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REVIEW

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Understanding Transcriptional Networks Regulating Initiation of Cutaneous Wound Healing

Rafik Boudra[a,b] and Matthew R. Ramsey[a,b,*]

[a]*Brigham and Women's Hospital Department of Dermatology, Boston, MA;* [b]*Harvard Medical School, Boston, MA*

The epidermis has an essential function in creating a barrier against the external environment to retain proper fluid balance and block the entry of pathogens. When damage occurs to this barrier, the wound must quickly be sealed to avoid fluid loss, cleared of invading pathogens, and then keratinocytes must re-form an intact barrier. This requires complex integration of temporally and spatially distinct signals to execute orderly closure of the wound, and failure of this process can lead to chronic ulceration. Transcription factors serve as a key integration point for the myriad of information coming from the external environment, allowing for an orderly process of re-epithelialization. Importantly, transcription factors engage with and alter the chromatin structure around key target genes through association with different chromatin-modifying complexes. In this review, we will discuss the current understanding of how transcription is regulated during the initiation of re-epithelialization, and the exciting technological advances that will allow for a more refined mechanistic understanding of the re-epithelialization process.

INTRODUCTION

The skin is the largest organ in the human body and is responsible for preventing fluid loss, blocking pathogens, and providing a physical barrier against the external environment. Efficient and rapid wound healing following skin injury is therefore critical to maintain proper homeostasis. This requires coordinated action between the many cell types that make up the skin (Figure 1a). Cutaneous wound healing can be divided into three major

*To whom all correspondence should be addressed: Matthew R. Ramsey, PhD, Brigham and Women's Hospital, 77 Ave Louis Pasteur, HIM 668, Boston, MA 02115; Tel: (617) 525-5775, Fax: (617) 525-5571, Email: mramsey@bwh.harvard.edu.

Abbreviations: PDGF, Platelet-Derived Growth Factor; EGF, Epidermal Growth Factor; TNF-α, Tumor Necrosis Factor-α; IL-6, Interleukin-6; TGF-β, Transforming Growth Factor-β; IGF-1, Insulin Like Growth Factor 1; IFN γ, Interferon γ; KGF, Keratinocyte Growth Factor; HF-SC, hair follicle stem cell; IFE, interfollicular epidermis; EMT, Epithelial-to-Mesenchymal Transition; PRC2, Polycomb Repressive Complex 2; lncRNA, long non-coding RNA; FAK, Focal Adhesion Kinase; TAK1, transforming growth factor-β-activated kinase 1; HIF, Hypoxia-Inducible Factor; KLF5, Kruppel Like Factor 5; SOX9, SRY-Box Transcription Factor 9; NF-κB, Nuclear Factor Kappa B; STAT3, Signal Transducer and Activator of Transcription 3; JAK, Janus Kinase; ERK, Extracellular Signal-Regulated Kinase; MAPK, Mitogen-Activated Protein Kinase; C-Jun, Jun Proto-Oncogene; EGLN1, Egl-9 Family Hypoxia Inducible Factor 1; KDM6B, Lysine Demethylase 6B; WAKMAR1, Wound and Keratinocyte Migration-Associated LncRNA; TET, Tet-eleven translocation Methylcytosine Dioxygenase; DNMT, DNA Methyltransferase; 5-hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytosine; 5caC, 5-carboxylcytosine; scRNA-seq, Single cell Ribonucleic Acid sequencing; ChIP-seq, Chromatin Immunoprecipitation-sequencing; snmC-seq, Single-cell DNA methylome sequencing; ATAC-seq, Assay for Transposase-Accessible Chromatin using sequencing.

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stages, the Inflammatory Phase, the Proliferative Phase, and the Matrix Remodeling Phase [1-4]. Immediately following wounding, vasoconstriction and recruitment of platelets quickly occurs, resulting in the formation of a fibrin clot that effectively stops hemorrhage. In addition to re-establishing hemostasis, platelets secrete cytokines such as Platelet-Derived Growth Factor (PDGF), which trigger the inflammatory response by attracting immune cells to clear wound bed from pathogenic agents and cell debris [5]. Release of cytokines and growth factors from infiltrating immune cells then help to initiate the Proliferative Phase, where keratinocytes at the wound edge break down their hemidesmosomes, severing their secure attachment to the basement membrane and adopt an activated state, characterized by a change in cytoskeletal network, cell surface receptors, the expression of integrins αVβ5, αVβ6, and α5β1, and of cytokeratins KRT6, KRT16, and KRT17 [4,6]. These leading edge keratinocytes switch to a non-proliferative, migratory mode and move to fill in the damaged epidermis while keratinocytes further away from the wound proliferate to replace the migrating cells [3,4]. When the keratinocytes have covered the wound area, they re-form a stratified epidermis, while coordinated action of macrophages, fibroblasts, and endothelial cells results in the formation of the granulation tissue beneath the wound [3,4].

As humans age, the ability to heal wounds decreases [7], and improper control of wound healing can lead to persistent, chronic ulceration and significant morbidity, which is the largest driver of skin disease costs at ~$15 billion per year [8]. One population at particularly high risk is patients with diabetes, as non-healing ulcers led to lower limb amputations in approximately 73,000 adults in 2010 [9], a number that rose to 108,000 in 2014 [10], correlating with the rise in incidence of diabetes [9,10]. A variety of therapeutic strategies have been developed seeking to improve wound healing and prevent ulceration. New methods of debridement and novel wound dressings incorporating matrix components [11,12] allow for induction of the natural wound response and protection from the external environment, but do not address the underlying pathology of chronic ulceration. Alternatively, many studies have sought to modulate the wound microenvironment with the addition of cytokines and growth factors, an approach that has shown promise in pre-clinical models [13-16]. Unfortunately, only one growth factor based drug (rPDGF-BB, Ortho-McNeil) has been FDA approved for non-healing ulcers, and the clinical benefit of this treatment is modest [17-19]. In addition, post-marketing retrospective studies found increased cancer incidence from systemic treatment, resulting in a FDA black box warning [20], suggesting that new therapeutic approaches are needed. While the mixture of cytokines and growth factors in the wound bed is complex, all the information they convey is integrated by a set of transcription factors to elicit a transcriptional response that allows keratinocytes to migrate across the wound bed. Thus, understanding the regulation of the transcriptional network controlling re-epithelialization may uncover new therapeutic targets that can promote re-epithelialization and proper wound healing.

TRANSCRIPTIONAL PLASTICITY OF EPIDERMAL STEM CELLS IN WOUND HEALING

Sequence-specific transcription factors serve as integration hubs for various cellular signaling pathways, allowing for ordered changes in sets of transcripts required for different physiological functions. Transcription factors often work together as regulatory modules [21], and the regulatory regions for genes that contain binding sites for multiple different transcription factors are concentrated into small stretches of DNA with high information content [22]. During wound healing, there is coordinate regulation of target genes by multiple transcription factors, which changes over time [23-25] allowing for integration of external signals to generate the appropriate physiological output. The ability of many transcription factors to bind to these regulatory regions in different cell types [26], or in response to stimuli [27] is often determined by pre-existing genome-wide chromatin accessibility. Transcriptional networks are remarkably cell-type specific [22], and even in different cell types with similarly accessible chromatin, a subset of transcription factors have sequence specific binding discrimination [28].

Keratinocyte migration into the wound bed is the earliest event of re-epithelialization, and starts about 24 to 48 hours after injury [11]. The numerous paracrine factors released by immune cells present in the wound bed triggers profound phenotypic changes in keratinocytes. In particular, Epidermal Growth Factor (EGF), Tumor Necrosis Factor-α (TNF-α, Interleukin-6 (IL-6), and Transforming Growth Factor-β (TGF-β) are important to fine tune the balance between keratinocyte migration and proliferation [29] (Figure 1b). Recent work has revealed the presence of at least two distinct populations of keratinocytes around the wound site: cells that are proximal to the wound edge that do not proliferate and actively express pro-migratory genes, and cells distal to the wound edge which proliferate, therefore supplying a new pool of cells that can in turn migrate into the wound [23]. These cells can come from a variety of stem and progenitor cell populations found in the hair follicle (HF-SC) [24,30-34], the interfollicular epidermis (IFE) [24,34-36], and the upper isthmus [32]. Importantly, spatial confinement of the stem cell populations is lost during wound healing, allowing for their recruitment to the injury site and their

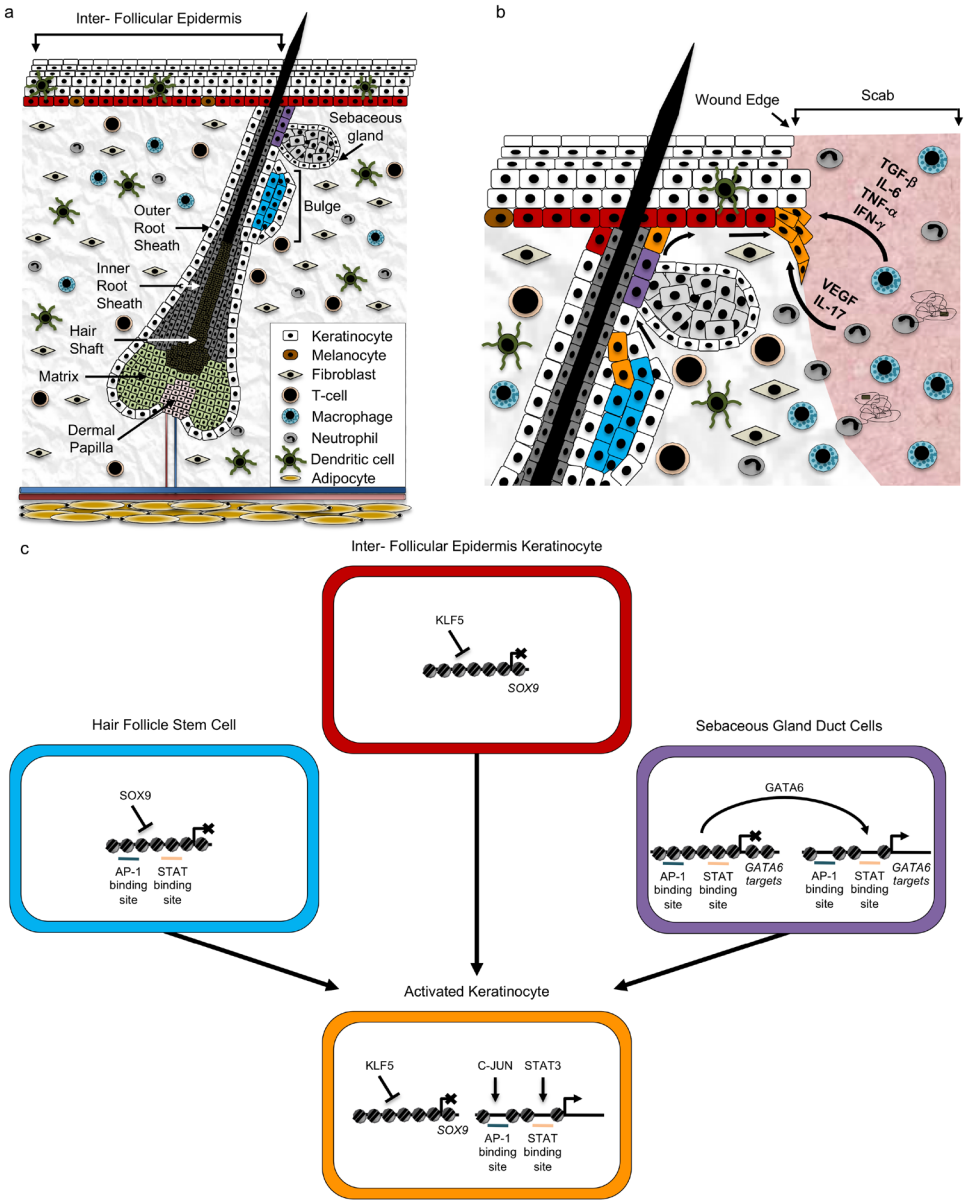

Figure 1. Changes in the skin microenvironment during cutaneous wound healing. (**a**) Diagram of major structures and cell types in the skin. Hair Follicle Stem Cells (HF-SC) are indicated in blue, Sebaceous Gland Duct Cells are indicated in purple, and basal Interfollicular Epidermis (IFE) cells are indicated in red. (**b**) Movement of key keratinocyte stem cell populations in response to skin wounding. Cytokines released from wound-infiltrating macrophages and neutrophils induce activation of keratinocytes from the Interfollicular Epidermis (red), Sebaceous Gland Ducts (purple), and Hair Follicles (blue). These populations acquire a common transcriptional program of activated wound keratinocytes (orange) and begin migration toward the wound edge. Direction of migration is indicated by black arrows. (**c**) Key transcriptional programs in keratinocyte stem cells converge on a common activated keratinocyte transcriptional program following wounding. In HF-SCs (blue), Sox9 facilitates maintenance of closed chromatin, blocking access to AP-1 and STAT binding sites and keeping key wound healing genes turned off. In contrast, IFE cells (Red) express the transcription factor KLF5, which represses SOX9 transcription. Although it is associated with Sebaceous Gland lineage commitment, GATA6 can also as a pioneer factor in Sebaceous Gland Duct cells (purple) to open up the chromatin around AP-1 and STAT binding sites. After wounding, all these cells express KLF5, which represses SOX9, and open up chromatin regions around AP-1 and STAT binding sites, to help initiate the wound repair process.

participation in epidermal regeneration [25] (Figure 1b). Genetic labeling experiments have shown that stem cell lineage plasticity is important for proper re-epithelialization [37]. When activated upon injury, HF-SCs transiently express the IFE-specific transcription factor KLF5, which suppresses the HF-SC transcription factor SOX9, establishing an IFE-like transcriptional state. This effect is mediated by the increased accessibility of binding sites for the transcription factors AP1 (JUN/FOS) and STAT3, which have increased expression around the wound [37]. This plasticity can also be seen in terminally differentiated cells, such as GATA6+ cells from the sebaceous gland duct, which can migrate into the wound bed and acquire IFE cell characteristics. In this case, GATA6 was proposed to act as a pioneer factor, opening up chromatin and revealing binding sites for AP1 and STAT transcription factors [38]. Thus, as stem cells exit their different niches, they converge on common transcriptional programs, which allows for the ordered repair of the epidermis (Figure 1c).

INFLAMMATORY SIGNALING DRIVES EARLY TRANSCRIPTIONAL RESPONSES

Inflammation is one of the earliest events in wound healing and is critical not only to prevent infections but also to trigger keratinocyte activation and re-epithelialization. Platelets and leukocytes present in the wound release numerous chemical signals, including IL-1α, IL-1β, IL-6, IL-8, TNF-α, PDGF, and TGF-β [39]. Besides their role as a feedback loop regulating immune cell infiltration, activity and inflammation, these cytokines have a profound impact on keratinocytes. TNF-α can activate NF-κB in keratinocytes (Figure 2), which increases the expression of genes involved in integrin signaling, cell adhesion, and motility (*ITGB6, ITGAV, ITGA5, NINJ1, RANBP9, NEF3, MMP9, MMP10, MMP13*) [40], while suppressing cell proliferation (reduced *CDC25, MCM3*) [41].

Cytokines and growth factors released into the early wound environment such as EGF and IL-6 can activate another key signaling pathway, the JAK-STAT pathway (Figure 2) [42,43]. Interestingly, increased accessibility of STAT binding sites in cells at the edge of the wound [37,38] is consistent with previous studies implicating STAT3 in wound healing. Keratinocyte-specific deletion of *Stat3* in mice causes a severely reduced rate of wound closure *in vivo*, and reduced mitogen-dependent migration *in vitro* [44], but has little effect on proliferation. STAT3 can also directly activate a variety of genes involved in migration and matrix remodeling, such as *THBS1*, *TIAM1*, *SERPINE2*, and *LOXL2* [45]. In addition, IL-6-mediated activation of STAT3 induces direct up-regulation of *Skint2/3* genes resulting in recruitment of dendritic epidermal T-cells [16], which can improve re-epithelialization in part by secreting Insulin Like Growth Factor 1 (IGF-1) and Keratinocyte Growth Factor (KGF) [46]. Increased STAT3 activity also serves to re-enforce early chromatin changes [37,38] and promote AP-1 dependent transcription by repressing *SOX9* and inducing transcription of *GATA6*, *FRA1*, *JUNB*, and *JUN* (Figure 1c) [45].

TRANSCRIPTIONAL CONTROL OF KERATINOCYTE MIGRATION

Re-epithelialization requires migration of keratinocytes, and there are many potential ways cells can move through tissues. One of the most studied mechanisms leading to individual cell migration is Epithelial-to-Mesenchymal Transition (EMT), in which polarized epithelial cells lose their adhesion to basement membranes and to each other and acquire mesenchymal cell properties. On the opposite end of the spectrum is collective cell migration, in which cells maintain most of their epithelial identity genes and move as a tightly connected sheet of cells [47,48]. While many studies have worked to elucidate what controls the EMT switch, there is an emerging appreciation that EMT is not an "either/or" process, but is a continuum [29,47-49].

One of the major pathways regulating migration in wound healing is the TGF-β pathway (Figure 2). When bound to ligands, TGF-β superfamily receptors phosphorylate and activate receptor-regulated SMAD proteins (SMAD2, SMAD3, SMAD5, SMAD8), which in turn bind and activate SMAD4. The heterodimer then translocates to the nucleus where it regulates the expression of genes involved in cell motility and EMT. This pathway is also negatively regulated by SMAD7, which inhibits TGF-β induced signaling through receptor-regulated SMAD protein degradation or inhibitory interaction with TGF-β receptors [50].

The role of the TGF-β pathway in wound repair has been somewhat confusing, likely due to differing effects of TGF-β signaling on the various cell populations in the wound area. Transgenic mice overexpressing the Tgf-β1 ligand under control of a *Keratin 14* promoter display a greater rate of re-epithelialization in partial thickness ear wounds, where only the epidermis is injured [51]. In contrast, full thickness back skin wounds had slower re-epithelialization [51], consistent with independent results seen with burn-induced skin wounds [52]. One potential explanation is that TGF-β1 increases keratinocyte migration, providing an advantage in partial thickness wounds, but impairs inflammation and dermal repair. This is supported by data from *Smad3*$^{-/-}$ keratinocytes, which have reduced migration in response to Tgf-β1. Interestingly, full thickness wounds in *Smad3*$^{-/-}$ mice actually heal fast-

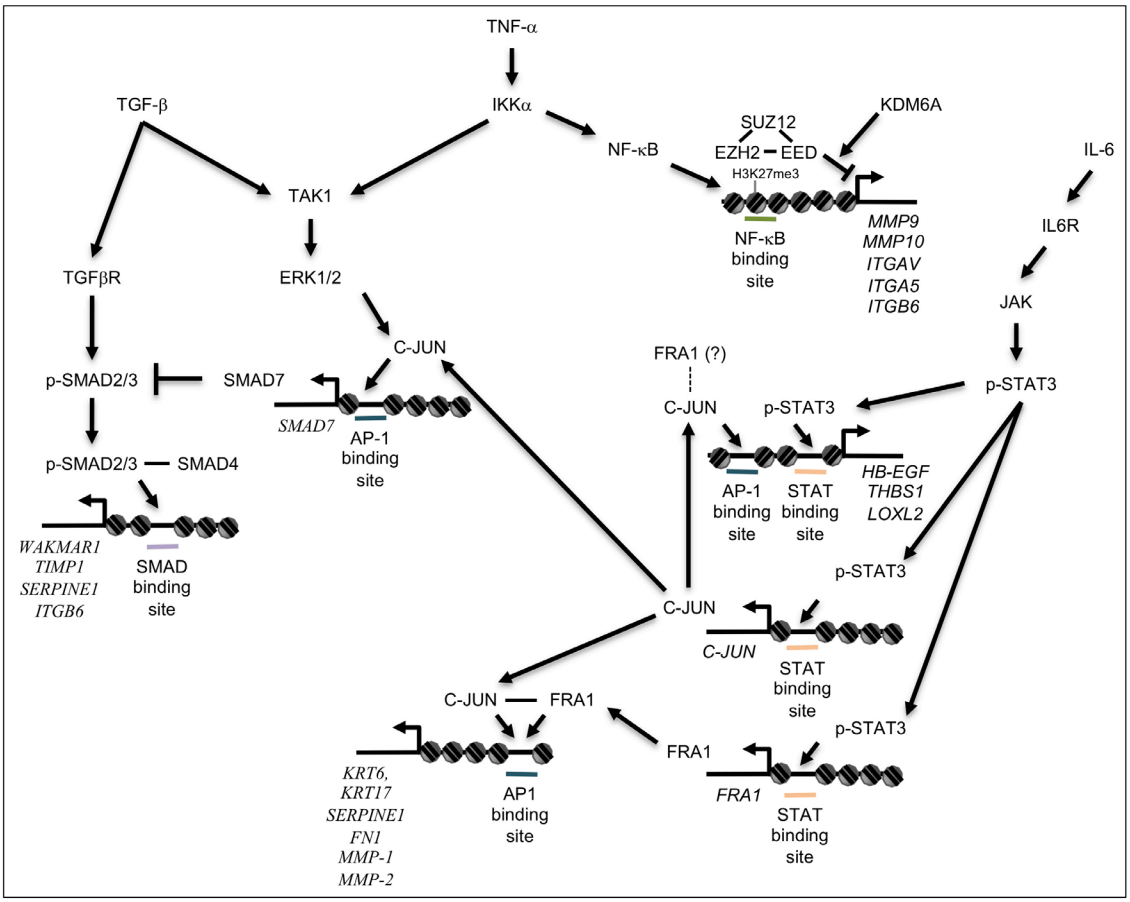

Figure 2. **Transcriptional network regulating initiation of re-epithelialization**. Schematic diagram of signaling pathways activated by cytokines and growth factors present in the wound bed, leading to initiation of re-epithelialization. TGF-β, TNF-α, and IL-6 released into the wound environment activate interconnected pathways, which collaborate with AP-1 transcription factors expressed in wound edge keratinocytes to activate transcription of target genes (Italics) required for matrix re-modeling and initiation of keratinocyte migration.

er than in *Smad3*$^{+/+}$ mice, an effect attributed to reduced monocyte infiltration in granulation tissue [53].

The TGF-β pathway inhibitor Smad7 also plays a role in cutaneous wound healing, where expression gradually increases and peaks approximately halfway through the healing process before returning to baseline levels [54]. Smad7 expression is stimulated by pro-inflammatory cytokines, such as IL-1, Interferon γ (IFN γ) and TNF-α which are present in the wound environment [55], and is transcriptionally activated by AP-1 transcription factors [56]. Interestingly, transforming growth factor-β-activated kinase 1 (TAK1) stimulates NF-κB activity [57], which can directly activate expression of SMAD7, resulting in negative feedback and attenuation of TGF-β pathway signaling [58]. Overexpression of Smad7 in Keratin 14-expressing cells accelerated wound healing and was associated with increasing proliferation and migration. Intriguingly, migrating cells with Smad7 overexpression also expressed Keratin 14 and had activation of Erk signaling, and increased migration was dependent on MAPK pathway activation [54].

While activation of the TGF-β pathway can induce EMT in some situations, such as cancer metastasis, the role of EMT in wound healing is less clear [59]. In epidermal wounds, leading edge cells appear to maintain the epithelial markers Keratin 5 and 14 during wound closure [23,60,61], and recent data suggesting an extending shield re-epithelialization mechanism [61] supports a role for collective migration. TGF-β has recently been shown to promote collective cell migration in cells, which is correlated with high activity of Erk1/2 [62]. Importantly, Erk1/2 activity is required for early migratory events induced by TGF-β1 [63,64], and potentiates TGF-β pathway signaling by enhancing Smad2-dependent transcriptional activity [65]. In addition, transcription factors downstream of Erk1/2 such as JunB [66] can modulate

the transcriptional effects of TGF-β1. These data highlight the importance of signal integration in modifying the TGF-β pathway response to promote a collective migration phenotype during cutaneous wound healing.

AP-1 FAMILY TRANSCRIPTION FACTORS IN EARLY RE-EPITHELIZATION

A key step in re-epithelialization is the activation of AP-1 family transcription factors, some of which (c-Jun, JunB, JunD, and c-Fos) are induced around 8 hours after wounding in the leading edge of migrating keratinocytes [67]. Jun family transcription factors (c-Jun, JunB, JunD) can form both heterodimers and homodimers, while Fos family proteins (Fos, FosB, Fra-1, and Fra-2) form heterodimers with Jun family proteins [68]. While this mix-and-match feature of AP-1 transcription factors has presented challenges in dissecting the role of individual Jun and Fos family proteins, knock-out experiments have clearly demonstrated roles for c-Jun and JunB in wound healing. Mice lacking *c-Jun* in either Keratin 14 [69] or Keratin 5 [70] expressing cells show a reduced basal proliferative rate in the skin, and severely reduced proliferation *in vitro,* which was attributed to decreased Epidermal Growth Factor (EGFR) signaling due to reduced expression of EGFR ligands such as HB-EGF. Interestingly, while there was slightly reduced wound healing with *c-Jun* loss in Keratin 14 expressing cells [69], this was not seen with *c-Jun* loss in Keratin 5-expressing cells [70]. It is important to note that this discrepancy may be due to the fact that the reported effect on wound healing was modest [69], and unlike human skin, wound healing in mice occurs largely by contraction, which may obscure effects on migration if murine models do not employ splinting [71,72]. Another potential confounding factor in these genetic experiments is that c-Jun expression is bi-phasic in the leading edge, which has little c-Jun protein at early timepoints (before 8 hours), and increases later during migration [67], while expression remains unchanged away from the leading edge. This is consistent with the opening of chromatin around AP-1 binding sites at early timepoints, allowing for later AP-1 regulated transcription [37,38]. *In vitro* experiments show more support for the role of c-Jun in migration, which is significantly reduced following scratch assay with loss of *c-Jun*, in conjunction with reduced EGF-dependent phosphorylation of Focal Adhesion Kinase (FAK) [69]. Loss of *c-Jun* causes an increase in stable focal contacts [73], consistent with the role of FAK in promoting turnover of focal adhesions. In addition, c-Jun directly activates transcription of *Src*, which along with FAK, also promotes turnover of focal adhesions and promotes migration [73]. These data point to a key role for c-Jun in promoting migration early in the process of re-epithelialization.

JunB has also been shown to contribute to epidermal wound healing using mice lacking *JunB* in keratinocytes and fibroblasts [74]. Mice lacking *JunB* in the skin demonstrate reduced keratinocyte migration as well as epidermal hyper proliferation, sustained inflammation and increased granulation tissue after wounding [74]. Interestingly, paracrine signaling by $JunB^{-/-}$ fibroblasts may cause some of these phenotypes, such as epidermal hyperproliferation [75]. In addition, these differential effects of *JunB* loss compared to *c-Jun* loss may be simply due to expression patterns [67,76], as genetic knock-in of JunB to the *c-Jun* locus can rescue many of the phenotypes seen in $c\text{-}Jun^{-/-}$ mice [77].

Beyond c-Jun and JunB, data on the functions of AP-1 family members in normal keratinocytes during wound healing is generally lacking. While it is likely that Fos family members form heterodimers with Jun family proteins during wound healing, this is yet to be rigorously tested *in vivo*. Intriguingly, Fra1 is activated by JAK-STAT signaling [37,38,45], and it was shown to Fra1 can activate transcription of the pro-migratory genes *KRT6, KRT17*, *FN1, SERPINE1, MMP-1, MMP-2,* and *MMP-12*, when co-expressed with c-Jun. Fra1 overexpression can also accelerate migration of immortalized HaCAT keratinocytes in scratch assays [78], making this an important transcription factor for further study in the context of re-epithelialization.

THE HYPOXIC RESPONSE TO TISSUE INJURY

Tissue injury causes vascular disruption and vasoconstriction, and coupled with high oxygen consumption by cells, the resulting wound environment becomes hypoxic [79]. The Hypoxia-Inducible Factor (HIF) transcription factors play a central role in the adaptation to low oxygen levels. In normoxic conditions, HIF proteins are hydroxylated by the HIF Prolyl-hydroxylase EGLN1, targeting it to the pVHL (Von Hippel-Lindau tumor suppressor protein) E3 ubiquitin ligase, ultimately leading to its degradation via the proteasome [80-82]. In hypoxic condition, EGLN1 is inhibited resulting in HIF protein accumulation and activation of its transcriptional program [83]. In cutaneous wounds, HIF1α protein is increased at the wound margin, as well as in the leading edge of migrating keratinocytes *in vitro* [84]. This is consistent with observations that hypoxia causes an increase in keratinocyte migration [85]. Keratinocyte specific deletion of *Hif1α* significantly impairs closure of *in vivo* punch wounds, an effect primarily attributed to a lack of migration, as proliferation, apoptosis, and inflammation are similar to controls. In addition, knock-down of *HIF1α* reduced migration of keratinocytes *in vitro* as assessed by scratch assays [84,86]. This phenotype is at least in

part due to activation of *SERPINE1, LAMA3*, *LAMB3*, *LAMC2*, *ITGA6*, and *ITGB1* by HIF1α, which promote migration [84,86-89]. In addition, secreted factors such as *VEGFA*, which is activated by HIF1α, are likely to contribute to proper wound healing by promoting angiogenesis [90]. Further support for a key role for HIF1α in promoting wound healing comes from the finding that mice lacking *Egln1* in the skin, resulting in stabilized Hif1α, have accelerated *in vivo* wound healing and *in vitro* migration of keratinocytes [91]. The HIF2α protein is regulated by oxygen in a similar manner to HIF1α, but has non-redundant functions in wound healing [84-86]. Interestingly, loss of *Hif2*α in keratinocytes results in accelerated wound healing [85], and combined *Hif2α/Vhl* loss leading to stabilized HIF1α also have accelerated wound healing, consistent with previous data suggesting opposing roles for HIF1α and HIF2α [92].

EPIGENETIC CONTROL OF RE-EPITHELIALIZATION

Transcription factors do not act in isolation, but form complexes with histone-modifying enzymes, which facilitate changes in chromatin accessibility to mediate activation or repression of transcriptional programs. It is now widely appreciated that histone proteins that serve as a scaffold for DNA are not passive bystanders in gene regulation, but active players [93-95]. The nucleosome core consists of a dimer of the tetrameric unit containing histones H2A, H2B, H3, and H4 that directly interacts with 146bp of DNA that is wrapped around it [96]. The post-translational modification of histone proteins on their exposed tails facilitates the opening or compaction of the chromatin allowing for the activation or repression of transcription. The modification of histones by acetylation, methylation, and ubiquitylation has been well studied, giving insight into the chromatin structures associated with different transcriptional states [95,97]. In general, acetylation of Histone H3 at lysines 9 and 27, as well as methylation at lysines 4, 36, and 79 have been strongly associated with genes that are active. Conversely, tri-methylation of Histone H3 at lysines 9 and 27, mono-methylation of Histone H4 at lysine 20, and monoubiquitylation of H2A lysine 119 are all associated with the repressive heterochromatin state [98-102]. Interestingly, a subset of genes that are not actively transcribed sit in a "poised" state and exhibit both active ($H3K4me^3$) and repressive ($H3K27me^3$) marks, allowing for developmentally appropriate activation during lineage specification and differentiation [103,104].

While a variety of chromatin modifiers have been shown to regulate physiological processes important for wound healing, such as proliferation, migration, and differentiation [103,105-107], relatively little is known about epigenetic regulation in the specific context of re-epithelialization. The regulation of $H3K27me^3$ is the most well studied and is controlled by the dynamic balance between the PRC2 complex and KDM6A/KDM6B (Figure 2). The core Polycomb Repressive Complex 2 (PRC2) subunits EZH2, EED, and SUZ12 have significantly reduced protein expression in the leading edge of migrating keratinocytes, correlating with reduced $H3K27me^3$. Functionally, EZH2 has been found to repress the *Ink4a/Arf* locus [108], and upregulation of $p16^{Ink4a}$ is associated with increased migration [109]. Somewhat paradoxically, loss of EZH1 and EZH2 results in delayed closure of split-thickness wounds, although single knockouts were not examined [108]. This might be explained by the differential expression of EZH2 in leading edge cells, where expression is reduced, and the proliferative hub cells, which still express EZH2 [110]. Alternatively, loss of EZH2 may cause a shift in lineage or increase differentiation, similar to what is seen in during pre-natal epidermal development [111].

The $H3K27me^3$ histone demethylase KDM6B is a crucial NFκB coactivator in keratinocytes. Its expression is increased in the wound edge where it helps activate the transcription of matrix metallo-proteinases, which promote matrix remodeling and help keratinocytes carve their way through the wound bed [112]. In addition, the KDM6B-NFκB axis is necessary for the expression of various cytokines by keratinocytes, including IL-6 and TNF-α, which may promote a positive feedback loop and install a molecular crosstalk between keratinocytes and other cell types within the wound. In contrast to PRC2 proteins, KDM6A/B expression is increased early in wounding, and then reduced at late stages [110,113]. Loss of KDM6B has been shown to reduce migration and is required for optimal induction of NF-kB target genes (*IL-1β, IL-6, IL-18, TNF-α, MMP-3, MMP-9, MMP-14, HB-EGF*) [112]. KDM6B also activates *NOTCH1* transcription and induction of NOTCH1 target genes (*RHOU* and *PLAU*) promoting loss of focal adhesion and migration [113]. PRC2-mediated repression is also countered by the activity of the Histone Methyltransferase ASH1L, which methylates H3K36 in the bodies of actively transcribed genes [100]. ASH1L inactivation leads to increased proliferation and delayed re-epithelialization during wounding [114], but the mechanism remains unclear.

Methylation is not restricted to histone proteins, and indeed a major epigenetic regulator of gene expression is DNA methylation. Early during embryogenesis, *de novo* methylation of DNA is facilitated by DNA methyltransferases DNMT3A and DNMT3B [115]. After establishment of methylation patterns, DNMT1 functions to maintain DNA methylation patterns following replication by binding to hemi-methylated CpG regions, and adding a methyl group to the newly synthesized strand [116]. DNA

methylation at promoter CpG islands can promote the formation of heterochromatin and transcriptional repression [117]. DNA methylation facilitated by DNMT proteins is opposed by the activity of the Tet Methylcytosine Dioxygenase protein family (TET1, TET2, and TET3), which convert 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC), then 5-formylcytosine (5fC), and finally 5-carboxylcytosine (5caC), ultimately leading to the removal of cytosine methylation [118]. Interestingly, many transcription factors implicated in wound healing (JUN, JUNB, E2F1, E2F4, ETS2, MYC) have altered affinity when the CpG regions of their binding sites are methylated [119-121], suggesting that DNA methylation changes are important for the wound healing response. Somewhat paradoxically, both proliferation and migration were reduced following DNMT1 knockdown, but it is unclear if the effects on migration are due to specific regulation of migration, or are secondary effects of reduced cell division in the proliferative hub causing a lack of new cells to fill in behind the leading edge cells as they migrate [122]. Further evidence for the potential role of DNA methylation in regulating wound healing comes from a recent study on the skin-specific long non-coding RNA (lncRNA) WAKMAR1, which is expressed in leading edge keratinocytes and promotes migration [123]. LncRNAs can regulate transcription in many ways, such as physically interacting with chromatin-modifying complexes like PRC2 [124]. In human keratinocytes, WAKMAR1 was found to bind to DNMT1, and block inactivating methylation of the E2F promoter [123]. While this seems to contradict the results seen with DNMT1 knock-down [122], lncRNAs have been proposed to target DNMT1 to specific loci [125], and DNMT1 may have gene-specific effects during wound healing. It is also possible that DNMT1 is required only in the proliferative hub, and suppression of DNMT1 activity is required for migration in leading edge cells, which do not proliferate. Given that WAKMAR1 is the first lncRNA shown to be involved in the regulation of wound healing, it is likely that future studies will uncover a much more robust network of non-coding RNAs that play significant roles in transcriptional control during re-epithelialization.

CONCLUSIONS AND OUTLOOK

A major challenge in understanding transcriptional regulation in wound healing remains the complex spatial and temporal changes that occur during the re-epithelialization. Key cell populations, such as the leading-edge cells that interface directly with the wound, are too small to capture using bulk population analysis, leaving the spatio-temporal dynamics of transcription unclear. Recently however, advances in single-cell analysis including RNA profiling (scRNA-seq) [126], DNA binding protein mapping (ChIP-seq) [127,128], methylation profiling (snmC-seq2) [129], chromatin accessibility (scATAC-seq) [130-132], and spatial positioning [133] along with new computation methods of integrating this complex data [134,135] have opened up new ways to explore the different transcriptional states of individual cells during the wound healing process. A key feature of these emerging technologies is the ability to integrate large-scale information from many unique cell populations that change over time. This will allow for the development of a high-resolution map of the movement and transcriptional changes of all cells involved in the repair of wounds and facilitate dissection of the functional interplay between the many cell types that contribute to wound healing. Indeed, these approaches are already being applied to wound healing model systems to examine the differential contributions of hair follicle bulge stem cells (Lgr5+) and Interfollicular Epidermis (IFE) cells (Lgr6+) stem cells. Lineage tracing experiments combined with scRNA-seq identified at least eight distinct cell states associated with unique transcriptional profiles during the course of wound regeneration, which were previously unappreciated. Interestingly, Lgr5+ cells converge on an IFE-like state [24], suggesting that different stem cell populations converge on a common transcriptional program that is necessary with proper repair. This is consistent with data suggesting lineage plasticity in hair follicle stem cells during wound healing, where transient co-expression of the transcription factors Sox9, Klf5, Tcf3, and AP2γ occurs in early phases of repair, then become lineage-restricted again after completion of repair [37]. Thus, single-cell analysis of transcriptional changes in $Lgr5^+$ and $Lgr6^+$ cells has both confirmed previous studies and identified new transcriptional states in cells that contribute to the re-epithelialization process.

A thorough understanding of the changes that occur in each cell during the wound healing process is essential for determining the key events that initiate re-epithelialization and identifying how these events are altered in chronic wounding. Currently, most wound healing studies assess the essential functions of genes in wound healing employing whole-body knock-outs or more restricted tissue specific inducible alleles [25]. The approach of using germline knock-out mice has the major drawback of affecting multiple different cell types, some of which may have opposing functions, thus obscuring major effects. Another confounding factor in many of these studies is that the proliferative hub cells have a very different function than migrating cells, yet both populations are often targeted in knockout mice, even when using tissue restricted Cre transgenes. The ability to analyze experiments at the single cell level may allow for dissection of the effects of gene disruption on these different populations. In addition, a more refined understanding of

gene expression could allow for more precise targeting to cells of interest. Moving forward, these new technologies are likely to dramatically improve our understanding of the mechanisms of transcriptional control regulating the many complex processes that occur in multiple cell types that contribute to proper cutaneous wound healing.

The ageing population and increased occurrence of metabolic diseases worldwide will fuel a continuous rise of the chronic wound epidemic, and finding better therapies is urgent. Current therapies include surgical or enzymatic debridement of necrotic tissue, specific dressings aimed at preventing infections while maintaining adequate moist levels, autologous skin grafts, extracellular matrix protein, and growth factors treatment such as PDGF [11,19]. While these treatment advances have made meaningful differences in the lives of patients, therapies that effectively correct the underlying causes of non-healing and chronic wounds remain elusive. Transcription factor complexes integrate the myriad signals emanating from the wound to control proper re-epithelialization, and as such represent promising therapeutic targets. By dissecting the mechanisms of transcriptional regulation underlying the normal wound healing process, a more complete picture of pathologic wound healing can emerge. Importantly, drugs inhibiting epigenetic regulators, such as EZH2, have been developed [136], and given the reductions in EZH2 activity in leading edge cells [110], inhibition of EZH2 activity could potentially induce keratinocyte migration and re-epithelialization. The targeting of the enzymatic activity of transcriptional complexes in pathological states such as cancer shows great promise [137,138] and such approaches could offer new ways to more precisely promote the process of re-epithelialization in normal and pathological wound healing.

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