, nonresolving inflammatory phase, causing proinflammatory signaling cascades to persist. This results in higher levels of proteases that work to destroy ECM components and other molecules beneficial to wound healing, preventing the proliferation and tissue remodeling phases from advancing normally.^{59,88} Studies have identified HIF-1 signaling as one of the underlying causes behind many nonhealing wounds. Common examples of chronic wounds include diabetic wounds, pressure ulcers, and aged wounds.⁸⁹

HIF signaling in the aged and potential treatments

As stated before, the structure and pathophysiology of aged skin differs greatly from the skin in younger individuals. These discrepancies are responsible for the delayed healing found in aged

skin, many of which are the result of irregular HIF-1 signaling. Over the past decade, extensive studies have examined the expression levels of HIF-1 in the aged compared with the young, and how regeneration was affected.

One of the major observations of aged wound healing is that there is significantly lesser neovascularization following ischemia, impeding recovery.⁹⁰ Neovascularization in humans occurs through both angiogenesis, the sprouting of new vessels from old ones, and vasculogenesis, the formation of new vessels by migration and aggregation of endothelial progenitor cells.^{91,92} HIF-1 α signaling has been shown to regulate both of these processes through transcription of cytokines such as SDF-1 α .⁵² Interleukin-1 β (IL-1 β) is another important cytokine involved in wound healing and was posited to regulate SDF-1 α expression independently of the HIF pathway. Thus, murine studies were performed to investigate the role HIF-1 α signaling played in neovascularization in both aged and young mice. Aged mice displayed delayed wound healing and lesser granulation tissue when compared with their younger counterparts, as expected. Aged mice displayed lesser CD31⁺ expression following immunofluorescence (IF) staining as well, indicative of lesser neovascularization. Real-time polymerase chain reaction (qPCR) exhibited significantly reduced SDF-1 α prevalence in aged wounds, complementing the CD31⁺ staining results. Western blot analysis found decreased HIF-1 α levels in aged wounds, whereas neither aged mice nor young mice displayed major changes in IL-1 β expression. Moreover, the only cells that displayed a significant response to IL-1 β were fibroblasts found in young mice, and this response was nullified following mutation of HIF-binding sites.⁵² Thus, HIF-1 α was found to be more critical with regard to SDF-1 α transactivation following skin injury. As such, HIF-1 α is integral to neovascularization in wound healing, and reduced HIF-1 α expression is likely a major hindrance to regeneration in the aged.

With disturbances in HIF signaling having been correlated with delayed wound healing in the aged, several studies have attempted to manipulate its expression. As such, Zimmerman *et al.* examined the role of PHD-2 in aged wounds. Prior studies had found that PHD-2 inhibition brought about several responses to hypoxia, such as stimulation of angiogenesis, protection from metabolic stress, HIF-1 α stabilization, and the induction of hypoxia-inducible genes.⁹³ Furthermore, PHD-2 has been implicated in the regulation of angiogenesis independent of HIF signaling as well.⁹⁴ With PHD-2

being unregulated and found in higher proportion in aged animals, it became a target of interest.

Thus, epidermal- and dermal-specific PHD-2 knockout mice were split into several groups to further investigate the role of PHD-2. Heterozygous K14-Cre/homozygous floxed PHD-2 and heterozygous Col1 α 2-Cre-ER/homozygous floxed PHD-2 mice were bred and had fibroblasts and keratinocytes harvested for analysis. These cells were analyzed with quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) and western blotting. Results displayed notably reduced mRNA levels of PHD-2 in knockout mice, with normal levels found in wild-type animals. Both knockout groups also displayed significantly reduced levels of PHD-2 and increased levels of VEGF and HIF-1 α when compared with the wild type following western blot analysis. Thus, animals separated into groups were members from both knockout groups that were wounded with an excisional wound model or an ischemic pedicle flap model.⁹⁵

Both of the PHD-2 knockout mice groups displayed significantly shorter time to wound closure when compared with wild-type mice in the excisional wound model, with appreciable differences being observed as early as day 4 postinjury. In addition, the ischemic pedicle flap model was used to observe tissue perfusion. Both knockout groups displayed significantly less necrosis and ischemia when compared with wild-type mice. Furthermore, IF staining for PHD-2, HIF-1 α , VEGF, and CD31 were performed on tissue taken from animals used in the excisional wound model, with both knockout groups displaying significantly less PHD-2 expression. Concurrently, HIF-1 α , VEGF, and CD31 were all higher in the knockout groups⁹⁵ (Fig. 4).

Duscher *et al.* found similar findings when carrying out a study examining the effects of fibroblast-specific HIF-1 α knockout mice, specifically that new vessel formation was hindered in knockout animals, further suggesting that HIF-1 α deficits may be particularly deleterious in chronic wounds.⁹⁶ Altogether, these results implicate PHD-2 as a strong inhibitor of HIF-1 α as well as confirming that HIF-1 α accelerates wound closure and improves local tissue perfusion. Targeting specific genes and molecules involved in the HIF pathways, such as PHD-2, may provide a solid foundation for future treatment aged wounds.

Other studies have investigated the role of ROS in the exacerbation of delayed wound healing in aged individuals. Among the molecules that has garnered interest is superoxide dismutase 3 (SOD3), an extracellular enzyme that contributes

to oxygen homeostasis by consuming superoxide anions and has been shown to encourage neovascularization in response to ischemia.^{97,98} With decreased neovascularization underlying many of the complications found in aged wounds, Fujiwara *et al.* examined the effects of SOD3 activity on regeneration in the aged. SOD3 knockout mice were wounded using an excisional wound model and their healing rate was found to be comparable to aged, wild-type mice. Oxidative stress levels were found to be similar between the knockout group and aged group, both of which were significantly higher than the young, wild-type group. Furthermore, protein analysis of the wounds found significantly less SOD3 expression in the aged mice as early as day 3 postwounding. This ultimately led to lesser growth of vasculature in both the aged mice and knockout groups when compared with the young mice.⁹⁸

Previous studies have demonstrated that many cell types are responsive to ROS, including neutrophils and fibroblasts. Specifically, ROS have been shown to incite an inflammatory response, which serves to recruit neutrophils to the site of injury.⁹⁹ Furthermore, studies have displayed that fibroblast function can be influenced through ROS activity as well.^{100,101} Both the aged and knockout groups in the Fujiwara *et al.* study displayed increased neutrophil infiltration and reduced fibroblast proliferation when compared with the young mice. Furthermore, reductions in TGF- β 1 and α -SMA were found to correspond with SOD3, suggesting myofibroblast differentiation is affected by high levels of ROS due to reduced SOD3 activity⁹⁸ (Fig. 5).

Duscher *et al.* performed another study investigating the effects of several drugs on both diabetic and aged wounds. Previous studies had found that the FDA-approved iron chelator, deferoxamine (DFO), stabilized HIF-1 α by chelating free radicals and preventing hydroxylation.¹⁰² Thus, DFO was applied to the wounds of diabetic and aged mice and resulted in accelerated wound healing. This was correlated with increased HIF-1 α binding under hypoxic conditions in addition to greater neovascularization.¹⁰³ Bonham *et al.* had similar findings when applying DFO to the wounds of aged mice as well as pressure ulcers through a newly developed transdermal delivery system (DFO-TDDS).¹⁰⁴ Thus, DFO provides another means to correct HIF-1 α deficiencies in the aged.

While there are certainly many aspects in molecular signaling that underlie the problems associated with wound healing in the aged, there are also discrepancies with regard to the cellular behavior

Figure 4. (A) qRT-PCR for PHD-2 KO in heterozygous Col1 α 2-Cre-ER/homozygous floxed PHD-2 fibroblasts compared with wild-type fibroblasts (* p < 0.05). (A') Western blot data indicating lesser PHD-2 expression in fibroblasts of heterozygous Col1 α 2-Cre-ER/homozygous floxed PHD-2 mice than wild type. (B) Western blot data displaying greater HIF-1 α expression in fibroblasts of heterozygous Col1 α 2-Cre-ER/homozygous floxed PHD-2 mice than wild type. (C) Western blot data displaying greater VEGF expression in fibroblasts of heterozygous Col1 α 2-Cre-ER/homozygous floxed PHD-2 mice than wild type. (D) qRT-PCR for PHD-2 KO in heterozygous Col1 α 2-Cre-ER/homozygous floxed PHD-2 keratinocytes compared with wild-type fibroblasts (* p < 0.05). (D') Western blot data indicating lesser PHD-2 expression in keratinocytes of heterozygous Col1 α 2-Cre-ER/homozygous floxed PHD-2 mice than wild type. (E) Western blot data displaying greater HIF-1 α expression in keratinocytes of heterozygous Col1 α 2-Cre-ER/homozygous floxed PHD-2 mice than wild type. (F) Western blot data displaying greater VEGF expression in keratinocytes of heterozygous Col1 α 2-Cre-ER/homozygous floxed PHD-2 mice than wild type.⁹⁵ KO, knockout; qRT-PCR, quantitative reverse transcriptase–polymerase chain reaction; VEGF, vascular endothelial growth factor. Color images are available online.

Figure 5. (A, B) Reduced fibroblast to myofibroblast transition in aged and SOD3 KO mice. (C, D) At the baseline, collagen type-III α -I and α -SMA RNA expressions are similar on the day of wounding. Compared with young control wounds their expression decreased in aged and SOD3 KO wounds at 7 days after. Y=young mice, A=aged mice, S=SOD3 KO mice. (E) Significant reduction of α -SMA in aged and SOD3 KO wounds on a protein level (western blot and immunohistochemistry). In both control conditions and when exposed to xanthine oxidase, fibroblasts from aged and SOD3 KO mice release significantly lower levels of TGF- β 1, compared with fibroblasts from young mice. * $p < 0.05$. Scale bar = 100 μ m.⁹⁸ α -SMA, α -smooth muscle actin; SOD3, superoxide dismutase 3; TGF- β , transforming growth factor beta. Color images are available online.

and function. These alterations in cellular function impact regeneration of the skin in various ways.

Effects of aging on stem/progenitor cell populations

Studies have shown that ASCs are proangiogenic in hypoxic conditions, producing high levels of VEGF.⁶⁴ Thangarajah *et al.* found that *in vitro* ASCs displayed an inverse relationship between oxygen concentration and VEGF production, with significantly higher levels of VEGF being produced in lower levels of oxygen.⁶⁵ Western blotting also found higher levels of HIF-1 α expression correlated with decreasing oxygen levels. Furthermore, hypoxia-treated ASCs were found to have a greater response to SDF-1 than normoxic ASCs, suggesting that hypoxia enhances their migratory capabilities. SDF-1 has been shown to mediate the migration of circulating stem cells to sites of neovascularization.¹⁰⁵ Additionally, ASCs displayed higher levels of proliferation under progressively lower levels of oxygen concentration. ASCs were also found to adopt endothelial cell characteristics, such as the ability to promote tubule formation when subjected to hypoxia in the presence of VEGF.⁶⁵ Altogether, these findings suggest that ASCs are integral to the reconstruction of blood vessels following injury (Fig. 6).

Building upon these findings, El-ftesi *et al.* examined ASCs in both aged and diabetic mice to determine how they differed from their younger counterparts. Using the surface markers Sca-1, CD90 and CD44 as positive identifiers, ASCs were

harvested from old, young, and diabetic mice and subjected to several analyses. ASCs in younger mice displayed significantly higher VEGF secretion under *in vitro*, normoxic conditions when compared with older and diabetic mice. While cells from each group displayed higher VEGF secretion under hypoxia than normoxia, ASCs from the younger mice produced VEGF at much higher levels under hypoxia than the other groups. ASC proliferation was also stunted under hypoxic conditions in aged and diabetic mice. Furthermore, a reduction in endothelial cell proliferation in the aged and diabetic groups correlated with lesser ASC proliferation, further implicating that ASCs are crucial to stimulating endothelial cell proliferation. Tubulogenesis was also found to be reduced in the aged and diabetic groups when compared with ASCs from younger animals.¹⁰⁶ These findings display significant impediments in ASCs as they age, with a notable loss in their ability to stimulate neovascularization through VEGF secretion. With how accessible ASCs are and given their role in facilitating wound healing, they are an attractive potential cornerstone for cell therapies and tissue engineering (Fig. 7).

Mesenchymal stem cells (MSCs) have also been noted to contribute to neovascularization and tissue repair throughout the body.¹⁰⁷ Studies have shown that MSCs, like all other cells, begin to lose function as a result of aging.¹⁰⁸ Duscher *et al.* performed a study observing how aging affects MSCs, finding that frequency within adipose tissue did not differ between aged animals and young

Figure 6. VEGF quantification by ELISA that displays ASC production of VEGF has an inverse relationship with oxygen present. Lower levels of oxygen lead to progressively higher output of VEGF by ASCs (modified from Thangarajah *et al.*⁶⁵). ASCs, adipose-derived stromal cells. Color images are available online.

Figure 7. (A) VEGF quantification by ELISA displays lesser VEGF production by diabetic and aged mice in both normoxic and hypoxic conditions when compared with their younger counterparts. (B) ASC proliferation measured with bromodeoxyuridine colorimetric cell proliferation assay is lower in aged and diabetic mice when compared with young mice. (C) Endothelial cell proliferation measured with bromodeoxyuridine colorimetric cell proliferation assay is lower in aged and diabetic mice when compared with young mice.¹⁰⁶ Color images are available online.

animals. This was consistent with previous studies performed by other groups.^{109,110} Subsequent *in vitro* seeding of these MSCs displayed that the aged MSCs did not have reduced proliferative capacity. However, single cell analysis found that a particular subpopulation of ASC, characterized by various genes related to stemness, tissue repair, and neovascularization, including HIF-1 α , was depleted in aged mice. This subset may be more pluripotent than other subsets and was implicated in several pathways, such as those involved with hypoxic signaling and cell differentiation. Furthermore, when hydrogels were seeded with aged ASCs and applied to the wounds of aged mice, there

was no observable difference in wound healing, whereas application of young ASC seeded hydrogels to aged wounds improved the healing rate. Finally, aged ASC-treated wounds displayed lower levels of SOD3, VEGF, and neovascularization when compared with young ASC-treated wounds.¹¹¹

SUMMARY

With so many variables it is difficult to ascertain which aspect of wound healing is of the greatest importance. Age-associated defects in both cellular function and molecular signaling have been shown to impede various processes in the aged. Recent research however, shows that targeting the signaling pathways may in fact ameliorate some of the problems in aged cells and normalize wound healing to that found in younger animals. Treatments such as genetic knockout and drugs such as DFO have proven to be useful and pave the way for optimal treatments that may reduce the burden of chronic and aged wounds in the future.

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Abbreviations and Acronyms

α -SMA = α -smooth muscle actin
 ASCs = adipose-derived stromal cells
 CAM = cell adhesion molecule
 DEJ = dermal–epidermal junction
 DFO = deferoxamine
 ECM = extracellular matrix
 HIF-1 = hypoxia-inducible factor 1
 HIF-1 α = hypoxia-inducible factor 1-alpha
 HIFs = hypoxia-inducible factors
 IF = immunofluorescence
 IL-1 β = interleukin-1 β
 MMP = matrix metalloproteinase
 MSCs = mesenchymal stem cells
 PDGF = platelet-derived growth factor
 PHD = prolyl hydroxylase domain
 ROS = reactive oxygen species
 SDF-1 α = stromal cell-derived factor 1-alpha
 SOD3 = superoxide dismutase 3
 TGF- α = transforming growth factor alpha
 TGF- β = transforming growth factor beta
 TIMPs = tissue inhibitors of metalloproteinases
 UV = ultraviolet
 UVA = ultraviolet A (long-wave)
 UVB = ultraviolet B (shortwave)
 VEGF = vascular endothelial growth factor