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Cellular and molecular facets of keratinocyte reepithelization during wound healing

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Abstract

Cutaneous wound healing is a highly coordinated physiological process that rapidly and efficiently restores skin integrity. Reepithelization is a crucial step during wound healing, which involves migration and proliferation of keratinocytes to cover the denuded dermal surface. Recent advances in wound biology clarified the molecular pathways governing keratinocyte reepithelization at wound sites. These new findings point towards novel therapeutic targets and provide suitable methods to promote faster tissue regeneration in vivo.

Keywords: Keratinocyte; Wound healing; Reepithelization

Skin wound healing: an overview

Skin wound healing represents a dynamic and well-ordered biological process [1,2]. Tissue injury causes vascular vessels disruption and extravasation. A temporary fibrin clot reestablish homeostasis and provides a transient substrate for platelets that secrete growth factors (GFs), cytokines and extracellular matrices (ECM). These mediators of the inflammation response recruits macrophages and neutrophils that secrete a battery of specific factors, orchestrating the following phase of tissue reepithelization [3]. Reepithelization is the resurfacing of a wound with new epithelium and consists of both migration and proliferation of keratinocytes at the periphery of the wound.

Migrating keratinocytes undergo subcellular modifications including (i) disassembly of hemidesmosomal links between epidermis and BM; (ii) retraction of intracellular tonofilaments and keratin filaments; (iii) dissolution of most desmosomes; (iv) formation of peripheral cytoplasmic actin filaments (lamellipodia) and focal contacts [4].

As epidermal migration moves on, keratinocytes at the wound margin begin to proliferate behind the actively migrating cells. The resulting dense hyperproliferative epithelium feeds the migrating sheets at the wound margins [5]. These events are regulated by the crosstalk of three main molecular actors: GFs, integrins and metalloproteases (MMPs) (Fig. 1).

Activation of integrin receptors by migrating keratinocytes allows the interaction with a variety of ECM proteins interspersed in the provisional wound bed and at the wound margin. Moreover, expression and activation of MMPs promotes degradation and modification of extracellular matrix proteins at the wound site, facilitating cell migration [6]. Then, BM proteins reappear in a very ordered sequence from the margin of the wound inward, in a zipper-like fashion. Epidermal cells revert to their normal phenotype, once again firmly attached to the reestablished BM and underlying dermis.

Here we report the molecular and cellular mechanisms that regulate keratinocyte reepithelization at wound sites. Genes regulating keratinocyte migration and proliferation during reepithelization, orchestrate growth factor receptors,

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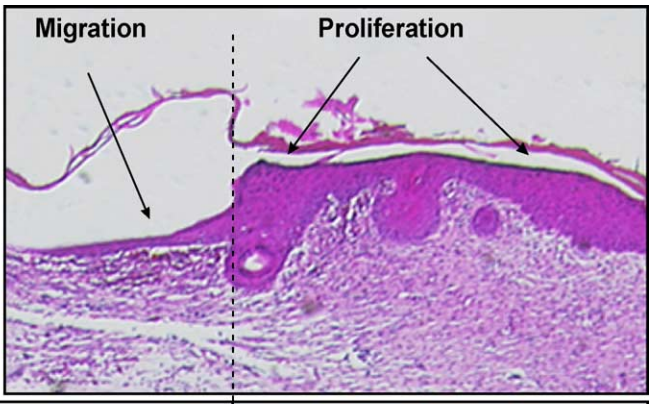

	Reepithelization (migration)	Reepithelization (proliferation)
Growth factors	MSP, TGFb1	EGFs, TGFa, KGF, GM-CSF
Integrins	a3b1, a6b4, avb6	avb3/b5, b4, b1
ECM	Laminin-5	Laminins and collagens
MMPs	MMP1, 2, 9, 10	MMP3

Fig. 1. GFs, ECM, integrins and MMPs involved in the migration and proliferation of skin keratinocytes at wound sites. Hematoxylin–eosin staining of mouse skin section during the reepithelization process (see text for details).

integrins, extracellular matrices (ECMs), and matrix metalloproteases (MMPs) in the cooperative effort of wound healing and skin regeneration.

Molecular and cellular events during keratinocyte migration at wound sites

In normal skin, keratinocytes are assembled in a quiescent epidermal tissue, tied up by cell junctions, which include desmosomes at cell–cell contacts and hemidesmosomes (HDs) at cell–substrate contacts (Fig. 1). Desmosomes are one of the most abundant types of cell–cell junctions in epidermis [7]. In keratinocytes of confluent cell sheets (resting phenotype), desmosomes are Ca^{2+}-independent, by contrast to those of subconfluent epidermal cells (motile phenotype) that become Ca^{2+}-dependent [8,9]. PKCα plays a key role in the rapid conversion from Ca^{2+}-independent to Ca^{2+}-dependent desmosomes, and is required to allow cell–cell dissociation, as part of the signaling pathway modulating desmosomal adhesion in response to wounding [10].

HDs are specialized cell–ECM junctions stabilizing adhesion of epithelial cells to the underlying BM. HD nucleation is triggered by binding of α6β4 integrin to laminin-5 and by following organization of a multiprotein intracellular complex [11]. The critical role for HDs stems from its ability to assemble stable and rigid structures at the basal cell surface, by linking the intermediate keratin filament cytoskeleton with the BM [12]. The importance of HDs for epithelium integrity is highlighted by the devastating blistering skin disease junctional epidermolysis bullosa (JEB), linked to mutations in the genes encoding the protein component of the HD [13–15].

Integrins and extracellular matrices (ECM)

To heal an injured skin epithelium, cells undergo HD dissociation, to release the contacts with BM and cell–substrate contacts reorganization, to allow migration. Critical for modulating HD and focal contacts are integrins, α–β heterodimeric transmembrane cell surface receptors, which bind ECM proteins via the extracellular portion and interact with cytoskeletal structures via the intracellular regions [16]. Eighteen α and eight β chains are encoded by different genes and each α–β array defines its own binding specificity and signaling properties [17,18]. To date, at least 24 αβ different heterodimers, composed by 18 different α and 8 different β subunits, are known [16]. Many integrins have overlapping ligand-binding functions and certain cell types can express more than one of the integrins, with the same ligand-binding specificity, although their signaling function appear to be different (e.g., keratinocytes can express at least three different fibronectin receptors, αvβ1, αvβ6 and α5β1). In addition, functional diversity may result from alternative splicing isoforms of α and β subunits.

Interaction of integrins with their ECM ligands leads to receptor clustering and recruitment of multiple intracellular signaling proteins. Interaction with p130Cas, paxillin, vinculin, “GTP exchanger” factors and protein kinases, such as focal adhesion kinase (FAK) or integrin-linked kinase (ILK) elicits “outside–in” signals that promote lamellipodia, filopodia and actin fibers formation. These

process have a direct impact on wound healing, since migrating keratinocytes extend long filopodia/lammelipodia-like cytoplasmic projections in the wound area and use actin filament to pull wound edge together [19]. On the other hand, the extracellular binding activity of integrins can be regulated by "inside–out" signaling [20]. In wound healing, the intact BM is degraded and migrating keratinocytes use a provisional matrix in the wound bed, which is rich in fibrin, fibronectin, vitronectin and laminin-5. During reepithelization, keratinocytes makes also contact with dermal collagens [21].

A mandatory step before keratinocyte migration over the provisional matrix is HDs disassembly. Integrins detach from laminin-5 via "inside–out" signaling, releasing basal localization and connection to the keratin cytoskeleton. Then, they relocate to F-actin-rich structures, such as lamellipodia, at the leading edge of migrating keratinocytes [22]. This mobilization is mediated by PKCα-mediated serine/threonine phosphorylation of the intracellular portion of β4. This phosphorylation induces the binding of 14-3-3 proteins to the connecting sequence (CS) of the β4 that contains putative phosphorylation sites, possibly important for the biology of α6β4 [23,24]. Several reports show that phosphorylation of serine/threonine and tyrosine residues in the β4 CS are critical for HD regulation and disassembly [24,25]. MSP (Macrophage-Stimulating Protein) and PMA (Phorbol Myristate Acetate) can modulate PKCα-mediated α6β4 phosphorylation and HD disassembly, therefore starting keratinocyte migration [24,26]. It has been suggested that also EGF (Epidermal Growth Factor)-dependent tyrosine phosphorylation of the β4 cytoplasmic domain may be a mechanism for HD disassembly. However, mutation of the major tyrosine phosphorylation sites provides only a partial protection from EGF-induced HD disassembly, suggesting the involvement of other concomitant mechanisms [25,27].

Migrating keratinocytes express several ECM receptors at wound sites, such as the α5β1 fibronectin-binding integrin, α3β1 and α6β4 integrins both binding laminin-5, the α2β1 collagen receptor, the αvβ1 fibronectin receptor, the αvβ5 and αvβ6 vitronectin-binding integrin and the α9β1 tenascin C receptor [19,28].

Keratinocyte migration

Laminins are large heterotrimeric molecules, composing the lamina lucida of BM. In particular, laminin-5 is synthesized, within hours after wounds, by skin keratinocytes as a precursor heterotrimeric protein (α3,β3,γ2) that undergoes processing of α3 and γ2 subunits after being secreted [29]. Consistently, while laminin-5 is differentially expressed during human wound healing, the precursor laminin-5 is detected in the wound site, but not in the flanking homeostatic tissue [30]. Moreover, laminin-5 expression is sustained by TGF-β and INFγ present in the wound fluid [31].

Current models of wound reepithelization propose that different cleavage of this type of ECM affect the preference for interactions with the two laminin-5 integrin receptors, α3β1 and α6β4. The precursor form of laminin-5 has a major binding affinity for α3β1, while the processed form associate preferentially to α6β4. This is the primary adhesive integrin of the adult epidermal basement membrane (BM) and supports cell adhesion by forming HDs after laminin-5 binding [11]. On the other hand, α3β1 is located in focal contacts and regulates cell spreading and migration [32]. The complementary roles for α6β4 and α3β1 in keratinocyte reepithelization can be explained through the control that α6β4 exerts on α3β1 integrin [23,30]. The interference by α6β4 integrin on α3β1-mediated migration on laminin-5 has been recently described in HaCat keratinocytes [33]. This "negative" control by one integrin on another has been observed also in vivo and is known as "transdominant" inhibition [34,35]. When keratinocyte start migrating, a switch from α6β4 to α3β1 integrin for laminin-5 binding occurs at wound site and, in parallel to α6β4 phosphorylation (see above), this promotes HD disassembly and relocation of the α6β4 integrin from HDs to lamellipodia. Concomitantly, α3β1 becomes "unlocked" and move to focal contacts regulating actin-myosin-driven processes required for cell spreading and migration (Fig. 2). Accordingly, laminin-5 expression are responsible for a shift in integrin dominance, hence modulating keratinocyte migration during skin reepithelization [30] and the interplay among α3β1, α6β4 and different laminin-5 isoforms is crucial to lead keratinocytes for recruiting α3β1 at focal contacts and mediating migration over the provisional matrix, but also to reform HDs component at later stages of wound healing [30,31,36,37]. The switch from α6β4 to α3β1 in migrating keratinocytes does not complete the overall mechanism. Deposition of precursor laminin-5 is necessary but not sufficient to stimulate motility via α3β1 integrin and wound closure. Other soluble factors, mainly growth factors, are possibly present at wound sites to promote full keratinocyte reepithelization.

While the wounding process goes on, integrin expression is up-regulated by keratinocytes, which also start to express novel integrins, including αvβ6 and αvβ5. A role for the fibronectin-binding αvβ6 integrin in tissue repair and remodeling has been suggested, but a clear function of αvβ6-integrin in wound healing is debated. Increased αvβ6 expression can be observed in wound keratinocytes, while in cultured cells αvβ6-integrin facilitates cell adhesion and migration on fibronectin, vitronectin and tenascin, which are components of the early wound provisional matrix [28,38]. Early expression of αvβ6 integrin during keratinocyte migration into the wound site suggests that αvβ6 integrin may regulate this process also in vivo [2]. However, β6 integrin-deficient mice showed no changes in wound closure rate [39]. Although αvβ6 integrin is expressed by migrating keratinocytes in early wounds, the maximal expression coincides with the formation of basement

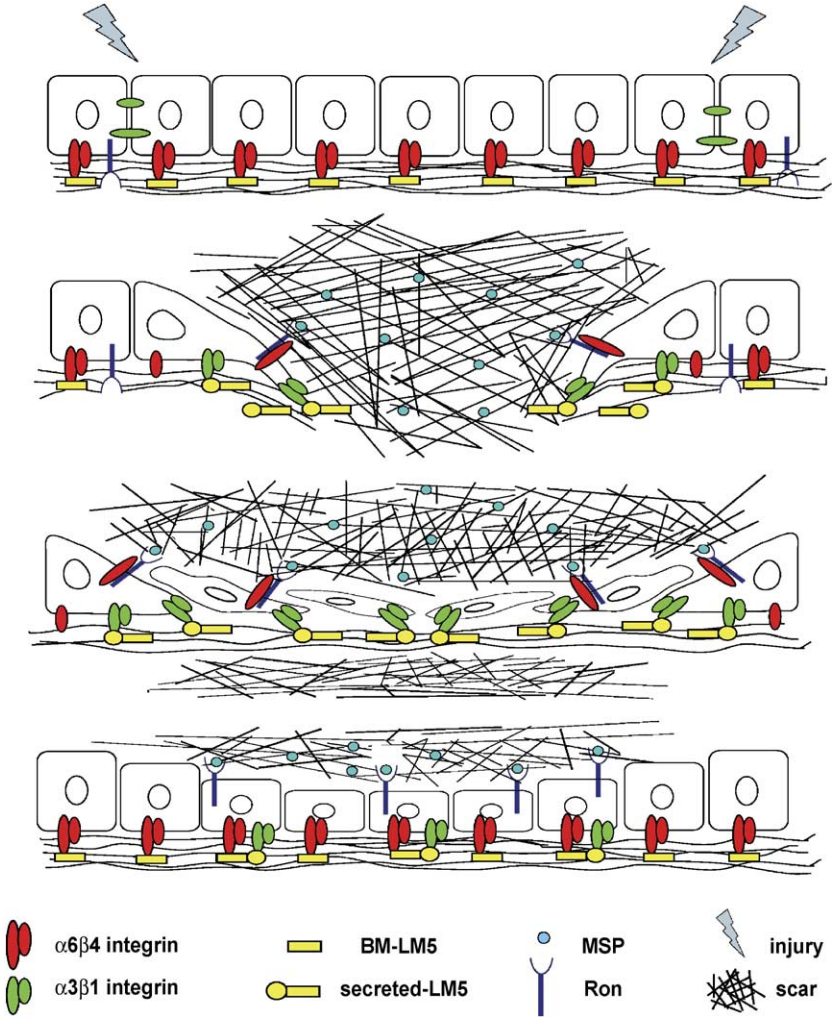

Fig. 2. Schematic representation of the events occurring at molecular level in skin keratinocytes after wounding. (1) Deposition of precursor laminin-5 in the provisional BM of wounds by leading keratinocytes. (2) Switch of integrin dominance for laminin-5, participation of α2β1, α3β1 and deposition of laminin-5 during migration on collagen. (3) Regulation of adhesion-dependent cell signalling on collagen through deposition of laminin-5 by leading cells. (4) Promotion of gap junction intercellular communication (GJIC) in following cells by laminin-5. (5) The need for laminin-5 for assembly of HDs in quiescent cells.

membrane and granulation tissue, when the migrating edges of the wound epithelium have joined. The temporal localization of αvβ6 integrin in regenerating epithelium suggests that αvβ6 integrin may also be involved in epithelial cell differentiation, regeneration of basement membrane, regulation of inflammatory reaction and formation of granulation tissue. Interestingly, heterologous expression of αvβ6 in oral keratinocytes led to enhanced MMP-9 secretion, functional in mediating cell migration [40]. The integrin β5 subunit is known to pair up only with the integrin αv subunit, forming the vitronectin receptor αvβ5 [41]. Expression of the integrin β5 subunit has been investigated in cutaneous wound healing and in incisional wounds. In suction blisters increased immunoreactivity for β5 complex has been detected in suprabasal cell layers of the regenerating epidermis [42]. However, as for β6, in β5-deficient mice, the healing rate of cutaneous wounds is not altered, although keratinocytes harvested from these mice demonstrate impaired migration on and adhesion to vitronectin [39]. The expression of the integrin αvβ5 subunit has been extensively studied in human cutaneous wound healing. Increased immunoreactivity for the αvβ5 complex has been detected predominantly in basal keratinocytes during excisional wound healing in human skin [2].

Integrins can also associate with several transmembrane proteins, such as IAPs (integrin-associated proteins), tetraspan/TM4SF proteins, cell surface proteoglycans and syndecans [43,44]. All of these proteins may critically regulate integrin functions especially during keratinocyte migration. The IAP protein CD98 through the binding to the α3β1 integrin can regulate its function [45]. The tetraspannin proteins CD151 form a stable complex with α3β1 integrin, which is required for α3β1 function and cell migration [46–48]. However, it has been found that CD151 can also associate to α6β4 integrins [49]. Another tetraspannin protein, CD9, associates with α3β1 in keratinocytes to form a complex involved in regulating cell motility [50,51]. Syndecans are transmembrane proteoglycan that

localizes to the focal adhesions of adherent cells and binds to a range of extracellular ligands, including growth factors, integrins and extracellular matrix proteins. Syndecans have a single transmembrane domain, a short cytoplasmic domain, and a larger extracellular domain that bears three to five glycosaminoglycan chains, mostly heparan sulfate [52]. Among the four mammalian syndecans, syndecan-1, -2 and -3 are the major syndecans of epithelial, fibroblastic and neuronal cells, respectively, whereas syndecan-4 is unusual, appearing as an ubiquitous component of most cells. Engagement of syndecan-4 is essential for adhesion formation in cells adhering via certain integrins, and for cell proliferation and migration in response to growth factors. The cytoplasmic domain of syndecan-4 interacts with a number of signaling and structural proteins, and syndecans acts as an organizing center for transmembrane receptor and is anchored to the actin cytoskeleton [53]. Hyaluronan is a glycosaminoglycan present in most extracellular matrices, including that between the vital cells of the epidermis. It forms a loose, highly hydrated, gel-like matrix that contributes to the maintenance of the extracellular space and facilitates nutrient diffusion. But it also works by interacting with specific receptors, such as CD44 and receptor for hyaluronan-associated motility, which activate intracellular locomotory signals [54]. Hyaluronan is involved in cell proliferation and differentiation, produces an environment favorable for migration, and stimulates cell locomotion. In skin epidermis, the narrow extracellular space surrounding keratinocytes contains a high concentration of hyaluronan, as do other stratifying squamous epithelia. Elevated tissue levels of hyaluronan occur during embryonic growth of tissues and organs and wound healing [55]. Recently, it has been shown that hyaluronan synthesis is up-regulated in cultured keratinocytes stimulated to migrate with EGF and KGF [56,57].

Growth factors and growth factor receptors

Skin injury represent the stimulus starting the migration of epidermal cells at wound sites and some authors suggest that the absence of neighbor keratinocytes at the margin of the wound, due to the mechanical cut off of the epidermal sheet, may be a signal for migration [4]. During the inflammatory phase, the local release by plasma, fibroblasts and macrophages/neutrophils of growth factors appears to be very important for activating keratinocytes at the wound margins.

Platelet-derived growth factor (PDGF)

PDGF has been widely recognized as having a significant role in the process of wound healing [58]. The role of PDGF during skin wound healing seems to be related to the following reepithelization phases of granulation tissue formation and contraction of the wound. Nanomolar concentrations of PDGF-BB and, at lesser extent PDGF-AA, accelerate the formation of granulation tissue both in vitro and in vivo [59]. PDGF is a chemotactic factor for cells migrating into the healing skin wound, such as neutrophils, monocytes, and fibroblasts [58]. In addition, it enhances proliferation of fibroblasts and production of extracellular matrix by these cells during wound contraction and extracellular matrix reorganization. Finally, it stimulates fibroblasts to contract collagen matrices and induces the myofibroblast phenotype in these cells [2]. The patterns of PDGF and PDGF receptor expression suggest a paracrine mechanism of action, since the ligands are predominantly expressed in the epidermis, whereas the receptors are found in the fibroblast of dermis and granulation tissue. PDGF may be involved in wound healing abnormalities. In some individuals, wound healing can lead to hypertrophic scar or keloid formation, characterized by an overabundant extracellular matrix and consistent with differences in growth factor profiles of both the epidermis and the dermis. Immunocytochemical studies revealed that augmented epidermal PDGF production leads to increased formation of the dermal matrix in hypertrophic scars [60]. On the other hand, PDGF and PDGF receptors expression significantly reduce during wound healing in healing-impaired genetically diabetic *db*/*db* mice, indicating the beneficial effect of exogenous PDGF in the treatment of wound-healing disorders [61]. Moreover, human keratinocytes genetically modified to overexpress PDGF-A, transplanted to full-thickness excisional wounds on the back of athymic mice, revealed that PDGF-A overexpression improves graft performance. This provides a therapeutic application to increase the performance of bioengineered skin substitutes as a strategy for skin repair [62]. PDGF is currently approved for use in human medicine [63].

Epidermal growth factor

A series of experimental and clinical studies have demonstrated a positive effect of EGF, TGF-α and HB-EGF on wound repair, suggesting that the endogenous growth factors are also involved in the healing process [1,64]. In particular, they are considered as key regulators of keratinocyte proliferation at wound site, released in abundance at the wound site mainly by eosinophils and macrophages. In addition, epidermal keratinocytes at the wound edge were identified as a source of TGF-α in partial-thickness murine burn wounds, with maximal levels during the phase of keratinocyte proliferation. EGF immunoreactivity was found to be associated with the presence of wound inflammatory cells and wound fibroblasts [65]. Finally, HB-EGF was localized in the advancing epithelial margin and in marginal surface keratinocytes of murine partial-thickness burn wounds [66]. Interestingly, HB-EGF was shown to act synergistically with insulin-like growth factor (IGF)-1, another growth factor present in wound fluid, in stimulating keratinocyte proliferation in vitro [67]. EGF, TGF-α and HB-EGF exert their function via binding to the EGFR, a transmembrane protein tyrosine kinase that is expressed on many different cell types [68]. Consistent

with the expression of the ligands during reepithelization, EGFR were also detected in the hyperthickened wound epidermis and in all appendages, but was absent from leading epithelial margins [69], suggesting a role for the EGF receptor in transducing a proliferative signaling response at wound sites during the reepithelization phase.

At least other two growth factors are central players in this process: TGF-β and MSP.

Transforming growth factor β (TGF-β)

The TGF-β superfamily encompasses a diverse range of structurally related but functionally distinct mammalian members, i.e., TGFβs and BMPs. Their biological effects are mediated by heteromeric receptor complexes [70]. The TGF-β ligand initiates signaling by binding to type I and type II receptor serine/threonine kinases on the cell surface and by high affinity to "nonsignaling" type III receptor, which presents TGF-βs to type II receptor [71,72]. This allows receptor II to phosphorylate the receptor I kinase domain, which in turn propagates the signal through phosphorylation of Smad proteins. There are eight Smad proteins, constituting three functional classes: the receptor-regulated Smad (R-Smad), the Co-Smad (Co-Smad), and the inhibitory Smad (I-Smad). R-Smads once phosphorylated and activated by the type I receptor kinases undergo homotrimerization and formation of heteromeric complexes with the Co-Smad, Smad4. The activated Smad complexes are translocated into the nucleus and, in conjunction with other nuclear cofactors, regulate the transcription of target genes. The I-Smads, Smad6 and Smad7, negatively regulate TGF-β signaling competing with R-Smads for receptor or Co-Smads and by targeting the receptors for degradation.

TGFβ1 is the most important ligand for starting migration of skin epithelial cells during the reepithelization process [73,74]. TGFβ1 can inhibit cell proliferation and start cell migration because it is a potent stimulator of gene expression, in particular of ECM components, MMPs and integrins in different target cells [71,75].

Platelets of the blood clot, immediately after wounding, facilitate the formation of the hemostatic plug and release large amounts of TGFβ1 [76]. This growth factor has two major target cells: macrophages and keratinocytes. Active TGFβ1 promotes migration and activation of monocyte–macrophage cells in the wound site for clearance of microorganisms and ECM fragments. Also, latent TGF-β1 is produced and sequestered within the wound matrix, allowing a sustained release by proteolytic enzymes. This combination of different cellular sources and temporary storage ensures a continuous supply of TGF-β1 throughout the repair process [73]. Later, TGFβ1 stimulates expression of some integrin subunits that promote keratinocyte migration on the provisional extracellular matrices of the wound bed [75,77]. Interestingly, TGF-β was shown to stimulate angiogenesis, fibroblast proliferation, myofibroblast differentiation and matrix deposition, critical for an efficient reepithelization [73,78]. This is supported by a series of studies in several animal models that demonstrate a beneficial effect of exogenous TGF-β for wound repair, in terms of both rate of healing and strength of the healed wound [73].

Interestingly, integrin αvβ6 can localize transforming growth factor (TGF)-β1 to cell surface by binding to the latency-associated peptide leading to TGF-β activation presumably by a nearby cell. TGF-β1 plays an important role in wound repair by regulating re-epithelialization, suppressing inflammation, and promoting connective tissue regeneration and scar formation. In the absence of αvβ6 integrin, there seems to be a deficiency of TGF-β1 activation by the epithelium leading to exaggerated inflammation in response to injury or infection. Transgenic mice with targeted deletion of β6 integrin developed excess of lung inflammation that was reversed by restoring β6 expression. Moreover, they were compromised in their ability to produce the inflammatory response to experimental infection in the gut. Activated TGF-βs may also regulate other cellular functions critical for wound healing, including integrin expression, cell migration, proliferation, differentiation and, matrix synthesis and degradation, in an autocrine or paracrine manner [75].

Macrophage stimulating protein (MSP)

MSP belongs to a protein family that includes plasminogen and HGF. MSP molecule is characterized by multiple copies of a highly conserved triple disulfide loop, named "kringle" [79], and is released by hepatocytes in circulating blood as a biologically inactive form, pro-MSP. Pro-MSP is proteolitically processed and activated at extravascular sites by specific serine proteases, such as trypsin-like proteases, enzymes of the coagulation cascade and a specific convertase located on the plasma membrane of macrophages [80,81]. MSP promotes its effects on target cells through binding to the Ron tyrosine kinase receptor [82,83]. MSP stimulation induces Ron dimerization and receptor autophosphorylation on several critical tyrosine residues in the kinase domain and in the C-terminal tail, generating specific-binding sites that interact and recruit SH2-containing adaptor/docking proteins. Ron activates a variety of intracellular signaling pathways including Ras/Erk, PI3K/Akt, JNK, FAK and NF-kB. It is known that these pathways are essential for cell growth, migration, survival and differentiation [84,85]. The MSP receptor is expressed in epithelial tissues, including human foreskin keratinocytes, and in specific hematopoietic cells [86–91]. Recently, several reports suggest a specific role for MSP and Ron in wound healing [24,92,93]. The involvement of MSP-Ron in skin wound repair is supported by several experimental evidences: (i) MSP, pro-MSP and an MSP convertase have been found at wound sites in human wound fluids; (ii) MSP and Ron expression was shown in full-thickness excision wounds; (iii) Ron is up-regulated in migrating keratinocytes at the wound edge and in dermal macrophages; (iv) an autocrine loop of MSP is observed in

migrating keratinocyte during reepithelization in vivo and in vitro; (v) wound treatment with exogenous MSP enhance reepithelization in mice; (vi) Ron promotes primary keratinocyte reepithelization and activate a specific gene expression program necessary to support full wound closure. The role of MSP and Ron in wound healing has been clarified at molecular level (Figs. 2 and 3). In basal keratinocytes normally expressing Ron, MSP-mediated activation of Akt and PKC protein kinases promote serine phosphorylation of both Ron and α6β4 receptors, generating in both molecules 14-3-3 binding sites. Due to their dimeric structure, 14-3-3 proteins promote the formation of a Ron-α6β4 complex, which reduced laminin5-α6β4 affinity and disassemble HD structures. These molecular events block α6β4 transdominant function promoting α3b1 integrin activation and keratinocyte migration at the wound sites. Finally, Ron activation and 14-3-3 binding are responsible for a shift in integrin dominance, hence modulating keratinocyte migration during skin reepithelization [24].

MSP/TGFβ1 synergism

Recent studies demonstrated that Ron activation results in increased expression of the Smad 2 proteins and directly causes its phosphorylation [85]. Smad 2 is a signal molecule responsible for TGF-β-induced EMT function [72]. The fact that Ron activation mediates Smad 2 expression and phosphorylation suggests that in epithelial cells exist a Ron-mediated TGF-β/Smad signaling pathway. Our recent studies further demonstrated that keratinocytes stimulation by MSP and TGF-β1 results in increased migration rate and EMT transition during in vitro wound healing assays (Santoro MM, unpublished results). Since both MSP and TGF-β are involved in regulating epithelial wound healing in vivo, the synergism in signaling between Smads and Ron might be essential in regulating migration of keratinocytes during reepithelization, as well as promotes de novo expression of integrin, ECM and MMP at wound site.

Small GTPases

Homeostasis of normal tissues require the regulation of actin cytoskeleton and of adhesive properties, which are both deeply influenced by the Rho family GTPases and their effectors [94]. The Rho family proteins are central regulators of cell protrusions and cell polarity, controlling the formation of lamellipodia and filopodia, by interactions with different downstream effectors, which are protein and lipid kinases, as well as activators of the Arp2/3 complex [95]. For instance, the balance between proliferation and differentiation in human keratinocytes has been related to the activity of the GTP-binding protein RhoA, through its signal mediators ROCK serine/threonine kinases: ROCK-I and ROCK-II. Pharmacological inhibition of ROCK blocks primary human keratinocyte terminal differentiation and induces cell proliferation. In contrast, activation of ROCK-II by expressing a conditionally active form of ROCK-II results in cell cycle arrest and an increase in the expression of a number of genes associated with terminal differentiation [96].

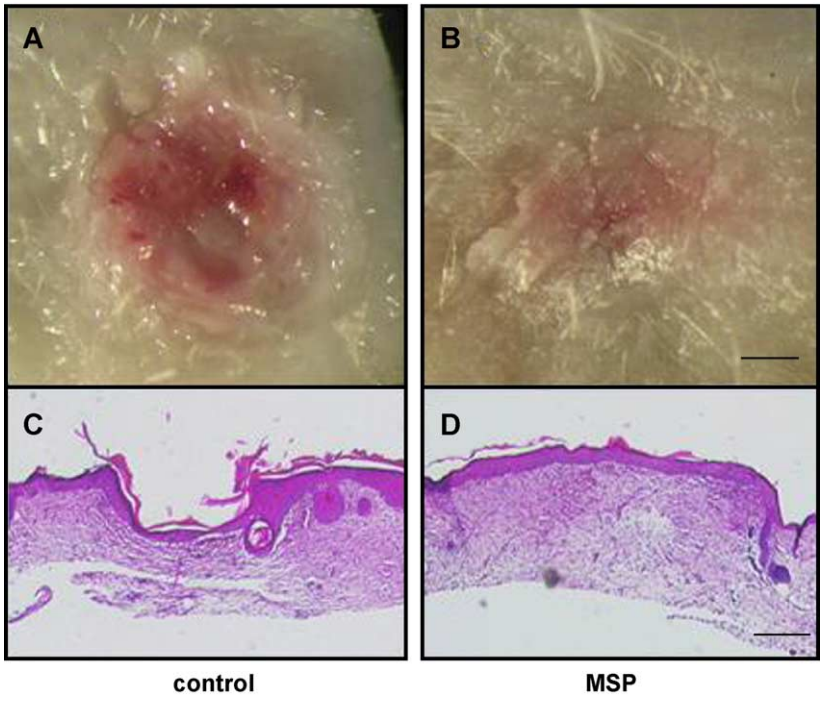

Fig. 3. MSP promotes wound healing in vivo. Full-thickness wounds treated with PBS + matrigel (A) with MSP + matrigel (B). (A, B) Wound appearance at day 4 after MSP treatment. The scale bar represents 1 mm. (C) Reepithelization completes after 10 days in MSP-treated wound. Representative pictures are presented.

An interesting example of the role of small GTPases in keratinocyte reepithelization involves the expression of extracellular metalloproteinases such as MMP-2 MMP-9, whose activity is required for cell migration and extracellular matrix remodeling. During response to keratinocyte injury, MM-9 is up-regulated upon the mechanical stress created by injury itself and depends by a cross-talk between signaling of RhoGTPases, $p38^{[MAPK]}$ and JNK. In particular, Rac1 and/or Cdc42 control the activation of $p38^{[MAPK]}$, while RhoA activity leads to stimulation of JNK [97].

Moreover, the role of small GTPases in keratinocyte reepithelization is confirmed by the role in α6β4 regulated keratinocyte chemotaxis. α6β4 integrin and laminin-5 interaction drives chemotaxis by sustained activation of the Rho family GTPase Rac1, possibly via activation of Phosphoinositide-3-kinase (PI3-K) [98] and redirection of α3β1 away from basal focal contacts. In absence of α6β4 activation, chemotaxis is mediated by an alternative pathway based on RhoA activity [99].

Matrix metalloproteinases

MMPs are a family of zinc-dependent enzymes whose catalytic activity is tightly regulated: They are secreted as inactive proenzymes (zymogens) and the activation occurs in the extracellular compartment [100]. In general, MMPs are not expressed constitutively in vivo, but are tightly regulated at the transcriptional and post-transcriptional levels as well as controlled at the protein level via activators, tissue-derived inhibitors (TIMPs) and cell surface localization [6].

During wound healing, degradation of ECM components by MMPs is required to remove and reorganize provisional matrices and to allow cell migration [37] and basal keratinocytes at the migrating front of reepithelization are the predominant source of MMPs. Human keratinocytes synthesize and secrete mainly MMP-1, MMP-2, MMP-9 and MMP-10, and this expression is required to regenerate the injured tissue.

Following injury, MMP-1 is consistently expressed by migrating keratinocytes bordering the wounds, but it is not express by proliferating keratinocytes [101]. MMP-1 expression is abundant at the wound edge and progressively decreases away from it [102]. It has been demonstrate that both TNF-α and TGF-β1 induce in vitro expression of MMP-1 in keratinocyte via a p38-dependent pathway [103]. In keratinocytes, MMP-1 expression is driven by contact with type-I collagen, mediated by α2β1 integrin [104]. In reepithelization, keratinocytes migrate over the dermal connective tissue, composed mainly by type I collagen, and lose contacts with BM, due to injury-mediated BM disruption. Loss of contact with BM and the interaction with type I collagen in the dermis promote α2β1 integrin-mediated MMP-1 expression in migrating keratinocytes. In turn, MMP-1 expression sustains keratinocytes migration on type I collagen, also associating MMP-1 it self and α2β1 integrin. This interaction provides a molecular mechanism for bridging together matrix, integrin and the protease. This results in controlled and localized proteolysis at the injury site, facilitating cell migration [104,105]. Laminin-1, but not laminin-5, appears to be able to down-regulate MMP-1 expression in migrating keratinocytes. An intriguing possibility is that deposition of laminin-1 during BM reorganization is the signal conveyed to keratinocytes, to suppress MMP-1 expression and motility.

Both MMP-2 and MMP-9 play an important role in wound healing. MMP-2 expression is modulated in keratinocytes by TGF-β1 and MSP [24,106]. Expression of MMP-2 remains stable at wound healing, suggesting a role during the prolonged remodeling phase, by proteolysis of laminin-5 that modulates keratinocyte migration [107]. The cleavage of laminin-5 by MMP-2 generates an EGF-like fragment promoting cell migration on epidermal cells [108]. MMP-9 is expressed at wound sites by migrating keratinocytes and macrophages. In keratinocytes, MMP-9 is constitutively expressed, but can be up-regulated by TGF-β, TNFα and IL1β stimulation [109]. MMP-9 can activate inactive TGF-β ligands, generating a positive loop that sustains keratinocytes migration [110]. Finally, MMP-10 is expressed by migrating keratinocytes at the migratory front of the epithelium, where it colocalizes with MMP-1 [111]. MMP-10 appears to assist keratinocyte migration at the epithelial front and also contributes to remodeling of the newly formed matrix. MMP-10 can also activate MMP-9.

In summary, MMP activity has the potential to induce migration, but also to reverse the function of the matrix molecules and growth factors from stimulating migration to supporting stable anchorage. For these reasons, imbalance expression of MMPs at wound sites may contribute to the pathogenesis of poorly healing wounds [37].

Keratinocyte proliferation at wound sites

During the step of reepithelization, keratinocytes both migrate to cover the wound and proliferate to form a dense hyperproliferative epithelium feeding the epithelial tongue. To reconstitute full thickness skin and tissue integrity, proliferation is sustained by growth factors, in turn assisted by integrins and MMPs [112].

It is well established that three growth factors, (i) EGF (Epidermal Growth Factor), (ii) TGFα (Transforming Growth Factor-α) and (iii) KGF (Keratinocyte Growth Factor), are central players in the proliferation process [5].

Both EGF and TGFα, belonging to the EGF superfamily, are abundantly released at wound sites as key regulators of keratinocyte proliferation [113]. Eosinophils, macrophages and epidermal keratinocytes at the wound edge have been identified as sources of EGF and TGFα at maximal levels during the phase of keratinocyte proliferation [66]. EGF and TGFα exert their function via binding to the EGFR present in healing wounds and in particular in hyper thickened

wound epidermis as well in all appendages, albeit it is absent from leading epithelial margins [69].

Another important epidermal wound regulator is KGF (or FGF7), which acts on keratinocytes in wound healing through specific interactions with the FGFR2 splicing variant FGF2IIIb [114]. FGF7 is 100-fold up regulated within 24 h after wounding in dermal fibroblasts adjacent to the wound and in fibroblasts of the granulation tissue [115]. Exogenous KGF applied to skin wounds shows mitogenic activity on healing epidermis [116]. In addition, γδT cell receptor-bearing dendritic epidermal T cells (DETCs) were recently identified as a major source of FGF7 in murine skin wounds [117]. FGFR2IIIb, the only high-affinity receptor for FGF7, is expressed in keratinocytes of the normal and wounded epidermis of murine and human wounds in addition to ligands [115,118].

Furthermore, the GM-CSF cytokine has been demonstrated to promote keratinocyte proliferation as an important step during wound reepithelization [119]. Several studies demonstrated a strong increase in GM-CSF levels of skin extracts after generation of full-thickness excision wounds, suggesting a role in cutaneous wound repair for this cytokine [120]. Furthermore, transgenic mice overexpressing GM-CSF in the epidermis displayed accelerated wound reepithelization, as a result of increased keratinocyte proliferation [120,121].

Recently, it has been clarified that the role of growth factors and their receptors in promoting keratinocyte proliferation during wound reepithelization can be greatly influenced by integrins, ECM and MMPs. In particular, integrins and ECM can positively or negatively regulate growth factor receptor signaling. Integrin engagement by ECM can modulate growth factor signaling pathways, increasing the activity of growth factor receptor and their downstream intracellular mediators, leading to enhancement of their biological effects [122]. Integrins and growth factor receptors can cooperate at the signaling level, but they may also form macromolecular complexes on the cell membrane of epidermal cells [123–128]. Integrin association with Receptor Tyrosine Kinases (RTKs) might protect the latter against phosphatase activity and/or ensure the correct subcellular juxtaposition of cytoplasmic tails of the dimerized receptors. Moreover, *trans*-phosphorylation among the two molecules occurs [27,124,128]. In the context of wound healing, the synergy between integrin, ECM and growth factors is probably a key process in the regulation of epidermal proliferation and cell cycle progression. It is well established that cell cycle G1 progression and cell proliferation require both cell adhesion and interaction with extracellular matrix (ECM) proteins, as well as stimulation by growth factors. It has been proved that ECM can modulate keratinocytes sensitivity to soluble mitogens and regulate their proliferation [129,130].

Normal cells require matrix attachments not only to proliferate, but also to protect themselves against anoikis, a form of apoptotic cell death occurring upon loss of matrix attachment. It is clear that Ras/Erk and PI3K/Akt pathways activation provides the route eliciting resistance to anoikis [131]. In keratinocytes, MSP elicits anti-apoptotic effect both via Erk and PI3-kinase/Akt pathways [91]. Laminin-5 is required for keratinocyte migration, but also for cell survival at wound site. Accordingly, laminin-5 null keratinocytes display reduced motility and fail to survive in culture [132,133]. Since both integrin α6β4 [134] and α3β1 [135] have been linked to the Ras/Erk signaling pathway and cell-cycle regulation, laminin-5 may contribute to survival through one or both of these pathways. Consistently, β4 mutant mice show signs of apoptosis in epithelial cells [136] and display proliferative defects that are characterized by increased levels of the cyclin-dependent kinase inhibitor p27 [137]. ECM and BM contain inactive growth factors bound to the matrix molecules. At wound sites, the ECM-bound growth factor may be released form the matrix by proteases that can also cleave growth factor receptors on the cell surface. These processes provide important examples for spatial and temporal control of the release and activation of growth factor during regeneration of epithelial cells. For example, FGF-2, which is normally associated with cell-surface or BM heparin sulfate proteoglycans, must be processed by proteases to become biologically active for cell proliferation. It has been found that MMP-2 and plasmin can release FGF-2. MMP-2 can also cleave the extracellular domain of the FGFR-1 and contribute to modulate the mitogenic properties of FGFs [138]. MMP-3 is expressed by proliferating keratinocytes distal to the wound edge and from dermal fibroblasts [111]. The role of MMP-3 in wound repair has been evaluated in MMP-3 knockout mice, which show impaired skin wound healing [139]. Interestingly, MSP stimulation of primary keratinocyte promotes de novo MMP-3 expression in these cells via p38- and NF-κB-dependent pathways [24].

During reepithelization, the proteolytic activity may also release growth factors already stored in the extracellular matrix. Specific MMPs have also the capacity to cleave IGF-1 and TGF-β1 to increase availability of these growth factors during wound-healing [140,141]. Furthermore, the role of epidermal stem cells is currently under investigation during wound healing as a new way to reconstitute skin integrity after wound healing [142].

Conclusions

Here we report the molecular and cellular mechanisms that regulate keratinocyte biology at wound sites. The coordinated interactions among integrins, extracellular matrix molecules, metalloproteases and growth factor receptors play a key role in wound healing in particular in the reepithelization phase. Further elucidation and improvement of cellular and molecular mechanisms governing keratinocyte reepithelization are essential to establish

therapeutic targets and methods to improve tissue regeneration during skin wound healing.

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