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Review

Mechanisms of human skin cell motility

W. Li, J. Fan, M. Chen and D.T. Woodley
The Department of Medicine, Division of Dermatology and the USC/Norris Cancer Center,
The University of Southern California, Los Angeles CA and VA Greater Los Angeles Health System, USA

Summary. The extracellular matrix (ECM) in contact with the cells and the soluble growth factors (GFs) binding to their cell surface receptors are the two main signals that directly regulate cell motility. Human keratinocytes and dermal fibroblasts are two primary cell types in skin that must undergo migration for skin wounds to heal. In this cell migration, ECMs play an "active" role by providing the cells with both focal adhesions and a migration-initiating signal, even in the absence of GFs. In contrast, GFs cannot initiate cell migration in the absence of a pro-migratory ECM. Rather, GFs play a "passive" role by enhancing the ECM-initiated motility and giving the moving cells directionality. Inside the cells, the initiation signal of the ECM and the optimization signals of the GFs are propagated by both overlapping and discrete signaling networks. However, activation of no single signaling pathway by itself is sufficient to replace the role of ECMs or GFs. This review focuses on our current understanding of both the individual and the combined functions of ECMs and GFs in the control of skin cell motility. An abbreviation of the terminologies used in this article is provided.

Key words: Keratinocytes, Dermal fibroblasts, Migration, Signal transduction, Wound healing

Introduction

Cell migration is the result of repeated cycles of cytoskeletal-mediated protrusion and polarization, formation of adhesive contacts, cell contraction, and retraction at the trailing edge (Lauffenburger and Horwitz, 1996). Initiation of these sequential processes is triggered by two main extracellular micro-environmental cues, extracellular matrices (ECMs) and soluble growth factors (GFs) (Schwartz et al., 1995; Eliceiri, 2001). In response to these motility signals, cells begin to show an initial protrusion of the plasma membrane at the leading edge. These protrusions are formed by polymerization of actin filaments into membrane structures called lamellepodia and filopodia (Hall, 1998). These structures are further stabilized through the formation of adhesive complexes on the ECM substratum, which are the clusters of integrin receptors, actin filaments, and associated proteins at the plasma membrane. As a cell migrates, focal complexes at the leading edge of the cell develop into larger, more organized adhesive complexes, called focal adhesions (Gumbiner, 1996). The focal adhesions then serve as points of traction over which the body of the cell translocates. Subsequently, release from the ECM substratum at the rear of the cell allows the cell body to be displaced, achieving a net step in a forward direction (Mitchison and Cramer, 1996). While the details of these temporally and spatially coordinated processes are complex and remain poorly defined, it is clear that the "dual signaling" by GFs and ECMs and their synergistic efforts determine the optimal level of a specific cellular response. The mechanism of action by ECMs and GFs is beyond the scope of this article and has been excellently reviewed in the past few years (Schlessinger and Ullrich, 1992; Giancotti and Ruoslahti, 1999; Heldin and Westermark, 1999; Eliceiri, 2001; Miranti and Brugge, 2002; Schwartz et al. 2002).

The migration of human skin cells, including keratinocytes, melanocytes, dermal fibroblasts and dermal microvascular endothelial cells, is critical for skin wound healing and remodeling (Clark, 1996).

Offprint requests to: Dr. Wei Li, The Department of Medicine, Division of Dermatology and the USC/Norris Cancer Center, the University of Southern California, 1303 North Mision Road, Los Angeles CA 90033, USA. e-mail: wli@hsc.usc.edu

Abbreviations. BSA: bovine serum albumin; BMZ: basement membrane zone; ECM: extracellular matrix; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; ERK: extracellular signal-regulated kinase; FAK: focal adhesion kinase; FGF-7: fibroblast growth factor-7; GF: growth factor; HK: human keratinocyte; HDF: human dermal fibroblast; HGF/SF: hepatocyte growth factor/scatter factor; HS: human serum; HP: human plasma; IGF-1: insulin-like growth factor-1; IL-1: interleukin-1; KGF: keratinocyte growth factor; MAPK: mitogen-activated protein kinase; MI: migration index; MMP: matrix metalloproteinase; RTK: receptor tyrosine kinase; PDGF: platelet-derived growth factor; TGFα: transforming growth factor-alpha; TGFß: transforming growth factor-beta; TNFα: tumor necrosis factor-alpha

Among them, the migration of human keratinocytes (HKs) and human dermal fibroblasts (HDFs) are the best characterized and, therefore, are taken as the examples in this article. In response to an acute wound, HDFs undergo proliferation, migration into the wound bed, production of new ECMs and matrix metalloproteinases (MMPs). A certain population of HDFs expresses thick actin bundles and becomes myofibroblasts (Singer and Clark, 1999). Similarly, within hours after an acute wound, basal HKs at the margins of the wound and within cut skin appendages (hair follicles, eccrine gland ducts) enter a migratory mode and begin to migrate laterally across the wound bed. One hypothesis is that the changes at the wound site in either GF levels or ECM profiles or both are responsible for the transition of HKs and HDFs from a stationary state to a migratory state (Clark, 1985; Sarret et al., 1992a,c). For instance, HKs and HDFs in unwounded skin only encounter the infiltrate of plasma or interstitial fluid. When human skin is wounded, however, HKs and HDFs experience serum, rather than plasma, for the first time. Numerous vasoactive mediators and chemoattractents are generated following blood coagulation and serum formation. Indeed, we have recently shown that that serum, but not plasma, promotes HK migration (Henry et al., 2003). Moreover, in unwounded skin, the basal HKs are in contact with the ECMs of the basement membrane zone (BMZ) such as laminin isoforms and nidogen. During wounding, the basal HKs migrate off the BMZ onto the wound bed and come into contact with a completely new set of ECM molecules such as dermal collagen and fibronectin. These new sets of GFs and ECMs in the wound bed, plus the influence of inflammatory responses, likely account for the HKs' transition from a stationary mode into a migratory mode during wound healing. In contrast, the reverse is true for HDFs. We have recently found that human plasma promotes HDF migration, whereas human serum does not (B. Bandyopadhay, J. Fan, M. Chen, D. Woodley and W. Li, unpublished observation). We postulate that the differential responses of HKs and HDFs to plasma versus serum are part of the regulatory mechanisms of wound healing. In this review, we summarize the factors involved in HK and HDF migration and discuss the mechanisms of their actions.

Growth factors that regulate HK motility

The growth factors/cytokines, which have been reported to promote HK motility, include epidermal growth factor (EGF), transforming growth factor-α (TGFα), insulin-like growth factor-1 (IGF-1), insulin, fibroblast growth factor-7/keratinocyte growth factor (FGF-7/KGF), hepatocyte growth factor/scatter factor (HGF/SF), transforming growth factor-ß (TGFß), interleukin-1 (IL-1), and interleukin-8 (IL-8).

1) EGF/TGFα

Barrandon and Green reported that the EGF-stimulated increase of HK colony radius was due to an increased rate of cell migration (Barrandon and Green, 1987). In this paper, however, cell division and cell migration were not independently dissected and measured. Chen and colleagues demonstrated that EGF was indeed a "motogen" (as well as a "mitogen") when a cell migration assay was used to distinguish cell division and cell motility (Chen et al., 1993). The HKs, placed on a sub-optimal pro-motility matrix in the presence of increasing concentrations of EGF, exhibited enhanced motility. Further, the addition of either EGF neutralizing antibody or blocking antibody against the EGF receptor inhibited this effect. Similar observations were made by Ando and Jensen who showed that EGF, IGF-1 and insulin enhanced HK migration on colloidal gold particles coated with collagen IV or fibronectin, but not on uncoated surface (Ando and Jensen, 1993). In a later study, Cha and colleagues compared the "motogenic" properties of EGF and TGFα. They demonstrated that although both GFs used the same EGF receptor and had similar binding affinities for the receptor, TGFα was a more potent motogen for HKs (Cha et al., 1996). In concordance with the above studies, studies from several other laboratories have shown that EGF stimulates HKs to express migration-related proteins, including fibronectin (Delapp and Dieckman, 1990), alpha2 and beta4 integrin (Chen et al., 1993; Song et al., 2003), collagenase-1 (Pilcher et al., 1997; Petersen et al., 1989, 1990), and MMP-9 (McCawley et al., 1998; Zeigler et al., 1999). The HK-derived fibronectin and metalloproteinases have been shown to play an important role in HK migration (Pilcher et al., 1999). The roles of TGFα and EG,, may be redundants. Two genetic studies showed that mice with mutations in the TGFα gene have abnormalities in hair follicle and skin architecture and often develop corneal inflammation. However, wound healing is not impaired in these mice lacking the TGFα gene (Luetteke et al., 1993; Mann et al., 1993).

TGFß

The role of transforming growth factor-ß (TGFß) in HK motility remains controversial. The unresolved key issue is whether the primary effect of TGFß on HK motility is stimulatory or inhibitory. Nickoloff et al. reported that TGFß promotes HK migration by inducing fibronectin production (Nickoloff et al., 1988). Salo and colleagues reported that TGFß upregulates the expression of type IV collagenases (gelatinases A and B or MMP-2 and MMP-9) in cultured HKs (Salo et al., 1991). Similarly, Decline and colleagues reported a correlation between TGFß-induced HK motility and alpha2beta1 interaction with HK-secreted and unprocessed laminin-5 (Decline and Rousselle, 2001; Decline et al., 2003). Zambruno et al. suggested that TGFß regulates HK motility by modulating its integrin receptor repertoire. These authors showed that TGFß upregulates/induces the receptors for fibronectin, alpha5beta1 and alpha(v)beta6, and for vitronectin,

alpha(v)beta5. At the same time, TGFß also downregulates the alpha3beta1 receptor, which interacts with laminin-5, an HK migration inhibitor (Zambruno et al., 1995). None of these studies, however, proved that TGFß is a primary pro-motility factor that can directly drive HK migration on a pre-existing ECM. Rather, all these studies suggest that TGFß promotes HK migration by indirect mechanisms

In contrast to the results of the above studies, a genetic study in mice indicated that TGFß signaling instead slows down or plays a negative role in wound re-epithelialization. Ashcroft et al. reported that disruption of the Smad3 gene, a downstream signaling effector for the TGFß receptor, accelerated cutaneous wound healing in mice (Ashcroft et al., 1999). One possible mechanism discussed was a reduced monocytic response in the skin of Smad3-disrupted mice, since they found that Smad3 is required for TGFß-induced monocyte chemotaxis and TGFß expression by monocytes. However, the intracellular "migratory machinery" in the keratinocytes isolated from these mice appeared to be intact, because adhesion and migration of the cells in response to other growth factors were normal (Ashcroft et al., 1999).

KGF

Keratinocyte growth factor (KGF, or fibroblast growth factor-7) is a paracrine growth factor produced by mesenchymal cells but it acts on cells of epithelial origins. It is the most potent growth factor reported for HKs. Tsuboi and colleagues were first to show that KGF stimulates HK migration as potently as TGFα, but less potently than HGF/SF, in *in vitro* wound healing ("scratch") assays (Tsuboi et al., 1993; Sato et al., 1995). Putnins and colleagues also showed that KGF treatment of HKs increases HK attachment to selective ECMs, such as collagen and fibronectin, by stimulating beta1 integrin expression. Moreover, KGF stimulates HK migration on these ECMs (Putnins et al., 1999). Further, Sato et al. showed that pre-coating the plastic surface with ECMs had neither stimulatory nor inhibitory effects on KGF-stimulated HK migration (Sato et al., 1995). These data showed that KGF was capable of stimulating HK migration even when the cells were attached to an ECM-free surface. This would mean that ECMs are unnecessary for KGF-driven HK motility. However, these experiments were flawed by confounding HK division and HK migration. In these experiments, no growth inhibitors, such as mitomycin C, were included during the 16-hour scratch assay. Since KGF is a powerful mitogen for HKs, the results of their scratch assays could be a combination of cell division and cell migration. A study by Zeigler et al. suggested that KGF is a mitogen, but not a motogen, for HKs (Zeigler et al., 1999). Interestingly, KGF-knockout mice and even KGF and TGFa double knockout mice have no wound healing impairment (Guo et al., 1996).The results of this study suggest that there may be a great deal of redundancy in the control of HK growth and motility, and that KGF is not essential.

Interleukins

Interleukin-1 (IL-1) is an autocrine factor produced by HKs. Chen and colleagues showed that IL-1 stimulates HK migration on certain ECMs, such as collagen, and that this pro-motility effect was independent of the pro-motility effect of EGF. Moreover, their study indicated that the pro-motility effect of IL-1 and that of other HK pro-motility factors, such as TGFα, are additive when sub-optimal concentrations of both were used. However, the maximum migration achieved by using both IL-1 and TGFα never exceeded the maximum levels attained by using either cytokine alone (Chen et al., 1995). IL-8 is a cytokine known as a chemoattractant for neutrophils. O'Toole et al. showed that IL-8 can be produced by HKs and that an IL-8 autocrine loop promoted HK motility on collagen (O'Toole et al., 2000). Consistently, Gyulai et al. reported that IL-8, as well as IL-1, can act as a chemoattractant for freshly prepared, but not cultured, HKs in a Boyden chamber assay (Gyulai et al., 1994).

HGF/SF

Hepatocyte growth factor (HGF)/scatter factor is a secreted, heparan sulfate glycosaminoglycan-binding protein that stimulates mitogenesis and motogenesis in a variety of cell types. Matsumoto and colleagues tested the effect of HGF on HK migration and growth under either low Ca^{++} (0.1 mM, growth promoting) or physiological Ca^{++} (1.8 mM, differentiation promoting) conditions. They showed that HGF strongly stimulated HK migration, but not proliferation, under low Ca^{++}. Under the physiological Ca^{++} concentration, HGF stimulated both the migration and DNA synthesis of HKs (Matsumoto et al., 1991). One possible mechanism is that HGF stimulates MMP-9 production in HKs (McCawley et al., 1998) by stimulating a sustained activation of the MEK1/ERK1/2 pathway (Zeigler et al., 1999).

TNFα

Tumor necrosis factor-α (TNFα) stimulates production and secretion of MMP-9, but not MMP-2, from HKs (Makela et al., 1998). One mechanism is via TNFα-stimulated activation of the ERK1/2 cascade (Holvoet et al., 2003). Han and colleagues showed that TNFα and TGFß synergistically induced MMP-9 production in both an organ culture of human skin and isolated human HKs and HDFs (Han et al., 2001). These authors also showed that TNFα stimulation promoted proteolytic activation of pro-MMP-9 by converting the 92-kDa pro-MMP-9 into the 84-kDa active enzyme. This occuredonly in human skin organ culture and not in isolated HKs or HDFs. This is because TNFα promotes the proteolytic activation of MMP-9 by downregulating

tissue MMP inhibitors (Han et al., 2002). Besides its effect on MMP production, it is not clear if TNFα is a primary pro-motility factor for HKs.

Insulin and insulin-like growth factors

It was reported that insulin and IGF-1 stimulate HK migration on ECMs, but not in the absence of matrix coating (Ando and Jensen, 1993; Benolial et al., 1997). A recent study showed that IGF-1 and EGF control different aspects of keratinocyte migration. IGF-1 stimulates membrane protrusion and cell spreading via PI-3K, whereas EGF causes cell contraction via ERK1/2 (Haase et al., 2003). Moreover, IGF-1 was shown to bind vitronectin and then stimulates keratinocyte protein synthesis and migration (Hyde et al., 2004).

We have recently compared and quantitated the effects of all the above reported pro-motility factors on HK migration on collagen in both colloidal gold migration assay and *in vitro* wound healing assay. Our data show the following relative potency: TGF-α > insulin > EGF > IGF-1> HGF > IL-1 > KGF/FGF-7 > IL-8 (Li Y., Li W. and Woodley D., unpublished). It remains to be determined what constitutes the HK pro-motility activity *in vitro*.

In summary, despite the above studies on GFs' roles in HK migration, several important questions remain unanswered. Are all of the growth factors/cytokines involved in HK motility during wound repair? Do they act simultaneously or sequentially? Why is it necessary to require multiple growth factors/cytokines, instead of a single one, for motility of a given cell type?

Growth factors that regulate HDF motility

A number of growth factors and cytokines have been reported to promote HDF migration or chemotaxis, including PDGF-BB, PDGF-AA, FGF, EGF, IL-4, and TGFß1.

PDGF

Platelet derived growth factor (PDGF) is the best-characterized GF for HDF growth and motility, and is believed to participate either directly or indirectly in many of the wound healing processes. The three different isoforms of PDGF (AA, BB and AB) show variable effects, both quantitatively and qualitatively (Deuel et al., 1991; Heldin and Westermark, 1999). Adelmann-Grill and colleagues reported that HDFs were able to migrate in response to only a brief pulse of a PDGF-BB gradient in Boyden chambers. They suggested that the cell migration does not require a constant and stable concentration gradient of the chemoattractant, i.e. PDGF-BB (Adelmann-Grill et al., 1990). One mechanism, by which PDGF stimulates HDF migration, is to modulate cell surface integrin levels. PDGF-AB was shown to induce beta1 integrin synthesis in HDFs (Kirchberg et al., 1995). Clark and colleagues showed that PDGF-AA, BB and AB all cause a decrease in the level of the alpha1 subunit and an increase in the alpha5 subunit in HDFs (Gailit et al., 1996). Direct evidence that PDGF is the major pro-motility factor in human serum for HDFs comes from a recent study in our laboratory. We demonstrated that the addition of PDGF-BB alone was able to replace the full pro-motility effect of human serum for HDFs. Blockage of PDGF-BB action in human serum by an anti-PDGF-BB neutralizing antibody completely inhibited serum-induced HDF migration on collagen (Li et al., 2004). Although this finding does not exclude the possible participation of other growth factors with lower pro-motility activity, it suggests that PDGF-BB is the most potent pro-motility factor in human serum for HDFs.

TGFß

Similar to the confusion about the role of TGFß in HK migration, there are conflicting reports on the effect of TGFß on HDF migration. Postlethwaite and colleagues reported that TGFß is a potent chemoattractant and pro-motility factor for HDFs in modified Boyden chamber assays (Postlethwaite et al., 1987). Their study showed that the motility of HDFs exhibited a bell-shaped response to TGFß, in which the maximum stimulation of migration was achieved at ~12 pg/ml of TGFß. Deuel's group also reported a stimulatory effect of TGFß on HDF migration *in vitro* and in incision wound healing experiments (Pierce et al., 1989), although they used an almost two-million-fold higher amount of TGFß than Postlethwaite and colleagues. A recent study from Mustoe's group showed that TGFß increased migration of HDFs only from young adult donors under both normoxic and hypoxic conditions. In contrast, PDGF was unable to increase the motility of aged HDFs (Mogford et al., 2002). However, these data were confounded by the fact that the cells were never deprived of serum prior to the in vitro motility assays. Therefore, a "serum-priming" effect confounded the experiments when TGFß was added exogenously to the assays. Consistent with this notion, Mogforg showed that the best detectable TGFß-stimulated increase in HDF migration was no more than two-fold over the controls (Mogford et al., 2002). In contrast with the above studies, Ellis et al. used a three-dimensional collagen gel matrix assay and provided evidence that TGFß around 10 ng/ml is a potent antagonist of HDF migration in response to other pro-motility factors (Ellis et al., 1992). They argued that the different responses of HDFs to TGFß depend upon whether the microenvironment is a two-dimensional substratum or a three-dimensional matrix. The type of substratum or microenvironment may also be important in modulating the HDF response to other pro-motility cytokines (Ellis et al., 1992). A technical concern for these experiments is their failure to dissect cellular motility from cellular proliferation. Specifically, the experiments were conducted over 96 hours in the

absence of any cell proliferation inhibitors. Therefore, the HDFs may have had time to proliferate. If this were the case, it is difficult to distinguish whether the inhibition by TGFß was on migration, proliferation or both. Although HDFs cease dividing when they are transferred into a three-dimensional collagen lattice (Kono et al., 1990a,b), this inhibition of HDF growth occurs only after five days, which well surpasses the 96-hour period of the experiments of Ellis and colleagues.

TGFß is a "pro-matrix" growth factor by virture of its stimulation of fibroblast-derived ECMs and its inhibition of enzymes that degrade ECMs. Therefore, it is widely believed to be an important factor in the healing of skin wounds (see reviews by Moulin, 1995; O'Kane and Ferguson, 1997; Takehara, 2000; Cordeiro, 2002; Verrecchia and Mauviel, 2002). Nevertheless, additional studies are needed to clarify whether TGFß has a direct pro-motility effect on HDFs, or whether TGFß influences HDF motility secondarily by stimulating the expression of other factors, such as GFs, MMPs and ECMs, that could then influence HDF motility. The design of the experiments cited above could not address these questions. It appears to be clear, however, that when TGFß is co-present with other pro-motility factors, such as PDGF, it potently blocks other pro-motility factor-stimulated HDF migration. Recently, Fan and colleagues have shown that TGFß is not a primary motogen for HDFs. Serum-starved HDFs were subjected to migration assays on a collagen matrix under serum-free conditions in the presence or absence of TGFß. Further, mitomycin C was included to prevent cell proliferation. They found that TGFß (0.01 to 10 ng/ml) has little pro-motility effect on HDFs. Moreover, 0.1 ng/ml or higher concentrations of TGFß completely blocked PDGF-BB-stimulated HDF migration (Fan, Woodley, and Li, unpublished observations). These data suggest that TGFß does not have a direct pro-motility effect on HDFs.

EGF

Epidermal growth factor (EGF) was reported to play a role in both proliferation and migration of many different types of cells. It was reported that human wound healing is accelerated when EGF is applied to the wounds (Brown et al., 1986; Schultz et al., 1991). Adelmann-Grill and colleagues first reported that EGF stimulated chemotactic migration of HDFs in an in vitro migration assay. The EGF stimulatory effect could be attenuated by the presence of PDGF and TGFß in the medium (Adelmann-Grill et al., 1990). To ensure the specificity of the signaling via the EGF receptor, Shiraha et al. showed that mutations, which abolish the receptor protein tyrosine kinase activity, compromised EGF-stimulated migration and proliferation. Furthermore, they showed that the reduced motility response to EGF by aged HDFs correlated with decreased EGF receptor levels (Shiraha et al., 2000). Using EGF receptor autophosphorylation and c-terminal truncation mutants, Chen and colleagues reported that the motogenic and mitogenic signaling by the EGF receptor was separable at the receptor level. Certain EGF receptor mutants that remain mitogenic failed to mediate EGF motogenic signals (Chen et al., 1994). Using a colloidal gold migration assay and a computer-assisted quantitation, we observed that human EGF-stimulated HDF migration on collagen was less than 40% of the PDGF-BB or human plasma-stimulated HDF migration (Li et al., 2004a).

IL-4

Interleukin-4 (IL-4) is a T lymphocyte- and mast cell-derived cytokine that primarily targets blood cells and acts as a crucial modulator of the immune system. In contrast to its general stimulatory effects on lymphocytes, IL-4 shows an active antitumor agent and also a potent inhibitor of angiogenesis. There were only limited studies on the effect of IL-4 on human skin cells. Postlethwaite and Seyer reported that IL-4 induced HDF chemotaxis in the Boyden chamber motility assay and that anti-IL-4 neutralizing antibody blocked the chemotactic activity (Postlethwaite and Seyer, 1991). These experiments suggest that IL-4, which largely promotes a T helper 2 type of inflammatory response, also promotes HDF motility. Follow-up studies on the role of IL-4 in human skin cell migration have simply been absent.

Extracellular matrices for HK and HDF migration

Due to the fact that ECMs, integrins and MMPs are functionally related molecules in the control of cell motility, their participation in HK and HDF migration will be discussed together.

For HKs

Type I (dermal) collagen, type IV (basement membrane) collagen, and fibronectin are ECMs that promote HK motility (reviewed by Woodley, 1996; O'Toole, 2001). Guo et al. reported that HKs, freshly isolated from human skin, were immotile, and only became competent to migrate on type I collagen and fibronectin in the presence of EGF following in vitro culture (Guo et al., 1990). Kim and colleagues showed that HK migration on fibronectin was dependent upon RGD peptide (Kim et al., 1992; Sarret et al., 1992b). Consistently, EGF treated HKs showed an increase in the expression of the alpha2 integrin subunit at the HK surface and the expression of HK-derived MMP-9, both of which are associated with enhanced HK motility (Chen et al., 1993; Charvat et al., 1998). Besides MMP-9, MMP-1 has also been shown to play an essential role in alpha2 beta1-dependent HK migration on type I collagen. Inhibition of MMP-1 function completely blocked HK migration on collagen (Pilcher et al., 1997, 1999). Chen et al. showed that, rather than migrating in random directions, HK migration on type I collagen

appeared to be directional, since HK migration left behind long, linear tracks (Chen et al., 1994). The linear migration tracks appear to be specific for HKs on collagen or fibronectin, since migration of other cell types, such as HDFs, human melanocytes, endothelial cells and NIH3T3 mouse embryonic fibroblasts, do not generate linear migration tracks (Chen et al., 1994; Fedesco, Woodley, and Li, unpublished observations).

Vitronectin, also known as "serum spreading factor," increases HK motility in a concentration-dependent manner (Brown et al., 1991). Kim et al. showed that the pro-motility effect of vitronectin in HKs was mediated by the alphav beta5 integrin (Kim et al., 1994b). Although these in vitro experiments indicated the importance of vitronectin binding to beta5 for HK motility, mice lacking beta5 and beta3 expression developed normally and showed, unexpectedly, enhanced angiogenesis. There were no defects in wound healing in these mice, even though isolated keratinocytes from these mice failed to migrate on vitronectin in vitro (Reynolds et al., 2002). These results argue that vitronectin may not be essential for re-epithelialization of skin wounds.

The effect of laminin-5 on HK migration is controversial. Using the Boyden chamber assay, Zhang and Kramer reported that deposition of laminin-5 by the migrating HKs promotes HK migration in an alpha3 beta1-dependent manner (Zhang and Kramer, 1996). They showed that HKs migrated on laminin-5-coated filters in a dose-dependent fashion. Using the same assay, Decline and Rousselle showed that HKs do not migrate on purified laminin 5. However, the interaction between alpha2 beta1 at the surface of HKs and the laminin-5, secreted by TGFß-stimulated HKs, is required for HK migration on plastic, fibronectin, and collagen IV (Decline and Rousselle, 2001). Carter's group reported that quiescent HKs do not attach to collagens. HKs first adhere and spread on laminin-5 via apha3beta1 and alpha6beta4 in a PI-3K-dependent manner. This attachment then activates the RhoGTPase, which in turn triggers HK adhesion to other ECMs such as collagen (Nguyen et al., 2001). Furthermore, O'Toole and colleagues reported opposite effect of laminin-5 on HK motility. Using colloidal gold migration and *in vitro* wound healing assays, they showed that HKs migrate well on type I and type IV collagens and fibronectin, but do not migrate on laminin-1, laminin-5, or heparan sulfate proteoglycan (Woodley et al., 1988). The HK-secreted laminin-5, instead, inhibits HK migration on a collagen matrix. Moreover, if HK-derived laminin-5 is neutralized by the addition of an exogenous anti-laminin-5 antibody, the HKs exhibit enhanced and prolonged migration compared with what is found with the no antibody control or with the irrelevant antibody control (O'Toole et al., 1997).

The non-concordant studies may be explained by the observation that laminin-5 has so-called unprocessed and proteolytically cleaved forms (Rouselle et al., 1991; Marinkovich et al., 1992; Goldfinger et al., 1998; Vailly et al., 1994). These post-translational modifications have profound effects on the pro-motility activity of laminin-5. Quaranta's group showed that cleavage of the gamma-2 chain of laminin-5 by MMP-2 reveals laminin-5's pro-motility activity (Giannelli et al., 1997; Koshikawa et al., 2000). Consistently, mature laminin-5 with a processed alpha3 chain also strongly promotes cell motility and cell scattering via integrin alpha3beta1 (Tsubota et al., 2000). In contrast, Goldfinger and colleagues demonstrated that conversion of the 190-kDa alpha3 chain of laminin-5 to a 160-kDa peptide by plasmin eliminates its pro-motility effect and changes laminin-5 to an anchoring ligand in hemidesmosomes (Goldfinger et al., 1998).

HK migration on fibronectin appears to be triggered by unidentified mechanisms after wounding or separation of the cells from human skin (Grinnell et al., 1987; Guo et al., 1990). Keratinocytes freshly isolated from unwounded skin were found unable to attach and spread on fibronectin (FN)-coated culture dishes and unable to bind and phagocytose FN-coated beads. In contrast, keratinocytes isolated from healing wounds were able to attach and spread on fibronectin (Grinnel et al., 1987). Further, they showed that the adhesiveness of basal keratinocytes to FN substrata was activated during the culture process, and the activation was specific for FN, even when the cells were cultured on other ECMs or plastic (Grinnel et al., 1987; Guo et al., 1990). These authors reasoned that in intact skin the basal keratinocytes of the epidermis are attached to a basement membrane containing laminin and type IV collagen. During wound repair, however, these cells migrate over or through FN. In these adhered and migrating keratinocytes, there appeared to be an increase in the intensity of beta1 integrin subunit around and beneath the migrating cells, suggesting that changes in the distribution of beta1 integrin subunits is associated with gaining migratory competence (Guo et al., 1990). Consistently, Kim and colleagues provided evidence that HK migration on fibronectin was mediated by interaction between alpha5 beta1 integrin and the RGDS sequence in fibronectin. In contrast, HK migration on type IV collagen does not require an RGD sequence. HK migration on either type I or type IV collagen is mediated by alpha5beta1 integrin receptor (Kim et al., 1994).

For HDFs

Postlethwaite et al. first reported that fibronectin is a potent chemoattractant for HDFs, but not for human monocytes and neutrophils (Postlethwaite et al., 1981). HDFs attach to and migrate on fibronectin partially by binding to its RGD sites via the alpha(v) beta3 integrin (Gailit et al., 1997). Treatment of the cells with the protein synthesis inhibitor, cycloheximide, did not interfere with the attachment, suggesting that the alpha(v) beta3 integrin is a latent receptor. Type I collagen is the most plentiful ECM in the dermis (Singer

and Clark, 1999). Li et al. compared HDF migration on type I collagen, type IV collagen, fibronectin, vitronectin, and laminin-1 in the absence or presence of human serum or PDGF-BB. Their results showed that the migration indices (MIs) are 8-10 for type I collagen and 4-7 for the rest of the ECMs in the absence of growth factors. However, in the presence of human serum or PDGF-BB, the MIs of the cells dramatically rose to 30 on collagen and to 8-14 on the other ECMs (Li et al., 2004a,b). Therefore, type I collagen and fibronectin are the stronger pro-motility ECMs for HDFs, at least in the in vitro assays.

In summary, multiple GFs and ECMs show pro-motility/anti-motility effects on HKs and HDFs. Challenging questions for future research then are what the individual function of the different GFs might be *in vivo* and, more importantly, how they work together in the dynamic control of HK and HDF migration during skin wound healing. We have just begun to appreciate the fact that answers to these questions are critical for better treatment of non-healing skin wounds. The following sections discuss what we do and do not understand about these questions.

Haptotaxis versus Chemotaxis: Who Needs Whom?

An overlooked problem

ECMs and GFs are the two major extracellular pro-motility cues, and they act synergistically to stimulate optimal cell migration (Schwartz et al., 1995; Eliceiri, 2001; Schwartz and Ginsberg, 2002). *In vivo*, ECMs are substratum-immobilized molecules. In contrast, GFs are soluble and diffusible, sometimes membrane-bound, polypeptides. Previous studies have defined cell migration on ECMs alone or in GF-containing medium without an ECM. Cell migration towards a substratum-bound ECM gradient in the absence of growth factors is referred to as haptotaxis (Carter, 1967). Cell migration toward a gradient of soluble GFs is known as chemotaxis (Zigmond, 1974; Devreotes and Zigmond, 1988). One central and yet unanswered question is whether or not haptotaxis and chemotaxis occur independently of each other, or whether they depend upon each other for initiation and optimization of cell migration.

One confounding and confusing problem in the scientific literature is that a number of studies suggest that either ECMs of GFs alone are able to initiate cell motility without the presence of the other. For example, Satish et al., and Russell et al., have suggested that selected soluble GFs can stimulate HK migration without the presence of ECM. These studies have used the so-called "*in vitro* scratch" assay in which uncoated plastic or glass cover slips serve as the substratum upon which the cell migrate. Similar studies have been reported in HDFs (Javelaud et al., 2003; Satish et al., 2003), murine embryonic fibroblasts (Nobes and Hall, 1999; Bladt et al., 2003; Cuevas et al., 2003; McAllister et al., 2003), human lung epithelial cells (Galiacy et al., 2003), and smooth muscle cells (Hsieh, et al., 2003), just mention a few. A cursory reading of these papers could make one believe that cell migration can occur in the presence of GFs without the presence of ECMs. However, what is likely in the in vitro scratch assay is that the cells on plastic or glass surface have already synthesized and deposited on the plastic or glass cover slips their own pro-migratory ECM. Otherwise, without an ECM, how would these cells have been able to form focal adhesions that are a prerequisite for cell migration? However, since the secretion of ECMs and subsequent cell migration on the secreted ECMs were out of the experimental design and unregulated with regard to their identities, compositions and amounts, it certainly compromised the conclusions at end of the experiments.

In other studies, the investigators did pre-coat the dishes or coverslips with ECMs prior to plating cells on them, and the results of these studies implicated the essential role of an ECM substratum (Ando and Jesen, 1993; Jones et al., 1996; Pullar et al., 2003; Sadowski et al., 2003; Haase et al., 2003). However, none of these studies was designed to define the specific roles of ECMs versus GFs, or to explain whether there should be any difference if the surfaces were not pre-coated with ECMs.

The active versus passive signal for cell motility

There is an interdependent relationship between ECMs and GFs in the regulation of cell motility. Tucker at al. observed that fibroblast growth factor (FGF) and TGFα accelerated collagen-mediated dispersion and locomotion of NBT-II carcinoma cells. They postulated that a "substratum conditioning" was responsible for the GF effect (Tucker et al., 1991). Klemke et al. showed that human pancreatic carcinoma cells can migrate on collagen, but not on vitronectin, in the absence of GFs. Activation of the EGF receptor then enabled the cells to migrate on vitronectin in an alpha(v) beta5-dependent manner (Klemke et al., 1994). Ware and colleagues reported that EGF-stimulated migration speed and directional persistence of NR6 fibroblast cells could be influenced by ECMs. Their data suggested that there was coordination between the ECM and the GF (Ware et al., 1998). Despite these studies, it is also not clear what plays an active versus passive role during motility initiation.

Using the *in vitro* wound healing assay as an example, Li and colleagues carried out side-by-side comparisons of HK and HDF migration under four clearly defined conditions: 1) cells on an uncoated or a non-motile ECM-coated surface without GFs; 2) cells on an uncoated or a non-motile ECM-coated surface in the presence of GFs; 3) cells on a type I collagen matrix without GFs; and 4) cells on a type I collagen matrix in the presence of GFs (Li et al., 2004a,b). As shown in Figure 1, they found that HKs could not migrate on un-coated tissue culture plastic without (panels a and b) or

with (panel c) GFs. In contrast, the cells were able to migrate on collagen even in the absence of GFs (panel e vs. panel d). However, the collagen-driven cell migration without the absence of GFs was sub-optimal. The addition of GFs dramatically augmented cell migration (panel f).

Similar observations were made in both HKs and HDFs using a colloidal gold migration assay, which measures the collective distance of a single cell migration unconfounded by cell division (Albrecht-Buehler, 1977; Woodley et al., 1988). As shown in Figure 2, neither HKs nor HDFs could migrate on an uncoated colloidal gold surface even in the presence of GFs (panels a and d). In contrast, both HKs and HDFs modestly migrated on collagen even in the absence of GFs (panels b and e). The addition of GFs dramatically augmented the migration on collagen (panels c and f). These findings suggest that GFs do not have a primary pro-motility effect in the absence of a pro-motility ECM. The role of GFs is instead to optimize ECM-initiated cell migration. In contrast, ECMs alone can initiate and drive cell migration. Therefore, haptotaxis (ECM-driven) appears to be the prerequisite for chemotaxis (GF-driven). Chemotaxis cannot take place in the absence of ECMs. In contrast, haptotaxis can occur without GFs, as previously reported (Carter, 1967).

The nature of the initiation, augmentation and directionality signals

In a review article in Cell, Gumbiner stated: "An important issue to address is why there is a need for signaling by cell adhesion molecules, which are especially suited to mediate physical interactions between cells, when there exist plenty of traditional cell surface receptors dedicated to signal transduction....." and "it is apparent that there are functional reasons to couple certain signaling processes to specific cell adhesion events." (Gumbiner, 1996). A related question is why ECMs can initiate cell motility without any other

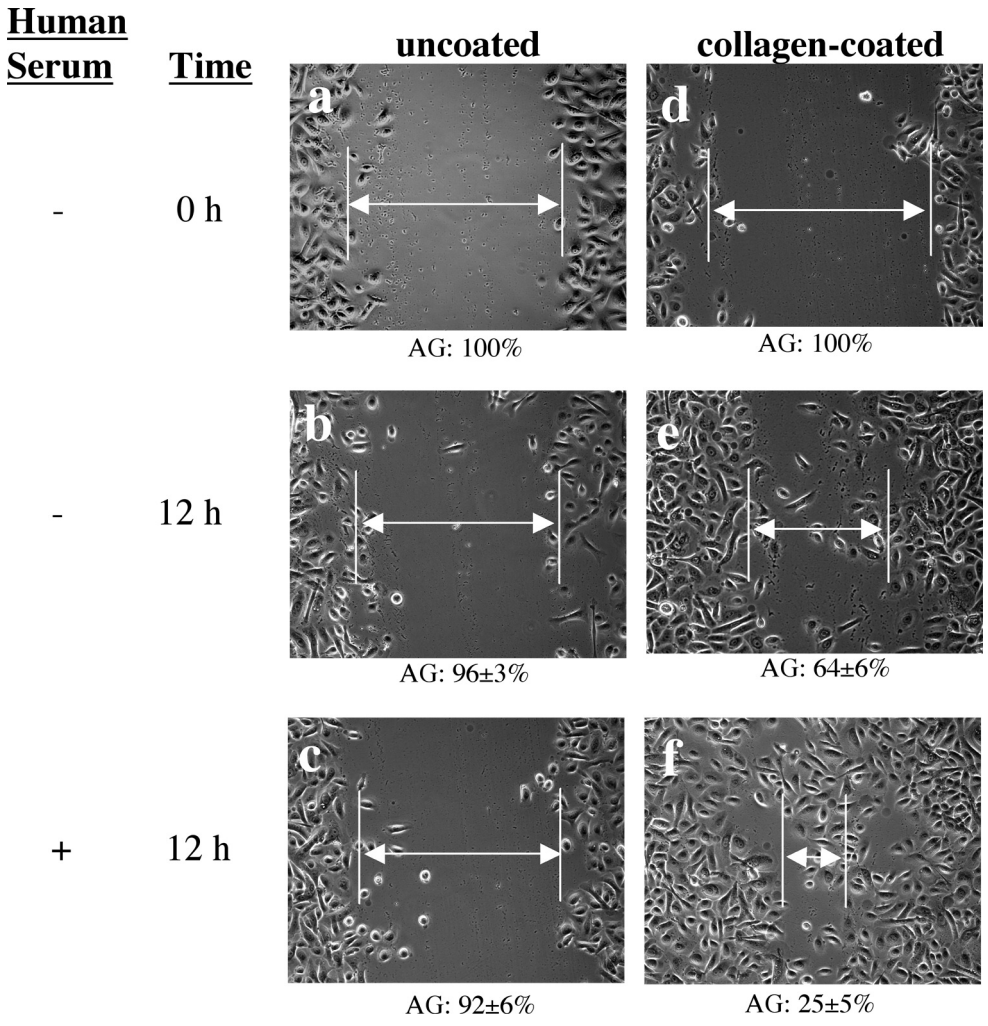

Fig. 1. Human keratinocyte migration in an *in vitro* wound healing assay. The detailed procedure of *in vitro* wound healing ("scratch") assay was as previously described (Li et al., 2004). It is emphasized that 1) the surface of the tissue culture wells was either uncoated or pre-coated with collagen prior to plating a confluent number of the cells on them, and 2) "wounds" were introduced immediately after cell attachment (within 4 hours). Cell migration was examined under the four conditions indicated. Average Gap (AG, i.e. unclosed gap of GF treated cells/control gap of untreated cells) was measured as described (Li et al., 2004).

"assistance," while GFs alone cannot. As previously mentioned, when cells bind to ECMs, integrin receptors become activated and engage in both parallel (local) and vertical (distant) signaling events. In parallel signaling, integrins cluster at the plasma membrane and are involved in the formation of focal adhesions. Focal adhesions allow the cells to polarize and generate contraction forces for subsequent cell body translocation. Integrins also activate the Rho-GTPases, Rac and Cdc42. This activation causes actin filament reassembly and the formation of lamellepodia and filopodia at the leading edge of the cell (Hall, 1998; Schwartz and Shattil, 2000; Ridley, 2001). Furthermore, integrin engagement can also activate receptor tyrosine kinases, such as EGFGR, PDGFR, VEGFR, HGFR, and the Ron receptor (Miyamoto et al., 1995; Wang et al., 1998; Moro et al., 1998; Sunberg and Rubin, 1996; Schneller et al., 1997; Woodard et al., 1998; Soldi et al., 1999; Danilkovitch-Miagkova et al., 2000). Under certain conditions, an integrin can trigger GF-independent activation of GF receptors. The co-activation of an integrin receptor and a GF receptor may be needed for optimal integrin signaling to downstream pathways (Moro et al., 1998, 2002).

In vertical signaling, integrins activate cytoplasmic signaling networks to induce expression of new gene products. For example, the important role of collagenase-1 (MMP-1) in HK migration on collagen may provide insight into the biochemical nature of the motility initiation signals. Pilcher et al. showed that HK attachment to collagen does not immediately initiate HK migration. Instead, collagen attachment first induces MMP-1 expression via an alpha2 beta1 integrin-dependent mechanism. Either membrane-bound or secreted MMP-1 cleaves native collagen into gelatin. Gelatin then initiates HK migration (Pilcher et al., 1997). These findings suggested that integrins, in addition to binding to ECMs to form focal adhesions and contraction forces, have also the ability of doing what GFs' can do, i.e. signaling to the nucleus to induce gene expression. These "dual" functions of integrins provide an explanation for why integrins possess the "basic powers" to initiate cell motility without a help of GFs. In contrast, GFs have only one of the two required powers.

Unlike ECMs, GFs alone are unable to initiate cell motility. At the molecular level, GFs stimulate optimal signal transduction only when the cells are attached to an ECM. It has been demonstrated that PDGF-BB and EGF stimulate stronger tyrosine phosphorylation of PDGFR, EGFR, and the downstream Ras-MAPK pathway in adherent cells compared with suspended cells (Miyamoto et al., 1996; Lin et al., 1997; Renshaw et al., 1997; Moro et al., 1998). Although GFs activate Rac in suspended cells, the activated Rac fails to couple to downstream effectors. In contrast, in ECM-attached cells, the ECM triggers the translocation of the activated Rac to the plasma membrane, where it connects with a downstream pathway (Del Pozo et al., 2000). Furthermore, in non-adherent cells, GF-stimulated JNK and the PI-3K-Akt pathway are suppressed (Khwaja et al., 1997; Short et al., 1998). Therefore, GFs have only one of the two required power sources for initiating cell motility. They provide "fuel"-like energy to enhance and optimize ECM-initiated cell migration, but they cannot

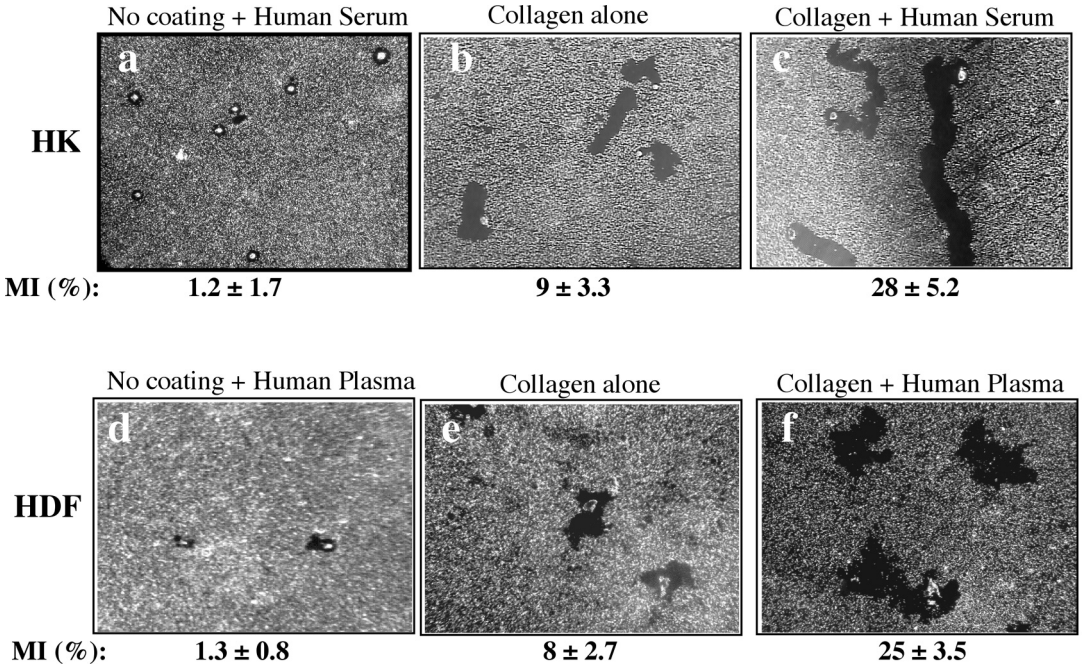

Fig. 2. Human keratinocyte and dermal fibroblast migration in a colloidal gold migration assay. The surface of the colloidal gold was either uncoated or pre-coated with collagen. Cells were plated and incubated under the four conditions indicated. Cell migration was observed by migration tracks and single cell migration tracks were quantitated as migration indices (MIs) (Woodley et al., 1988). Representative images under various conditions were shown. MIs were presented underneath. Arrows point out the cells responsible for the migration tracks.

substitute for ECM's "pavement-support" role during the migration initiation. Thus, ECMs are the primary pro-motility factors while GFs are not.

Instead, the roles of GFs are 1) to augment ECM-initiated motility and 2) to provide cell movement with directionality. There are several possible mechanisms by which GFs "fuel" the ECM-initiated cell migration. First, GF stimulation can provide cross-talk with integrins. It is a part of the so-called inside-out signaling needed to modulate the ability of cell surface integrins to bind and have altered affinity ECMs (Elliott et al., 1992; Hardy and Minguell, 1995; Maile et al., 2002; Cardo-Vila et al., 2003). Second, GFs stimulate *de novo* production of MMPs that modulate the ECM environment upon which cells will attach and migrate. In HKs, TGFß, TNFα, EGF, and HGF have been shown to stimulate the expression of MMP1, MMP-9, and MMP-13, which may play important roles in cell migration (Petersen et al., 1989, 1990; Johansson et al., 1997, 2000; McCawley et al., 1998; Sudbeck et al., 1999; Han et al., 2001). Similarly, in HDFs, PDGF and basic FGF synergize with cytokines such as IL-1α and TNFα to induce MMP-9 (Bond et al., 1998). Third, although the mechanism by which the cells migrate in certain directions in vivo remains unclear, in vitro GFs provide cell migration with directionality by the means of chemotaxis. That is, in response to a gradient of GFs, the cells polarize and migrate toward the source of GFs, resulting in directional migration. Such GF gradients likely exist in vivo in the skin wound bed, where blood coagulation converts plasma to serum and causes the secretion of a score of cytokines by platelets and inflammatory cells at the site. Based on these studies, we propose the following functional relationship between ECMs and GFs: the ECM signal plays an "active" role and the GF signal plays a "passive" role in cell migration. Together, ECMs and GF determine the optimal cell migration. This working model is summarized schematically in Figure 3.

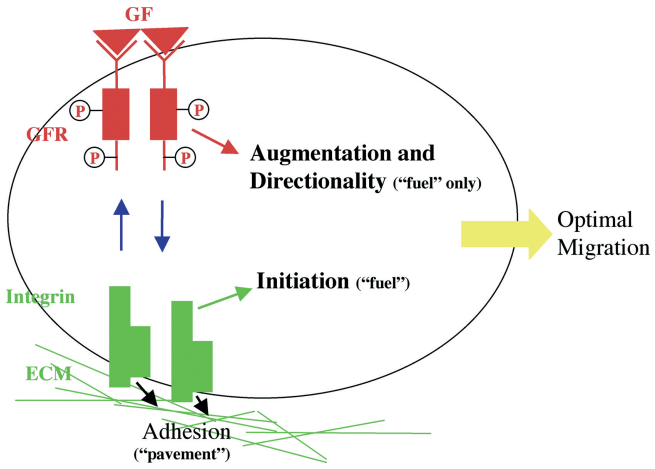

Fig. 3. A schematicrepresentation of "active" versus "passive" role of ECMs and GFs in cell motility Optimal cell migration requires the "dual signaling" of ECMs and GFs, which bind to integrins and growth factor receptors. ECMs provide both "pavement" and "fuel" effects and, therefore, are capable of initiating cell migration. GFs do not offer any "pavement" effect and only provide enhancement and directionality for ECM-initiated migration.

Future perspectives

A major challenge for skin wound healing research is to understand the mechanisms of gene expression, cell proliferation and migration of each individual cell type in the wound and to understand the dynamics of the sequential events involving multiple cell types. The administration of GFs to skin wounds in clinical trials have been overall disappointing. If ECMs are the key factors for initiating cell motility, treatment strategies to provide pro-motility ECMs first to non-healing wounds needs to be explored.

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