

Epithelialization in Wound Healing: A Comprehensive Review

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Significance: Keratinocytes, a major cellular component of the epidermis, are responsible for restoring the epidermis after injury through a process termed epithelialization. This review will focus on the pivotal role of keratinocytes in epithelialization, including cellular processes and mechanisms of their regulation during re-epithelialization, and their cross talk with other cell types participating in wound healing.

Recent Advances: Discoveries in epidermal stem cells, keratinocyte immune function, and the role of the epidermis as an independent neuroendocrine organ will be reviewed. Novel mechanisms of gene expression regulation important for re-epithelialization, including microRNAs and histone modifications, will also be discussed.

Critical Issues: Epithelialization is an essential component of wound healing used as a defining parameter of a successful wound closure. A wound cannot be considered healed in the absence of re-epithelialization. The epithelialization process is impaired in all types of chronic wounds.

Future Directions: A comprehensive understanding of the epithelialization process will ultimately lead to the development of novel therapeutic approaches to promote wound closure.



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SCOPE AND SIGNIFICANCE

KERATINOCYTES, THE MAJOR cellular component of the epidermis, are not only important for barrier maintenance but also for its restoration upon injury through a process known as epithelialization. This review will focus on cellular processes and mechanisms of their regulation during re-epithelialization. Many cellular features important for re-epithelialization, including mechanisms of transcriptional regulation, such as microRNA (miRNA) and histone modifications, as well as recent discoveries regarding epidermal stem cells (ESCs), immune, and

neuroendocrine functions of the epidermis will be discussed.

TRANSLATIONAL RELEVANCE

Epithelialization is defined as a process of covering denuded epithelial surface. The cellular and molecular processes involved in initiation, maintenance, and completion of epithelialization are essential for successful wound closure. A great deal of research effort has been focused on understanding these processes in both acute and chronic wounds. Recent advances in cell therapies for chronic wounds will also be reviewed.

CLINICAL RELEVANCE

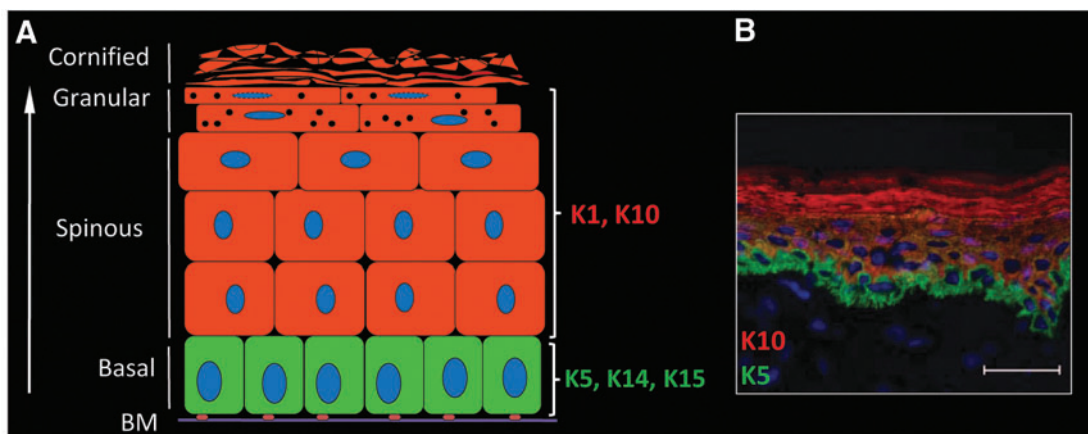
Epithelialization is an essential component of wound healing used as a defining parameter of its success. In the absence of re-epithelialization, a wound cannot be considered healed. Barrier breach provides a portal for wound infection. This process is impaired in all types of chronic wounds. Failure of keratinocytes to maintain the barrier may contribute to wound recurrence, which is another significant clinical problem. A better understanding of the epithelialization process may provide insights for new therapeutic approaches to accelerate wound closure.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

Epidermis and its role in barrier maintenance

Epidermis as a skin barrier. The epidermis is a stratified epithelium composed of several layers of keratinocytes, which provides a physical barrier between the environment and the organism, thereby protecting it from external agents and pathogens, and limiting the loss of fluids. This integument is continually maintained by keratinocytes that switch from a proliferative state in the basal layer to a differentiated state as they migrate through the granular layer, and finally become the flattened dead cell remnants of the cornified layer (Fig. 1). However, keratinocytes are not only important in maintaining the epidermis but also in restoring it after injury.

In the basal layer, keratinocytes are proliferative and characterized by an infrastructure composed of keratin (K) intermediate filaments, K5 and K14. The cells move toward the surface traversing layers known as the spinous layer, granular layer, and stratum corneum. As they ascend, they undergo the process of differentiation, characterized by the switch from synthesis of K5 and K14 in the basal layer to K1 and K10 in the suprabasal layers (Fig. 1).¹ Basal cells are attached to the basement membrane (BM) through hemidesmosomes and focal adhesions, and suprabasal cells attach to their neighboring cells through desmosomes, which must be disconnected to allow keratinocyte migration during the epithelialization process.² Formation of the barrier also requires the delivery of lipids and proteins contained in lamellar granules (in the granular layer) to the stratum corneum interstices, and the formation of high molecular weight polymers through the crosslinking of cornified envelope proteins (loricrin, involucrin, filaggrin, and other peptides).³ During terminal differentiation and formation of the cornified envelope, keratinocytes also become dehydrated and flatten into a polyhedron, referred to as the terminal corneocyte.⁴ Corneocytes are interconnected by corneodesmosomes, cell junction structures comprising the adhesive protein corneodesmosin. The lipid layer, secured to the protein structure around the corneocytes, is often referred to as the “mortar” in a “brick and mortar” analogy of the stratum corneum. It forms the water



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Figure 1. Structure of the epidermis. (A) Schematic illustration of epidermis. Keratinocytes are the major cell population of epidermis important for maintaining a barrier formation during homeostasis as well as restoring it after the injury. Mitotically active basal layer is adjacent to the basement membrane (BM). Keratinocytes in the basal layer are characterized by keratin (K) 5, K14, and K15. As they differentiate, keratinocytes form suprabasal layers known as the spinous, granular, and cornified layer. Differentiated keratinocytes express K1 and K10. (B) Immunolocalization of K5 (green) and K10 (red) in human epidermis. Nuclei are visualized with 4',6-diamidino-2-phenylindole–DAPI (blue). © 2005 Wiley. Modified with permission from Morasso and Tomic-Canic.⁵⁵ To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

barrier and serves to maintain the fluid balance in the epidermis.⁵

Regulation of keratinocyte differentiation. During the process of differentiation, the undifferentiated keratinocytes change into differentiated nondividing cells as they migrate upward to eventually give rise to the cornified envelope. Three major MAP kinase pathways are involved in the process of differentiation, activated by multiple stimuli, including calcium influx, epidermal growth factor (EGF), and tumor necrosis factor (TNF).⁶ This differentiation cascade also uses various protein kinase C (PKC) isoforms. Studies have shown that conventional PKCs, which are calcium dependent, are inhibitory, whereas the calcium-independent non-conventional PKC isoforms stimulate keratinocyte differentiation markers.⁷ The final step in differentiation is the activation of the proteolytic and nucleolytic activity, which leads to the destruction of cellular organelles and DNA. The increased intracellular calcium leads to transglutaminase activation and formation of the cornified envelope through envelope precursor crosslinking.⁴ Several structural proteins, including loricrin, involucrin, and filaggrin, become covalently crosslinked by calcium-dependent transglutaminase, with final attachment of insoluble lipids forming the outermost layer of the epidermis. Integral in maintaining the epidermal calcium gradient and epidermal barrier is Scarf (skin calmodulin-related factor). Scarf acts as both, a calcium sensor, and a regulator of target protein function in barrier maintenance and formation.⁸

Keratinocytes continually renew, traveling upward from the basal to differentiated layers. A continuous, dynamic renewal throughout homeostasis as well as during disruption of the epidermal barrier is maintained by ESCs.⁹

The role of ESCs in wound healing

The regenerative capacity of the skin relies on the local populations of ESCs. ESCs reside within specific microenvironments, stem cell niches, which regulate their activity and fate.^{10,11} There are three distinct ESC niches identified to date: bulge of the hair follicle (HF), the base of the sebaceous gland, and the basal layer of the interfollicular epidermis (IFE) (Fig. 2).^{12,13} Under normal homeostatic conditions, each discrete ESC niche behaves unipotently, replenishing its own respective tissue compartment. It has been proposed that within the IFE, a hierarchy exists, comprising slow cycling stem cells in the basal layer, which produce a population of transit amplifying cells that undergo a limited number of divisions before differentiating as they ascend through the suprabasal layers.¹⁴ However, an alternative model has been put forth in which a single progenitor has the ability to divide into either two undifferentiated basal cells, two terminal differentiating cells, or one of each type.¹⁴⁻¹⁶ Recent evidence supports the notion of a hierarchy and suggests that it is the slow-cycling cells of the IFE and not the transit amplifying cells that are involved as major contributors to long-term tissue repair.¹⁷

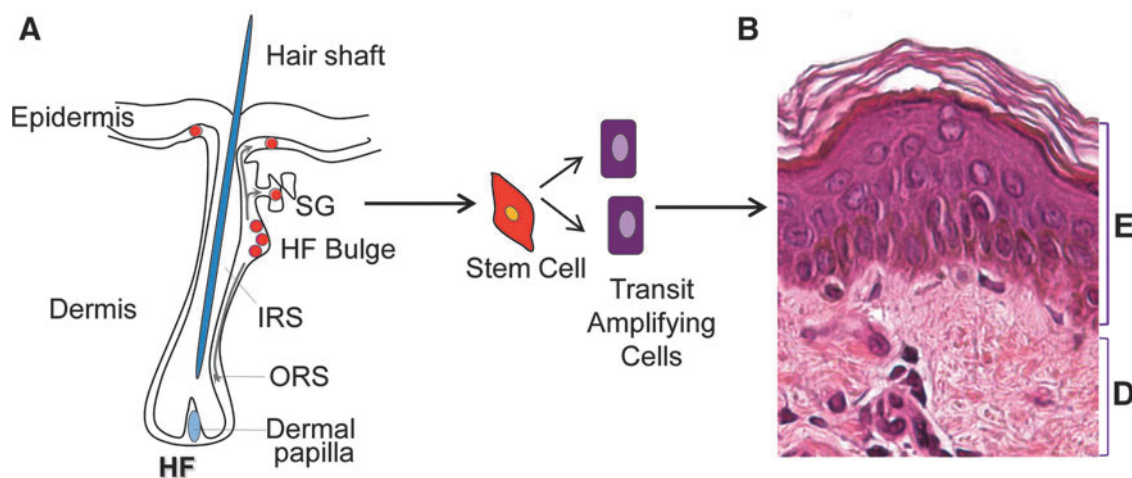


Figure 2. Regenerative capacity of the skin relies on local populations of epidermal stem cells. **(A)** Schematic representation of a hair follicle (HF) with the multipotent stem cells (red). Three distinct epidermal stem cell (ESC) niches are identified so far: bulge of the HF, the base of the sebaceous gland (SG), and the basal layer of the interfollicular epidermis. It has been shown that stem cells migrate from the HF and interfollicular stem cell niche to aid repair and epithelialization upon skin wounding.¹⁸⁻²¹ ESCs generate transit amplifying cells, which will differentiate to form the stratified epidermal layers. **(B)** Section of human skin stained with hematoxylin and eosin to distinguish epidermis (E) and dermis (D). ORS, outer root sheath; IRS, inner root sheath. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

In response to epidermal injury, both the HF and IFE niches participate in re-epithelialization of the wound defect.^{18–21} Ito *et al.* demonstrated that in a murine full-thickness wound model, HF bulge stem cells characterized by the expression of K15 migrate into the IFE initially postwounding. However, the contribution of K15-positive cells may be temporary, as evidenced by their absence from the IFE several weeks after healing.¹⁸ Interestingly, studies utilizing more recent bulge stem cell markers, such as LGR5²² and SOX9,²³ indicate the presence of bulge-derived ESCs in the repaired epidermis long after healing has taken place. The role of HF ESCs was further defined by Langton *et al.* who demonstrated a delay in the early phase of re-epithelialization when acute incisional wounds were created in HF-deficient mice.²⁰ Complete closure, however, was achieved presumably through recruitment of ESCs from the IFE niche. These findings suggest that in incisional wounds characterized by a minimal epidermal defect, HF ESCs provide an initial burst in the rate of healing, but are likely less important than in larger wounds, where re-epithelialization must occur over extensive surface areas.

The role of ESCs in wound healing as well as the mechanism, which orchestrate their function, has been studied predominantly in mouse models pointing to the need for confirmatory evidence in human skin. Further insight into the wound healing process will help focus research efforts to elucidate cellular defects contributing to nonhealing wounds aiming to improve current and developing novel stem cell-based therapies for wound healing disorders.

In addition to ESCs, somatic cell therapy is utilized to stimulate wound closure. These tissue-engineered human skin equivalents are FDA approved for the treatment of chronic wounds. It has been suggested that this cell therapy promotes healing through the release of various cytokines and growth factors when applied to a nonhealing wound after surgical debridement.²⁴ However, a recent study documented that even without tissue engineering, human allogeneic keratinocytes and fibroblasts in a spray formulation can be successfully used as a therapy for nonhealing venous ulcers.²⁵ Further insights into the mechanism of action of cell-based therapeutics are needed for better understanding and to determine the optimal use. A clinical trial to assess the added advantage of viable cells within the bioengineered constructs as compared with the matrix alone in the treatment of chronic wounds is currently ongoing.²⁶

Keratinocyte migration and proliferation during epithelialization

Upon acute skin injury, as the barrier is disrupted, neutrophils, monocytes, and macrophages are recruited to the site of injury.²⁷ Subsequently, keratinocytes become activated, and the activation process is achieved by expression of several cytokines and growth factors. The activated phenotype is marked by changes in the cytoskeleton network and cell surface receptors essential for re-epithelialization, namely, expression of K6 and K16, allowing keratinocytes to migrate into the wound to fill the defect.²⁸

Keratinocyte migration

To close the defect in the epidermis, keratinocytes at the wound edge must first loosen their adhesion to each other and to the basal lamina, and need to develop the flexibility to support migration over the freshly deposited matrix. This process is modulated sequentially beginning with disassembly of cell–cell and cell–substratum contacts maintained through desmosomes and hemidesmosomes, respectively.²⁹ This release allows keratinocytes to start migrating from the wound edge over the denuded area, whereas keratinocytes behind the migrating tongue begin to proliferate (Fig. 3).³⁰ Numerous regulators play a critical role in modulating the proliferation and migration of keratinocytes during epithelialization.

For the process of migration to begin, the cell–cell interaction needs to be dissolved. During this process, PKC α gets activated and leads to the conversion of calcium-independent to calcium-dependent desmosomes, thus decreasing their adhesive properties.³¹ Among multiple transcription factors involved in this process, the transcription factor Slug allows for additional release of keratinocytes by increasing desmosomal disruption.³² Hemidesmosomes linking the BM to the basal layer of keratinocytes need to be disassembled as well to allow for migration.³³ The $\alpha 6 \beta 4$ integrin expressed by basal keratinocytes binds to an isoform of laminin called laminin-5 (LN5) in the lamina densa of the BM, contributing to the adhesive property of hemidesmosomes. One proposed mechanism suggests that there is a differential affinity of integrins to laminin LN5, namely, the precursor form, nonproteolytically processed LN5. The nonproteolytically processed form has a major binding affinity for $\alpha 3 \beta 1$, while the processed form binds preferentially to $\alpha 6 \beta 4$. When keratinocytes start migrating, a switch from $\alpha 6 \beta 4$ to $\alpha 3 \beta 1$ integrin for LN5 binding occurs.³⁴ The inside-out modulation of $\alpha 6 \beta 4$ affinity for ligands by alterations of the

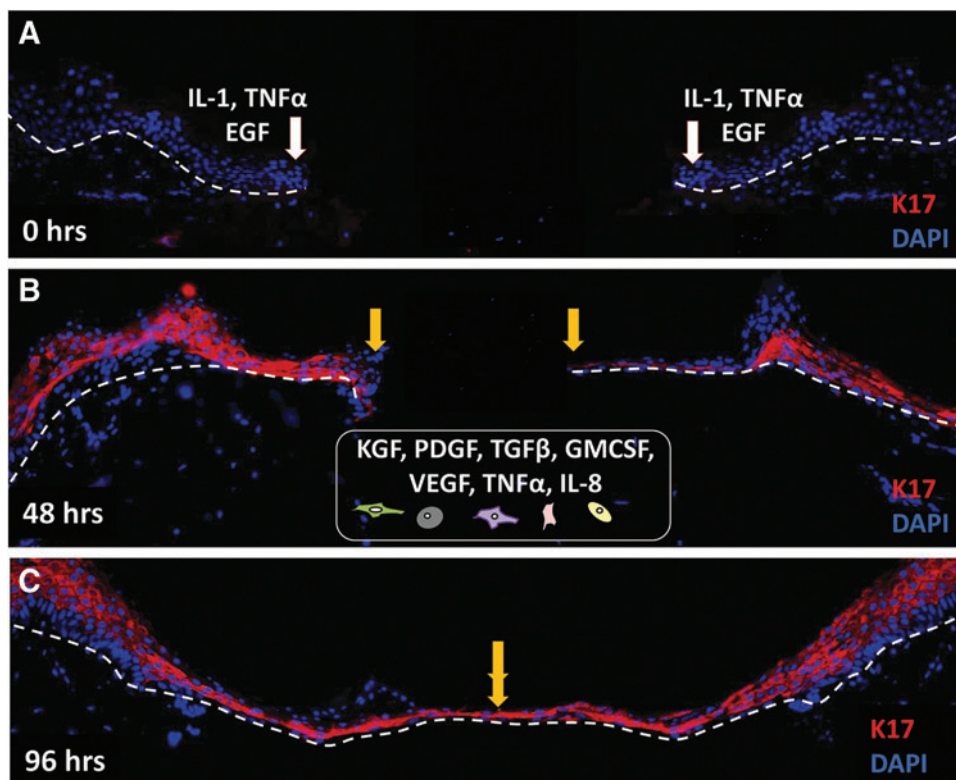


Figure 3. Epidermal keratinocytes migrate over the wound bed to epithelialize the wound gap. Immunofluorescence staining with keratin 17 (K17, red) antibody demonstrates epithelialization process in human *ex vivo* wound model. White arrows indicate wound edges after initial wounding, while yellow arrows point at the edges of the migrating epithelial fronts. K17 is not present at the time of wounding (0 h, **A**). Immediately after injury (**A**), keratinocytes release proinflammatory cytokines and growth factors, including interleukin 1 (IL-1), tumor necrosis factor α (TNF α), and epidermal growth factor (EGF). In response to these stimuli, keratinocytes become activated and start migrating over the wound bed. Migrating keratinocytes show an upregulation of K17 (48 h, **B**). Strong K17 staining persisted over 4 days after the wounding when the wound is completely closed (96 h, **C**). A well-balanced communication with other cell types, fibroblasts, neutrophils, endothelial cells, monocytes, and macrophages (schematically presented at the bottom, **B**) through various cytokines and growth factors (KGF, PDGF-bb, VEGF, GM-CSF, TGF β , IL-8), is necessary for successful epithelialization. Nuclei are visualized with DAPI (blue). White dashed lines indicate the dermal–epidermal boundary. KGF, keratinocyte growth factor; PDGF-bb, platelet-derived growth factor bb; VEGF, vascular endothelial growth factor; TGF β , transforming growth factor β ; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-8, interleukin 8. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

cytoplasmic portion of integrins is another proposed mechanism involved in keratinocyte migration;³⁵ namely, serine phosphorylation of $\beta 4$ subunits through action of PKC α increases keratinocyte motility by increasing disassembly of hemidesmosomes, while EGF and macrophage-stimulating protein found in the wound environment modulate this PKC α -dependent phosphorylation.³⁶ Migrating keratinocytes show an upregulation of K6, K16, and K17³⁷ keratins, which are hypothesized to increase viscoelastic properties of migrating cells,³⁸ and their expression is regulated by growth factors present within the wound environment.

Multiple regulators such as growth factors and cytokines, integrins, keratins, matrix metalloproteinases (MMPs), chemokines, and extracellular macromolecules modulate keratinocyte migration.^{27,39} Epidermal growth factors such as HB-EGF, EGF, and TGF- α , transactivate EGFR, which

directly stimulates keratinocyte migration and proliferation²⁷ and induces expression of K6 and K16.⁴⁰ On the other hand, our laboratories have shown that glucocorticoids (GCs) can block EGF-mediated migration by repressing transcription of K6/K16.⁴⁰ EGF signaling is not properly executed in the epidermis of chronic wounds. In contrast to membranous localization in normal epidermis, EGFR is primarily localized in the cytoplasm of keratinocytes of the chronic wound.⁴¹ The carbohydrate-binding protein, galectin-3, may play a critical role in the cytoplasmic localization of the EGFR, as our laboratories have demonstrated that in mice genetically depleted of galectin-3, wound healing is delayed and the EGFR is localized intracellularly in wound-edge keratinocytes, as opposed to normal, membranous localization in wild-type animals with normally healing wounds.⁴² In addition, non-specific disintegrin and metalloprotease 12 (ADAM12)

implicated in inactivation of HB-EGF- and IGF-binding proteins has been found induced in chronic wound epidermis.⁴³ Together, this suggests that one major impediment to healing of a chronic wound is the inability of the wound edge keratinocytes to respond to EGF family members, even if supplied externally. This may explain the clinical failure of topically applied EGF to improve healing in some human chronic wounds.⁴⁴

Another group of growth factors, fibroblast growth factors (FGF) -2, FGF-7, FGF-10, and FGF-22 have also been shown to stimulate epithelialization mostly through paracrine effects.⁴⁵ As an example, FGF2 (also known as KGF) is produced by fibroblasts and acts in a paracrine fashion through the KGFR2IIIb receptor found exclusively on keratinocytes, resulting in increased migration and proliferation during wound healing.⁴⁵ Cytokines, such as IL-1, IL-6, and TNF- α , can also modulate migratory phenotypes of keratinocytes. Among its multiple functions during wound healing, IL-1 increases secretion of FGF-7,⁴⁶ whereas IL-6 acts through the STAT3-dependent pathway, which allows keratinocytes to respond to mitogenic factors and stimulate migration.⁴⁷ TGF β 1 can also promote keratinocyte migration by stimulating MMPs.⁴⁸ For the wound to heal successfully, keratinocytes should be able to not only detach from the underlying basal lamina but also to move and migrate through the fibrin and newly synthesized extracellular matrix (ECM) of the wound, a process facilitated by MMPs. Involved MMPs are MMP-1, MMP-2, MMP-3, MMP-10, MMP-14, MMP-19, and MMP-28.^{49,50} MMP-1, which is expressed abundantly at the wound edges, sustains keratinocyte migration on type 1 collagen, mediated by the α 2 β 1 integrin.⁵¹ Cytokines and growth factors secreted during the wound healing process also stimulate MMP production. **More importantly, optimal keratinocyte migration during wound closure is dependent on tight regulation of MMPs and tissue inhibitors of metalloproteinases (TIMPs),**⁵² whereas deregulated MMP/TIMP ratios demarcate chronic nonhealing wounds.⁵³ Additional stimuli independent of the ECM, growth factors, and cytokines, such as electric fields, may also participate in wound healing by directing cell migration.⁵⁴ In intact human skin, current is limited by the very high resistance of stratum corneum. Disruption of epidermal integrity triggers the immediate formation of an endogenous electric field. A net flow of current through the low-resistance wound pathway occurs upon injury and results in the generation of a lateral electric field within or beneath the adjacent epidermis, thus contributing to keratinocyte migration.⁵⁴

Keratinocyte proliferation

Upon the advancement of migrating epithelial tongue, the first layer that covers the wound, keratinocytes start to proliferate to ensure an adequate supply of cells to encase the wound. The regulation of keratinocyte proliferation is dependent on the availability of growth factors, degree of cell differentiation, and cell attachment to the substrate. Only the basal keratinocytes have the ability to proliferate, while the terminally differentiated keratinocytes in the suprabasal layer lose this ability.⁵⁵

Growth factors that play a major role in the proliferative process during epithelialization include previously mentioned HB-EGF, EGF, TGF α , and KGF.⁵⁶ Another growth factor present in wounds, insulin-like growth factor (IGF)-1, was shown to act synergistically with HB-EGF in stimulating keratinocyte proliferation.⁵⁷ Increased keratinocyte proliferation has also been documented in transgenic mice overexpressing epidermal granulocyte-macrophage colony-stimulating factor, and this growth factor has been shown to accelerate wound closure.⁵⁸ MMPs, components of the ECM, and integrins, can work together to assist growth factors in promoting keratinocyte proliferation. MMPs work through their proteolytic activity to release growth factors from the wound matrix and they can also digest latent forms of growth factors, such as IGF-1, converting them to active forms.⁵⁹ The ECM can also engage integrins to modulate growth factor receptor pathways, leading to an increase in growth factor activity.⁶⁰ In summary, it is the synergy between growth factors, the ECM, and integrins that plays a pivotal role in regulation of keratinocyte proliferation during re-epithelialization. After being activated to repair an injury, ultimately, the deactivated keratinocytes return to their normal differentiation pathway. Once the wound is healed, defined as being fully epithelialized with no drainage, and covered by a keratinocyte layer, the proliferation signals cease and the stratification process begins.

Proliferation and migration of keratinocytes in the chronic wound. Keratinocytes at the nonhealing edges of chronic wounds are different both phenotypically and biologically than keratinocytes comprising intact epidermis or the edge of acute wounds. In contrast to normal skin, where mitotically active keratinocytes reside only in the basal layer, keratinocytes in chronic wounds undergo divisions throughout the suprabasal layers. This hyperproliferative epidermis is a result of c-myc activation and overexpression.⁶¹ Parakeratosis

(presence of the nuclei in the cornified layer) and hyperkeratosis (a thick cornified layer) are additional properties characterizing chronic wound keratinocytes⁶¹ (Fig. 4). Specific epidermal morphology can also be explained as a consequence of deregulation of differentiation and activation pathways.⁶² K1/K10 and a subset of small proline-rich proteins, together with the late differentiation

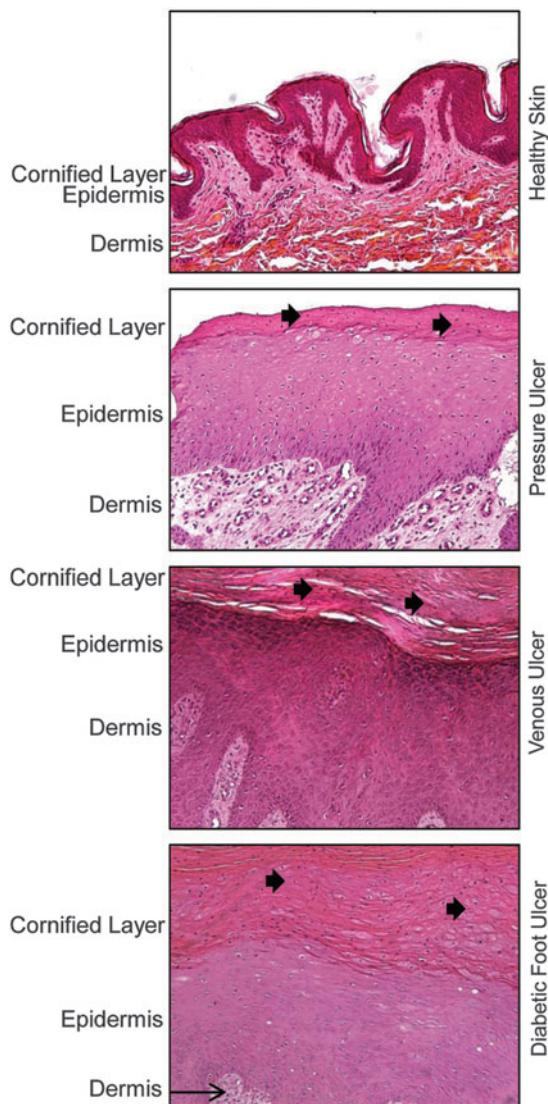


Figure 4. Epidermis of chronic wounds shows specific morphology that differs from epidermis of healthy skin. Healthy human skin is composed of several layers of keratinocytes. These cells lose their nuclei through the process of differentiation and form enucleated cornified layer. However, epidermis of chronic wounds, such as pressure ulcers, venous ulcers, and diabetic foot ulcers, has distinct morphology. They are characterized by hyperproliferative epidermis as a result of c-myc activation and overexpression.⁶¹ In addition, presence of hyperkeratosis (a thick cornified layer) and parakeratosis [presence of the nuclei in the cornified layer (black arrow)] are additional hallmarks portraying epidermal keratinocytes of chronic wounds.⁶¹ To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

marker flaggrin, were found to be suppressed, whereas late differentiation markers involucrin and transglutaminase 1 were induced in venous ulcers when compared with healthy skin. The epidermis of chronic nonhealing wounds has reduced expression of the precursor of the $\alpha 3$ chain of LN5,⁶³ contributing to impaired migratory capacity of these cells. Furthermore, β -catenin is present in the nuclei of chronic wound keratinocytes even in the cornified layer.^{61,64} All these factors yield to nonmigratory, hyperproliferative epidermis that fails to re-epithelialize and restore the barrier.

Cross talk between keratinocytes and other cell types during epithelialization

Complex interactions and cross talk between keratinocytes, fibroblasts, endothelial, immune cells, and other cell types during all three phases of wound healing are critical for successful wound closure and repair (Fig. 3). Upon injury, keratinocytes located immediately adjacent to the wound site undergo an activation process in which they alter their gene expression rendering them competent for migration over the wound bed. Once activated, keratinocytes produce signaling molecules that act in an autocrine and paracrine fashion, resulting in pleiotropic effects on multiple cell types. These cells, in turn, respond by producing several signaling molecules that regulate keratinocyte activation during wound closure.

The initial phase of healing results in a release of prestored IL-1 by keratinocytes. IL-1 functions in an autocrine fashion by increasing keratinocyte migration and proliferation, and by inducing the expression of K6 and K16 in keratinocytes migrating into the wound.⁶⁵ In addition to acting on the keratinocytes, IL-1 activates nearby fibroblasts and increases the secretion of KGF, which in turn promotes keratinocyte migration and proliferation as discussed above,⁴⁵ and triggers the inflammatory cascade. The other common initiator of keratinocyte activation is the proinflammatory cytokine, $\text{TNF}\alpha$.⁶⁵ Similar to IL-1, $\text{TNF}\alpha$ can also act in an autocrine fashion to stimulate keratinocyte migration, and in a paracrine fashion activating fibroblasts and increasing the secretion of proteins of the FGF family of signaling molecules.⁶⁶ This promotes fibroblast migration and deposition of ECM components conferring increased keratinocyte motility during re-epithelialization.⁶⁷ Another important signaling molecule produced by both keratinocytes and fibroblasts is $\text{TGF}\beta$. Its expression is upregulated upon wounding and has been shown to induce granulation tissue formation and myofibroblast differentiation facilitating contraction of

the collagen matrix and wound closure.⁶⁸ In addition, TGF- β acts as an important regulator in reverting the activated keratinocytes to the basal cell phenotype by inducing the basal cell-specific markers K5 and K14 and reducing proliferation.⁶⁹ However TGF- β signaling is suppressed in the epidermis of venous ulcers,⁷⁰ which together with additional mechanisms⁴¹ contributes to the nonhealing and nonmigratory phenotype of chronic wound keratinocytes.

The early response to wounding also results in the release of chemokines by keratinocytes that act as chemoattractants for migration of immune cells to the site of injury. Neutrophils arrive at the wound site within minutes of wounding and become the predominant cells in the wound for the first 2 days after the injury occurs. Neutrophils and platelets entrapped and aggregated in the blood clot release a wide variety of factors that amplify the aggregation response, initiate a coagulation cascade, and/or act as chemoattractants for cells involved in the inflammatory phase.⁷¹ Among other proinflammatory cytokines, IL-6 is produced by neutrophils and has been shown to be important in initiating the healing response; namely, IL-6 has both mitogenic and proliferative effects on keratinocytes and, at the same time, acts as a chemoattractant for neutrophils.⁴⁷ The inflammatory phase of wound healing continues with active recruitment of macrophages from blood vessels, which is orchestrated by growth factor signals from keratinocytes, and by foreign epitopes of microorganisms.⁷² The macrophage chemoattractant protein (MCP-1), a member of the CC family of chemokines, is induced in keratinocytes and promotes migration of both macrophages and T cells to the site of injury.⁷³ The significance of this chemokine is underscored in MCP-1-deficient mice in which re-epithelialization was significantly inhibited.⁷⁴

Signals released by keratinocytes and fibroblasts upon the wounding target also skin endothelial cells (ECs). Growth factors, cytokines, and cell-cell and cell-matrix interactions activate ECs. Activated ECs, platelets, macrophages, and fibroblasts release proangiogenic cytokines and growth factors, leading to the invasion and migration of ECs into the ECM, EC proliferation, and new immature vascular formation.⁷⁵ Early events in angiogenesis, particularly EC migration and proliferation, are promoted by vascular endothelial growth factor (VEGF) and the initial phase of wounding results in release of VEGF by platelets in response to hypoxia.⁷⁶ VEGF acts in a paracrine fashion not only on ECs but also on keratinocytes and immune cells promoting re-epithelialization and at the same time stimulating angiogenesis and restoring oxygen perfusion.⁷⁷

Taken together, growth factors and cytokines control wound healing events, and the balance of tightly regulated ligands and their receptors coordinates progression through all phases of healing.^{27,39} However, nonhealing wounds fail to progress and often remain in a prolonged inflammatory phase. As a consequence, imbalances in wound proteases and their inhibitors in chronic wounds may cause degradation of growth factors and cytokines in the wound site, inhibiting progression of the healing process.⁵³ Recent advances in the field of miRNAs and histone modifications have revealed new aspects and an additional level of complexity regarding the molecular mechanisms controlling normal and impaired wound healing processes, as detailed below.

Mechanisms of gene expression regulation during epithelialization

Role of miRNAs in wound healing. miRNAs are small noncoding RNAs that regulate gene expression at the post-transcriptional level by binding to the 3' UTR of mRNAs, repressing their translation or causing messenger degradation.⁷⁸ miRNAs are generated by sequential processing of long RNA polymerase II transcripts by two key RNase III proteins, Drosha and Dicer.⁷⁹ To target mRNA, miRNA binds mRNAs in a unique manner using Watson-Crick base pairing through a conserved 6- to 8-bp seed sequence. Importantly, one miRNA can target hundreds of genes, whereas a single gene can be targeted by multiple miRNAs.^{78,79}

The importance of miRNAs in epidermal development and adult skin stem cell maintenance has been established by experiments involving conditional depletion of either Dicer1 or Drosha in murine epidermis.^{80,81} When either one of these two genes were ablated in the epithelium, the barrier function of the skin was compromised.^{80,81} Furthermore, these studies identified several miRNAs that were expressed differentially or exclusively in murine epidermis as compared with other skin lineages. The miR-200 family (a, b, and c), miR-141, miR-429, and the miR-19/miR-20 family (miR-19b, miR-20, miR-17-5p, and miR-93) were preferentially expressed in epidermal lineage, while the miR-199 family was exclusively expressed in HF. ^{80,82} More recent studies compared the miRNA expression patterns of *in vitro* differentiated keratinocytes, with miRNA profiles of ESCs, transit amplifying keratinocytes and terminally differentiated keratinocytes isolated from human skin.⁸³ The results revealed eight upregulated miRNAs in differentiated keratinocytes (miR-23b, miR-95, miR-210, miR-224, miR-26a, miR-200a,

miR-27b, and miR-328), and one downregulated miRNA (miR-376a) both *in vivo* and *in vitro*, suggesting their involvement in the process of epidermal differentiation.⁸³

Based on the multicellular nature of such a complex process that depends on timed response and signaling control, it was reasonable to assume that miRNAs have a role in controlling wound closure.⁸⁴ This hypothesis was confirmed by several recent discoveries.^{85–88}

miR-203, a very abundant miRNA that targets p63 and contributes to barrier formation, was recently associated with wound healing.^{89,90} miR-203 is downregulated at the migrating epithelium of acute wound edges allowing the expression of p63, RAN (member of the G-protein superfamily), and RAPH1 (lamellipodin), hence contributing to re-epithelialization.⁹¹ In contrast, the epidermis of nonhealing chronic wounds has elevated miR-203 levels.⁸⁵ Another recent report has shown an antiproliferative effect of miR-483-3p in human keratinocytes, induced by wounding *in vitro* and *in vivo*.⁹²

The mechanisms by which deregulation of miRNAs contribute to the pathogenesis of chronic wounds have also been proposed. A set of miRNAs, miR-16, miR-20a, miR-21, miR-106a, miR-130a, and miR-203 were found to be upregulated in the hyperproliferative nonmigratory epidermis of chronic venous ulcers (Fig. 5).⁸⁵ Furthermore, miR-130a and miR-21 inhibited epithelialization *in vivo* and *ex vivo* by targeting multiple genes important for wound closure, including the early growth response factor 3 (EGR3) and leptin receptor (LepR).⁸⁵ Hypoxia-induced miR-210 has also been linked to decreased re-epithelialization in ischemic wounds.^{86,88} Together, these studies demonstrate that miRNAs contribute to both normal epithelialization and its inhibition in nonhealing wounds.

Epigenetics of the wound healing process. Different types of epigenetic regulation include differential methylation and hydroxymethylation of DNA, histone modifications, ATP-dependent chromatin remodeling, and higher order chromatin remodeling and positioning within the nuclear

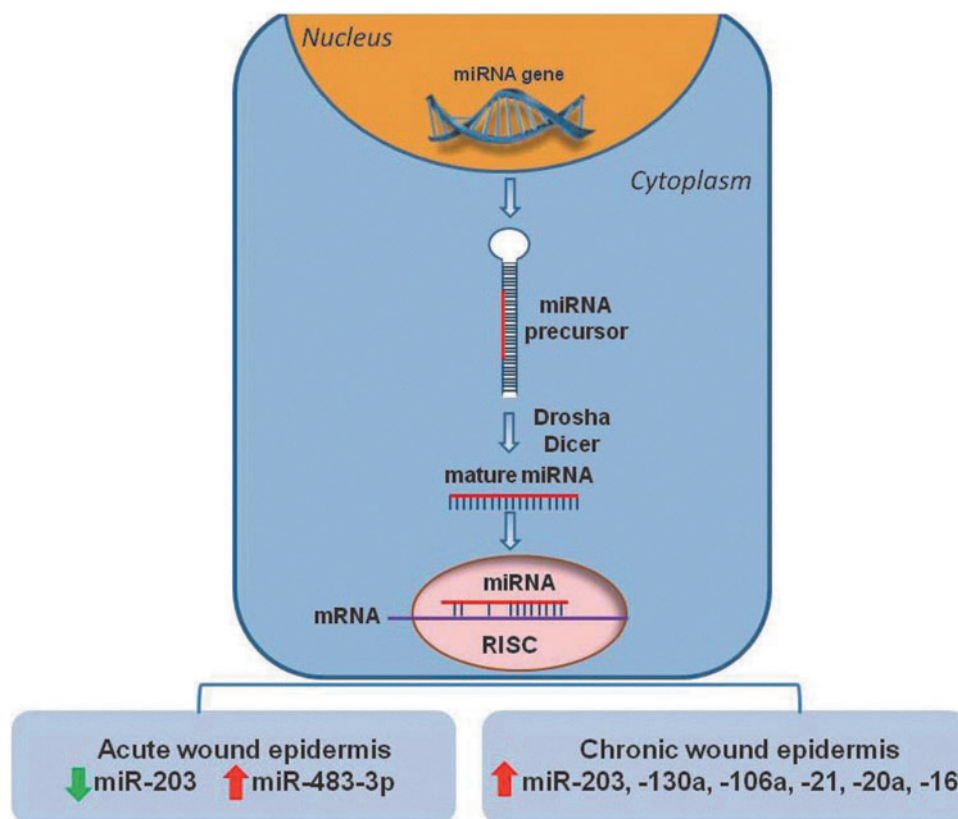


Figure 5. Role of miRNAs in epithelialization. Schematic representation of miRNA biogenesis: miRNAs are transcribed in the nucleus as 70-bp precursor products that are processed into the mature ~22-bp products by the Drosha and Dicer cytoplasmic enzymes. The mature miRNA interacts with the RNA-induced silencing complex (RISC) and binds to complementary sequences in target messenger RNAs (mRNA) leading to downregulation of gene expression. Suppression of miR-203 and induction of miR-483-3p promote keratinocyte migration during normal wound healing process. Induction of miRNA—203, -130a, -106a, 21, -20a, -16 in keratinocytes of nonhealing edges of chronic wounds aids to their pathogenesis by inhibiting epithelialization and wound closure. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

space. Each of these was found to participate in keratinocyte differentiation and barrier formation (reviewed in Botchkarev *et al.*⁹³) although their involvement during wound healing and epithelialization remains mostly unexplored. Reduction of histone H3 lysine 27 trimethylation (H3K27me3) occurs at the healing edges of full-thickness wounds in mice.⁹⁴ H3K27me3 is a silencing chromatin modification that can be set by the Polycomb Repressive Complex 2 (PCR2)⁹⁵ or removed by the specific H3K27 demethylases Jmjd3 and Utx.⁹⁶ Consistently, the components of the PCR2/Eed-Ezh2 complex were downregulated at the wound edges, whereas Jmjd3 and Utx were upregulated.⁹⁴ In addition, less Eed was bound to the promoter regions of two genes important for wound healing, EGFR and myc, up to 3 days postwounding suggesting the existence of tight epigenetic regulation of the epithelialization process.⁹⁴ Polycomb-group-mediated H3K27me3 also plays an important role in balancing HF stem cell quiescence.⁹⁷

Moreover, nuclear epigenetic changes and miRNA regulation of gene expression seem to be interconnected as miRNA expression can be regulated by histone modifications and DNA methylation. Conversely, miRNAs can target epigenetic regulators such as DNA methyltransferases and histone modifying proteins.⁹⁸ For example, the reepithelialization-related miR-203 is found to be silenced by DNA methylation of its promoter region in several types of tumors resulting in induced cell growth and invasion.⁹⁹ Furthermore, miR-203 can also target Bmi-1, a component of the polycomb repressor complex 1 involved in migration and invasion of cancer cell lines.¹⁰⁰ In addition, miR-205 overexpression, which targets lipid phosphatase SHIP2 and increases keratinocyte migration during wound healing,¹⁰¹ has been found to suppress the expression of Ezh2 (enhancer of zeste homolog 2) in prostate cancer, suggesting a possible contribution to histone modifications.¹⁰²

These new mechanisms of gene expression regulation during wound closure provide novel insights and additional levels of complexity to the wound healing process. Further exploration of these mechanisms may help us to understand how normal epithelialization occurs and why in chronic wounds this control is lost.

Epidermis as a neuroendocrine organ

As a sophisticated physical barrier exposed to a diverse range of stressors, the skin and epidermis in particular, have evolved as an independent neuroendocrine organ to efficiently sense and appropriately respond to the stress and changes in

the external environment. The dynamic capability of skin to communicate with the central nervous system through local production and systemic release of hormones, neuropeptides, neurotransmitters, and biological regulators establishes it as a neuroendocrine organ.^{103,104} This interdependent cross talk involves expression of specific receptors, synthesis, activation or inactivation of the hormones, and exertion of biological activity, which help maintain local and consequently systemic homeostasis.¹⁰³ Both keratinocytes and melanocytes share analogous properties with secretory neurons by expressing a highly organized hypothalamus–pituitary–adrenal axis, which includes corticotropin-releasing hormone, urocortin, pro-opiomelanocortin, with its products adrenocorticotrophic hormone, α -melanocyte-stimulating hormone, and β -endorphin.¹⁰⁵ In response to stress, skin cells can also produce vitamin D3,¹⁰⁶ cortisol,¹⁰⁷ parathyroid hormone-related protein,¹⁰⁸ precursors to biogenic amines,¹⁰⁹ and the neurotransmitters—catecholamines and acetylcholine (Table 1).^{104,110} Furthermore, the skin is a site for activation of steroid hormones such as converting T₄ to T₃ or testosterone to either 5 α -dihydrotestosterone or estradiol.¹¹¹ The production of these locally synthesized hormones and expression of their receptors is either constitutive or can be induced by specific stimuli.^{107,112,113}

The most prevalent cellular components of the neuroendocrine system in skin are keratinocytes, which not only express a vast repertoire of hormone receptors, but can also synthesize hormones to enhance or impair wound healing. Most importantly, keratinocytes possess the ability to synthesize cholesterol *de novo*, the precursor to all steroids.¹¹⁴ Hormones as well as intermediates in the cholesterol synthesis pathway, such as farnesyl pyrophosphate (FPP), affect epithelialization. FPP acts as an agonist for the GC receptor, thus inhibiting keratinocyte migration and wound closure.¹¹⁵ Sex steroid hormones are important for controlling skin turnover, and a large percentage of androgens and estrogens are synthesized locally from inactive precursors.¹¹⁶ It has been shown that estrogen has a mitogenic effect on keratinocytes and increases the rate of epithelialization postwounding in animal models.¹¹⁷ Exogenous systemic or topical estrogen treatment enhances healing by stimulating matrix deposition and reducing inflammation.¹¹⁷ On the other hand, testosterone possesses an inhibitory effect on skin repair and epithelialization.¹¹⁸ These results do not imply differences in the healing rates between males and females, but rather indicate the role of

Table 1. *Epidermis is a neuroendocrine organ*

Ligand	Receptor	Epidermal Layer	Function	Reference
Corticotropin-releasing hormone (CRH)	CRH-R1	Present throughout epidermis	Inhibits keratinocyte proliferation Enhances the production and secretion of the POMC peptides in keratinocytes, melanocytes, endothelial cells, and cutaneous nerves Stimulates production of steroids in the skin	112,176–178
Class II PTH/PTHrP	Class II PTH/PTHrP-R	Highly expressed between basal to granular layers	Inhibits keratinocyte proliferation and stimulates differentiation Restoration of epidermal homeostasis during wound healing	108,112
Vitamin D	Vitamin D-R	All epidermal layers except stratum corneum	Inhibits keratinocyte proliferation and stimulates keratinocyte differentiation	112,179
Testosterone dihydro-testosterone	Androgen-R	Restricted to basal layer In genital skin present in spinous and granular layers	Inhibits wound healing, promotes inflammation, regulates keratinocyte proliferation and differentiation, and reduces permeability barrier function of stratum corneum Androgen receptor antagonist: accelerates wound healing	180–182
Estrogen	Estrogen-R (nuclear ER β)	ER β : present in the spinous and basal layers	Stimulates keratinocyte proliferation and accelerates epithelialization Decreases inflammatory response and suppresses apoptosis	117,178,182,183
Glucocorticoid	Glucocorticoid-R	Present throughout the epidermis, but mainly expressed in basal layer	Inhibits epithelialization Represses expression of basal cell-specific keratins K5, K14, and wound healing-associated keratins K6, K16, K17 Prolonged exposure can defect the permeability barrier function	107,179,181
Mineralocorticoid	Mineralocorticoid-R	Present throughout epidermis	Aldosterone/MR affect sodium, potassium, or calcium currents in keratinocytes, leading to altered epidermal ion homeostasis	178,184
Acetylcholine	Muscarinic-R	Predominantly in the basal or suprabasal layers	Regulate keratinocyte proliferation, migration, and differentiation	112,185,186
Acetylcholine	Nicotinic-R	Throughout epidermis, but predominantly in the basal or suprabasal layers	Stimulates keratinocyte motility and differentiation The receptors represent functional ion channels mediating the influx of sodium and calcium, and the efflux of potassium, being essential for keratinocyte viability	112,185
ACTH	Adrenoreceptors MC1R and MC2R	MC1R: strongly expressed in suprabasal layer; MC2R: present throughout epidermis	Stimulates <i>de novo</i> cortisol synthesis in keratinocytes Regulates keratinocyte differentiation	112,187–189

POMC, proopiomelanocortin.

sex hormones in fine-tuning the wound healing process. Keratinocytes also express enzymes such as steroid 11 β -hydroxylase (CYP11B1) essential for cortisol synthesis and 11 β -hydroxysteroid dehydrogenase 1 (11 β HSD1), which catalyzes conversion of inactive to active cortisol.^{107,119} Inhibition of skin-specific GC synthesis by CYP11B1 inhibitor metyrapone, accelerates wound closure *in vivo*.¹⁰⁷ In addition, inhibition of 11 β HSD1 leads to increased proliferation of keratinocytes.¹¹⁹ Inhibition of GC synthesis in keratinocytes resulted in increased expression of proinflammatory IL1- β implying that skin-specific cortisol synthesis serves as a local negative feedback to prevent excessive inflammation upon initial injury and serves to regulate epithelialization.¹⁰⁷

Another type of hormone, the catecholamine epinephrine, can impair keratinocyte migration in a dose-dependent manner by binding to the beta2-

adrenergic receptor (beta2AR) leading to delayed epithelialization.¹¹³ Beta2AR antagonists increase keratinocyte migration by preventing the binding of endogenously or systemically synthesized epinephrine.^{104,113} These studies have clinical implications, providing a framework for understanding how chronic stress impairs healing and a therapeutic target for improving healing. Indeed, anecdotal case reports support the use of beta adrenergic antagonists as therapeutic agents to heal chronic wounds.¹²⁰ The unique ability of the skin to work as an independent neuroendocrine system allows for both local and systemic hormone-mediated responses in homeostasis and wound healing.

Keratinocyte immunity during epithelialization

A well-executed immune response is also essential for successful wound healing. The skin has

traditionally been regarded for its protective role as a physical barrier to foreign pathogens. More recently, however, several resident skin cell populations, including keratinocytes, have gained recognition for their active contribution to host immune responses during epithelialization.

Keratinocytes perform functions that directly influence both immunity and wound closure. Although wound re-epithelialization is driven primarily by keratinocyte migration and proliferation, in this process, keratinocytes simultaneously employ strategies to resist microbial entry. Keratinocytes recognize foreign pathogens and endogenous danger signals through their Toll-like receptors (TLRs), specifically, TLRs 1–6 and 9.^{121–123} TLR activation initiates effector immune cell recruitment, through the production of proinflammatory cytokines and chemokines, and has recently been implicated in the process of wound closure and re-epithelialization.^{124,125} In particular, TLRs 2, 3, 4, and 9 have been identified as key modulators of wound epithelialization. The increased expressions of TLR2 and TLR4 have been shown to contribute to impaired wound healing in animal models.^{125,126} Furthermore, an association between nonhealing chronic venous ulcers and chronic inflammatory cytokine expression secondary to persistent activation of TLR2 and TLR4 has been described.¹²⁷ More recently, TLR4 was shown to promote optimal early inflammation in wound repair. Mice deficient in TLR4 experienced significantly delayed wound closure as well as impaired inflammatory cell infiltration.¹²⁸ Similarly, TLR3, a recognized initiator of inflammation following injury,¹²⁹ and TLR9, have been reported to accelerate wound closure and epithelialization.^{130,131} The discrepancies in the above findings underscore the need to maintain a delicate balance and temporal regulation of TLR activation to return the skin to homeostasis following acute injury.¹²⁴ One such way to harness TLR-induced inflammation may involve commensal skin bacteria.¹²⁹

Following wounding and/or microbial challenge, keratinocytes are also stimulated to release antimicrobial peptides (AMPs), including human cathelicidins, hCAP 18 and LL-37, and human β -defensins.¹³² Ubiquitous in nature, AMPs are potent antimicrobial agents that are effective against both gram-positive and gram-negative organisms. These proteins have been shown to be critical in wound closure and epithelialization. Levels of AMPs increase significantly in early wound healing and subsequently decrease upon wound closure.^{133,134} However, expression of AMPs is deregulated in chronic wounds; keratinocytes in

nonhealing wounds show a constitutively high baseline expression of human β -defensin-2 (h β D-2) and psoriasin, while expression of RNase 7 and LL-37 is suppressed.^{133,135} Nevertheless, AMPs are still considered as potential antimicrobial therapeutics for chronic wounds; as an example, topical LL-37 has been reported to accelerate epithelialization in acute wounds in murine models.¹³⁶ In the porcine wound model, the cathelicidin protein PR-39 was reported to induce syndecans in acute wounds.¹³⁷ Syndecans have been suggested to play a key role in appropriate wound healing, as mice deficient in syndecan-4 genes were observed to have delayed wound closure.¹³⁸ Most recently, the critical role of regenerating islet-derived protein 3-alpha (REG3A) and regenerating islet-derived protein 3 gamma (RegIII γ) in wound epithelialization was described.¹³⁹ Reg3A and RegIII γ have been shown to function as antimicrobial proteins in the intestine, inhibiting microbial proliferation.¹⁴⁰ These proteins were also found to inhibit keratinocyte differentiation/proliferation, thus facilitating wound closure.¹³⁹

In response to various insults and injuries, keratinocytes can also activate inflammasomes.¹⁴¹ Inflammasomes are multiprotein innate immune complexes that produce and mature proinflammatory cytokines upon recognition of foreign pathogens. The role of inflammasomes in wound healing has recently been explored. Levels of epidermal caspase 8, a mediator of apoptosis, fluctuate greatly during the course of wound repair. The loss of epidermal caspase 8 led to the induction of aberrant cross talk among epithelial, mesenchymal, and leukocytic cells. This ultimately resulted in epidermal hyperplasia and the alteration of the wound repair process, highlighting the need for an appropriate inflammasome response for wound closure.¹⁴²

The immunologic function of the skin relies heavily on interactions among keratinocytes and other immune cells. Keratinocytes are the main protagonists of skin innate immunity, which further control the adaptive immune response during epithelialization and wound closure. Keratinocytes clearly participate in host defense against invading microorganisms in multiple ways during normal and impaired wound healing, and one approach to understanding the skin–bacteria interaction during wound closure includes recently advanced microbiome studies.

Skin microbiome and wound healing

A microbiome can be defined as the entirety of all microbes, their genomes, and interactions within a particular environment. Recent advances in high-

throughput sequencing technologies have facilitated studies of the complex microbial inhabitants of the human body by providing a more comprehensive identification of microbes than traditional culture methods.¹⁴³ These novel methods use 16S rRNA gene-based analyses to characterize the diversity of microbial communities present at specific tissues or body locations, including skin.¹⁴³ Although the complexity and diversity of healthy human skin microflora have been established,¹⁴⁴ a more thorough understanding of skin microbial inhabitants and pathogens can provide a foundation for future research regarding the interactions between microbes and epidermis, during normal, acute, and impaired wound healing. As the epidermis is the first line of host defense against the external environment, defining wound microbiomes conducive to the normal wound healing process is important and may greatly benefit patients with nonhealing wounds. The power of the 16s rRNA sequencing technology of microbiomes is shown by a recent study indicating a shift in both unwounded skin and wound microbiota in diabetic mice (db/db) that was used as a model of impaired wound healing.¹⁴⁵ Forty times more bacteria and different types were found on the skin of db/db mice and impaired healing coincided with a shift in microbial diversity, which changed over time during healing.¹⁴⁵ The human skin microbiome undergoes dynamic changes after barrier disruption as well. The most recent study suggested that the microbiome of the deeper stratum corneum layers plays an important role in the microbial re-colonization process of the human skin after injury caused by tape stripping.¹⁴⁶ It has also been suggested that microbiomes of chronic wounds play significant roles in impaired wound healing, in the presence or absence of clinical signs of infection.^{147–150} This polymicrobial bioburden in chronic wounds exists predominantly in the form of a biofilm,¹⁵¹ which can inhibit keratinocyte migration and epithelialization.^{152,153} The use of standard clinical microbial cultures to diagnose the wound bioburden has been shown to be of limited value as culture techniques typically detect only microorganisms that grow relatively quickly and easily in laboratory media,¹⁵⁴ while 16S rRNA gene-based analyses have revealed more complex wound microbiomes than those described previously in culture-based analyses.^{147–149} It has also been suggested that the diversity of the chronic wound microbiome is significantly reduced compared with neighboring healthy skin.¹⁴⁸ Depending on the study and the type of chronic wounds evaluated, among the most prevalent genus in chronic wounds were *Staphylo-*

coccus, *Pseudomonas*, and *Corynebacterium*, while obligate anaerobes *Bacteroides*, *Peptoniphilus*, *Fingoldia*, *Anaerococcus*, and *Peptostreptococcus* spp were identified as well.^{148,150} In addition, the prevalence of Oxalobacteraceae, Corynebacteriaceae, and Pseudomonadaceae was increased by the use of antibiotics.¹⁴⁹ Recent studies using animal models of wound infections have shown that polymicrobial infection with *S. aureus* and *Pseudomonas aeruginosa* inhibits epithelialization significantly more than infection with single species,¹⁵³ suggesting that the complex chronic wound microbiome may have even more of a detrimental effect on wound closure.

Despite these molecular advances, diagnosis of wound infection in the clinical setting is still based on standard microbiology culturing techniques, making detection of biofilm and anaerobic species impossible. A better understanding of the bacterial populations associated with clinical outcomes is essential to improve the management of chronic wounds. In the future, we can expect the development of quantitative PCR-based methods that will enable more detailed analyses of target taxons of the human cutaneous and wound microbiota, complementing high-throughput sequencing and enabling detection of wound pathogens that would influence treatment decisions to benefit patients with chronic nonhealing wounds.

Models to study epithelialization

To assess keratinocyte migration in response to wounding and specific treatments (*e.g.*, pharmacological agents), the scientific research community employs a range of models using keratinocyte cultures, cultured fetal, adult, or engineered human skin, as well as various animal models (Table 2).^{107,155–158} Due to space limitation, we will describe only the most frequently utilized wound healing models. It should be kept in mind that the etiology of human chronic wounds often includes a combination of multiple factors and underlying comorbidities, and that various models typically address a single factor (*e.g.*, diabetes, infection, and ischemia). In spite of research efforts focused on improving existing models and developing new ones, a model that fully replicates human non-healing chronic wounds remains missing.

A widely used *in vitro* assay to study epithelialization is a wound scratch assay, where human keratinocytes are grown to confluence in a tissue culture dish and then wounded by a scratch using a pipette tip.^{40,61,159} As the scratch wound gap closes, keratinocyte migration is microscopically recorded and quantified by image analysis. This model al-

Table 2. Summary of frequently used models to study wound epithelialization

	Advantage	Disadvantage	Reference
<i>In vitro</i>			
Wound scratch assay	Utilizes human epidermal keratinocytes By inhibiting proliferation, one can only assess keratinocyte migration and its underlying biology	Lack of cross talk with other cell types involved in the wound healing process	40,61,159
<i>Ex vivo</i>			
Human <i>ex vivo</i> acute wound model	Presence of full-thickness epidermis and dermis; Langerhans cells, pigment cells, and nerve endings	Absence of immune cells and blood supply Variation in wound depth	85,107,115,155,161,162
Human <i>ex vivo</i> linear excisional wound model	Standardized wound depth Presence of full-thickness epidermis and dermis; langerhans cells, pigment cells, and nerve endings present	Absence of immune cells and blood supply	164
Partial-thickness human skin culture model (incision)	Presence of full-thickness epidermis and dermis; langerhans cells, pigment cells, and nerve endings present	Absence of immune cells and blood supply	163
Murine and human skin equivalents	Ability for studying the interaction between keratinocytes and fibroblasts	Lack of immune cells and blood supply; simplified ECM	157,165
<i>In vivo</i>			
Tape stripping	Partial removal of epidermis Several levels of barrier disruption can be induced	Variation in depth of barrier disruption	190
Murine incisional/excisional wound model	Ability for studying the interaction and influence of different cell types on epithelialization Use of wound splinting in full-thickness wounds to minimize contraction Uncomplicated standardization and multiple replicates	Rodents heal by contraction of the subcutaneous muscle Variation in skin anatomy and physiology in rodents vs. humans Influence of the hair follicle phase on epithelialization	77,85,158,167,170,191
Porcine partial/full-thickness wound model	Greatest structural skin similarity and the wound healing process to the humans Multiple replicates within single animal	Lack of commercially available reagents for analyses	107,172,192,193
Rabbit ear wound model	Decreased blood flow Absent wound contraction Multiple replicates within single animal	Dermis firmly attached to underlying cartilage Avascular wound base	171,194

ECM, extracellular matrix.

lows for quantifying only migration, by inhibiting proliferation using, for example, mitomycin C pretreatment. As a caveat, however, data obtained using this technique should be corroborated using other model systems since *in vitro* conditions cannot mimic the complexity of cross talk between different cell types that occurs during the wound healing process *in vivo*.

However, there are obvious difficulties in sampling skin acute wounds in humans due to ethical concerns. To overcome this obstacle, a first report modeling the wound healing process in human skin using organotypic cultured skin was reported by Kratz.¹⁶⁰ In this model, both epidermal and dermal cells maintained their viability in incisional wounds created in *ex vivo* cultured skin, maintained for up to 14 days in either 2% or 10% fetal bovine serum (FBS). Skin explants maintained in low FBS did not show signs of epithelialization, whereas wounds maintained in high FBS showed complete epithelialization after 14 days.¹⁶⁰ Our laboratories developed an *ex vivo* acute wound model utilizing

human skin obtained from reduction surgeries in which we create acute wounds using a biopsy punch, maintaining them at the air-liquid interface to assess the epithelialization process.^{85,107,115,155,161}

Details of the wounding technique can be found in the cited publications.^{161,162} Recently, two new *ex vivo* wound models, a partial-thickness human skin culture model,¹⁶³ and linear excisional wound model¹⁶⁴ were described, further confirming the validity and usefulness of *ex vivo* human wound models. Having in mind the limitation of material that can be obtained from cosmetic surgeries, bioengineered skin substitutes offer the advantage of creating multiple wounds from the same tissue source, minimizing experimental variability. These bioengineered skin substitutes use keratinocytes grown on fibroblast feeder layers thus recapitulating skin morphology.

Advances in tissue engineering have facilitated the progress of the development of diverse models to study wound re-epithelialization and provided our research community with both murine and

human skin equivalents.^{157,165} Benefits such as uncomplicated standardization, a possibility of multiple repetitions, as well as following a wound progression through time are advantages that have promoted wide utilization of animal models for wound healing studies. The *Drosophila* wound model provided insight on how the cytoskeletal machinery is involved in the epithelialization process.¹⁶⁶ Furthermore, small mammals such as mice and rats allow scientists for genetic manipulation, utilizing overexpression or knockout technologies to specifically target keratinocytes and focus on the epithelialization process, or by mimicking diseases known to affect the wound healing process such as diabetes, dyslipidemia, and obesity.^{77,85,156} In studies utilizing small animals, usually a wound is created on the dorsum of the animal, by tape stripping, incisional or excisional wounding, and the rate of wound epithelialization is measured using histomorphometric analysis.^{77,85,158} One of the widely adopted full-thickness mouse models utilizes wound splinting. This model minimizes contraction, a mechanism by which murine wounds mostly heal, and allows wound healing to occur primarily through the processes of granulation and re-epithelialization.^{158,167} However, differences in skin composition (*e.g.*, thickness of skin and number of HF's per cm²) should be considered when obtained data are translated to the human skin. To address these dissimilarities, a model where full-thickness human skin is transplanted onto nude animals has been developed.^{168,169} In addition, recent work by Ansell *et al.* described the influence of the different hair-cycle stages on murine skin wound healing. The authors showed that murine skin wound healing is accelerated during the anagen phase of HF cycling, thus raising caution in regard to interpretation of obtained data when murine wound healing studies are employed.¹⁷⁰ A rabbit ear model is also used to assess re-epithelialization and limits wound contraction by virtue of the underlying cartilage splinting the wound.¹⁷¹ The porcine wound model, however, either with partial- or full-thickness skin wounds, has been shown to have the greatest similarity to the human wound healing process.^{107,172} Porcine skin is structurally most similar to human skin, including parameters such as epidermal thickness and dermal-epidermal thickness ratios, as well as similar patterns of HF's and blood vessels.¹⁷³ Por-

TAKE-HOME MESSAGES

- The epidermis is a stratified epithelium comprising several layers of keratinocytes.
- Keratinocytes are responsible for restoring the epidermis after injury through a process known as epithelialization.
- Keratinocytes continually renew during homeostasis as well as during disruption of the epidermal barrier.
- The regenerative capacity of the skin relies on the local populations of ESCs.
- Multiple regulators such as growth factors and cytokines, integrins, keratins, MMPs, chemokines, and extracellular matrices modulate keratinocyte migration during wound closure.
- The epithelialization process is impaired in all types of chronic wounds.
- Novel mechanisms of gene expression regulation, including miRNA and histone modifications, are important for re-epithelialization.
- Keratinocytes produce various hormones, neuropeptides, and neurotransmitters, and express their receptors that help in maintaining epidermal homeostasis and are implicated to have a role in wound closure.
- Keratinocytes actively participate in host defense against invading microorganisms during wound healing.
- A better understanding of the epithelialization process may ultimately lead to the development of novel therapeutic approaches for nonhealing wounds.

cine wounds heal primarily by epithelialization, whereas murine, rabbit, and dog wounds heal primarily by contraction.¹⁷⁴ Epithelialization in a porcine wound healing model can be also assessed using the salt-split technique in conjunction with histological evaluation.¹⁷⁵ Taken together, wound healing studies conducted on animals are extremely valuable and allow for initial preclinical evaluation for testing potential treatment modalities.

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Abbreviations and Acronyms

- 11 β HSD1 = 11 β -hydroxysteroid dehydrogenase 1
 ACTH = adrenocorticotropic hormone
 ADAM12 = disintegrin and metalloprotease 12
 AMP = antimicrobial peptides
 beta2AR = beta2-adrenergic receptor
 BM = basement membrane
 CRH = corticotropin-releasing hormone
 CYP11B1 = steroid 11 β -hydroxylase
 DP = dermal papilla
 ECM = extracellular matrix
 ECs = endothelial cells
 EGF = epidermal growth factor
 EGR3 = early growth response factor 3
 ESCs = epidermal stem cells
 Ezh2 = enhancer of zeste homolog 2
 FBS = fetal bovine serum
 FGF = fibroblast growth factors
 FPP = farnesyl pyrophosphate
 GCs = glucocorticoids
 GM-CSF = granulocyte-macrophage colony-stimulating factor
 H = histone
 H3K27me3 = histone H3 lysine 27 trimethylation
 HF = hair follicle
 h β D-2 = β -defensin-2 and psoriasin
 IFE = interfollicular epidermis
 IGF = insulin-like growth factor
 IRS = inner root sheet
 K = keratin
 LepR = leptin receptor
 LN5 = laminin-5
 MCP-1 = macrophage chemoattractant protein
 miRNAs = microRNAs
 MMPs = matrix metalloproteinases
 ORS = outer root sheet
 PCR2 = polycomb repressive complex 2
 PKC = protein kinase C
 POMC = proopiomelanocortin
 PTHrP = parathyroid hormone-related protein
 REG3A = regenerating islet-derived protein 3-alpha
 RegIII γ = regenerating islet-derived protein 3 gamma
 RISC = RNA-induced silencing complex
 SG = sebaceous gland
 TIMPs = inhibitors of metalloproteinase
 TLRs = Toll-like receptors
 TNF = tumor necrosis factor
 VEGF = vascular endothelial growth factor