Glu-Leu-Arg-Negative CXC Chemokine Interferon γ Inducible Protein-9 As a Mediator of Epidermal–Dermal Communication During Wound Repair

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Normal wound healing is a complex, highly regulated dynamic process that requires co-ordinate responses of both epidermal and dermal compartments. To accomplish the healing process several growth factors, chemokines, and matrix elements signal both cell proliferation and migration during the inflammatory and reparative phases and limit these responses during the remodeling phase. We have found that the Glu-Leu-Arg-negative CXC chemokines interferon γ inducible protein 10, monokine induced by interferon γ, and platelet factor 4, limit fibroblast responsiveness to growth factors, but the functioning of these factors in wound healing remains uncertain. We hypothesized that the keratinocyte-derived member of this Glu-Leu-Arg-negative CXC family, interferon γ inducible protein 9 (IP-9) CXCL11 (also known as I-TAC, β-R1, and H-174) signals to the dermal compartment to synchronize the re-epithelialization process. Interferon γ inducible protein 9 was produced after mechanical wounding of a keratinocyte monolayer, suggesting for the first time that this could be a wound response factor. Interferon γ inducible protein 9 limited epidermal growth factor (EGF)-induced fibroblast motility (57 ± 7%) by the same protein kinase A (KA)-mediated inhibition of calpain activation and cell de-adhesion as described for interferon γ inducible protein 10. Surprisingly, interferon γ inducible protein 9 enhanced growth factor-induced motility in undifferentiated keratinocytes (137 ± 19%) as determined in a two-dimensional *in vitro* wound healing assay, and interferon γ inducible protein 9 alone promoted motility in undifferentiated keratinocytes (49 ± 10% of epidermal growth factor-induced motility). A stimulated keratinocyte/target cell coculture system revealed that interferon γ inducible protein 9 acts as a soluble keratinocyte-derived paracrine factor for both fibroblasts and keratinocytes. Further, we found that in both fibroblasts and undifferentiated keratinocytes, interferon γ inducible protein 9 exerted its action through modulation of a cytosolic protease, calpain. Interestingly, interferon γ inducible protein 9 increased calpain activity in undifferentiated keratinocytes, whereas the same chemokine inhibited the calpain activity in fibroblasts. This provides for a model whereby redifferentiated basal keratinocytes could limit fibroblast repopulation of the dermis underlying healed wounds while simultaneously promoting re-epithelialization of the remaining provisional wound. ***Key words: cell motility/CXC chemokines/epidermal growth factor receptor/calpain/wound healing. J Invest Dermatol 120:1110–1117, 2003***

Wound healing is a complex process of tissue deposition and remodeling resulting in the regeneration of an intact skin barrier and functional organ (Gailit and Clark, 1994; Thomas *et al*, 1995). The complexity of this process is accentuated by the fact that skin is comprised of two distinct compartments, the ectodermally derived epithelial epidermis and the mesodermally derived mesenchymal dermis, which are separated by a basement membrane barrier. To regenerate this organ, numerous cellular, hormonal, matrix, and enzymatic activities in each compartment are required to act in a co-ordinated manner with the other compartment. Whereas precise mechanisms are undefined, a generalized picture has emerged. Healing of skin has two important but distinct aspects, fibroplasia and epithelialization. Closure of a wound requires fibroblast proliferation and migration into granulation tissue, followed by sequential depositions of specific matrix components, and finally contraction and remodeling. The epidermal barrier must be re-established by proliferation and migration of keratinocytes over the denuded area, followed by differentiation. Both closure and epithelialization are required to restore physical integrity of the skin and prevent untoward sequelae, chiefly infection. Data are emerging that demonstrate that these two processes are temporally linked; in some human wounds if keratinocytes fail to re-epithelialize, the underlying dermis remains in a granulation tissue state (Jaffe *et al*, 1999). This communication is thought to be critical for preparing the wound bed for remodeling and

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Abbreviations: ELR, Glu-Leu-Arg; Mig, monokine induced by interferon γ; Boc-LM-CMAC, t-BOC-Leu-Met-chloromethylaminocoumarin; EGF, Epidermal growth factor; IP, interferon γ inducible protein.

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maturation, thus prevent excess scarring. Whereas it remains unknown how the epidermis and dermis co-ordinate their respective regeneration, it has been hypothesized that select molecules signal the status of wound healing between these two compartments.

During the regenerative phases of wound repair, the cells of each layer must proliferate and migrate to repopulate the nascent wound. The basal keratinocytes undergo a transition that enables such repopulation, whereas the provisional matrix is invaded by fibroblasts as the first step in regenerating the future dermal layer. The numerous growth factors present throughout repair, including high levels of epidermal growth factor (EGF) receptor ligands, are thought to promote these mitogenic and motogenic responses (Kiritsky *et al*, 1993; Steenfos, 1993; Wells *et al*, 1998; Singer and Clark, 1999). While during the initial stages of wound repair, fibroblasts and keratinocytes are driven to proliferate and migrate into the wound bed, these processes must be limited late in wound healing to prevent fibroplasia or excess matrix deposition.

The transition to the remodeling phase is marked in the dermis by cessation of the fibroplasia response and in the epidermis by redifferentiation to a stratified squamous epithelium. Thus, as the basement membrane is re-established behind the leading edge of re-epithelialization (Gipson *et al*, 1988) the keratinocytes revert to a differentiated phenotype and the underlying dermis attains the profile of nonwounded skin (Martin, 1997). As this extends deep into the dermis, soluble factors have been suggested as the key communicators to the fibroblasts that the epidermis has been re-established. Whereas the signals that trigger this maturation are still not fully deciphered, the ELR (Glu-Leu-Arg)-negative CXC chemokines have been shown to be capable of inhibiting fibroblast motility by preventing rear de-adhesion (Shiraha *et al*, 1999) and thereby converting movement to matrix contraction (Allen *et al*, 2002). One of these chemokines, interferon γ inducible protein (IP)-10 (or CXCL10) appears during this late phase, being produced by the neovascular endothelial cells deep in the regenerating wound bed (Engelhardt *et al*, 1998). A related ELR-negative CXC chemokine, IP-9 [or β-R1 (Rani *et al*, 1996), H174 (Jacobs *et al*, 1997), I-TAC (Cole *et al*, 1998), or CXCL11], is produced by basal keratinocytes in response to immune-mediated injuries (Tensen *et al*, 1999). We propose that this also appears during the process of healing as a wound response factor. These ELR-negative CXC chemokines were originally found as modulators of cells of the hematopoietic lineage; however, recently, chemokine receptors also have been found expressed and functional on mesenchymal cells (Neote *et al*, 1994; Yue *et al*, 1994; Peiper *et al*, 1995; Mach *et al*, 1999; Sauty *et al*, 1999; Shiraha *et al*, 1999; Cockwell *et al*, 2002; Grone *et al*, 2002). The ELR-negative members of the CXC family of chemokines, all of which bind to a common CXCR3 receptor, inhibit endothelial cell proliferation and migration (Gupta and Singh, 1994; Luster *et al*, 1995; Strieter *et al*, 1995) and fibroblast migration (Shiraha *et al*, 1999). These appear to act dominantly over promitogenic and promotility chemokines and growth factors (Shiraha *et al*, 1999). Thus, these chemokines, which signal through the same CXCR3 receptor (Farber, 1997; Baggiolini, 1998; Flier *et al*, 2001; Loetscher *et al*, 2001; Podovan *et al*, 2002), are candidates to signal dermal maturation.

As the above data suggest, the ELR-negative CXC chemokine IP-9 would be well situated to stop the fibroblast repopulation of the dermal layer; however, the modulators of the keratinocyte progression are unknown, though thought to involve cell–cell contact inhibition (Martin, 1997). Still, due to proximity the re-epithelializing keratinocytes would be exposed to the same factors as the dermal fibroblasts. Thus, the question remains of whether this molecule also affects the adjacent keratinocytes that need to proliferate and migrate over the provisional wound matrix. In this study we determined that IP-9 modulates the phenotypic behavior of undifferentiated keratinocytes in a manner opposite to its effects on fibroblasts. Surprisingly, IP-9 induces keratinocyte migration in contradistinction to its inhibition of fibroblast motility. Unexpectedly these diametrically opposed behaviors, seen in different cell types derived from distinct lineages, were effected through the same biophysical process of calpain-regulated de-adhesion. Calpain, a ubiquitous intracellular protease (Johnson and Guttman, 1997; Sorimachi *et al*, 1997; Sorimachi and Suzuki, 2001), is required for rear de-adhesion during the motility of adherent cells (Glading *et al*, 2000; Glading *et al*, 2001). IP-9 elicited the same protein kinase A-mediated inhibition of EGF-induced calpain activation as IP-10 in fibroblasts. In undifferentiated keratinocytes, however, IP-9 activated calpain. Initial studies suggest that EGF and IP-9 induce cell migration via differential usage of calpain isoforms. These findings not only explain the diametrically opposite effects of the same ligand in different cell types and provide for contextually appropriate though divergent cell responses but also highlight the epigenetic controls of cell decision making during complex physiologic responses.

MATERIALS AND METHODS

Materials Hs68 normal human diploid fibroblasts were purchased from American Type Culture Collection (ATCC, Rockville, MD). Hs68 cells were used at passages 5–12, representing PDR 21–42, prior to any significant deficit due to *in vitro* aging (Shiraha *et al*, 2000). Human neonatal foreskin epidermal keratinocytes were obtained from Cascade Biologicals, Inc. (Portland, OR) and the cultures were used at passages 2–9. IP-9 and IP-10 were purchased from Peprotech (Rock Hill, NJ). Recombinant human interferon (IFN)-γ was obtained from R&D Systems, Inc. (Minneapolis, MN). Human recombinant EGF was obtained from Collaborative Biomedical Products (Bedford, MA). Antibody against I-TAC/IP-9 was obtained from Peprotech. Calpain inhibitor I ALLN (CI-1) was obtained from Biomol (Plymouth Meeting, PA). Rp-8-Bromo-cyclic adenosine monophosphate was purchased from Calbiochem (La Jolla, CA). Boc-LM-CMAC (t-butoxycarbonyl-leu-met-chloromethylaminocoumarin) was obtained from Molecular Probes (Eugene, OR). Mitomycin C was obtained from Sigma (St Louis, MO). Human monokine induced by interferon γ (MIG) antibody was obtained from R&D Systems, Inc. The use of the anonymized excess tissue specimens and human primary cells were deemed exempt by the University of Pittsburgh IRB upon review.

Fibroblast and keratinocyte cultures Hs68 fibroblasts were grown in Dulbecco minimal Eagle's medium with 10% fetal bovine serum, 1 × antibiotics (penicillin and streptomycin), 1 × nonessential amino acids, and 1 × sodium pyruvate (all from Life Technologies, Rockville, MD) (Shiraha *et al*, 1999). When the cells reached 80% confluence they were quiesced in Dulbecco minimal Eagle's medium with 0.1% dialyzed fetal bovine serum for 48 h; this media switch reduces cell proliferation and thymidine incorporation over 90% while maintaining viability (Shiraha *et al*, 1999). Human epidermal keratinocytes were grown in EpiLife medium [serum free medium with human EGF (10.2 ng per ml), hydrocortisone (0.18 μg per ml), bovine pituitary extract (0.2% v/v), bovine insulin (5 μg per ml), and transferrin (5 μg per ml)] (Cascade Biologicals) containing low calcium (0.06 mM) to maintain a dedifferentiated, proliferative, and migratory state. This state of the keratinocytes will be referred to as "undifferentiated" to distinguish them from polarized keratinocytes noted upon a switch to high calcium (0.37 mM) (Eckert and Rorke, 1989; Gibson *et al*, 1996). Cells were quiesced in EpiLife medium without EGF, bovine pituitary extract, and insulin for 48 h; this media switch abolishes cell proliferation while maintaining viability (cell number at 48 h under these conditions is 94 ± 6% of that at 24 h; p is not significant).

Keratinocyte–fibroblast cocultures Normal human neonatal foreskin keratinocytes were seeded on a Transwell clear inserts of polyester membrane with a 0.4 μm pore size and a diameter of 12 mm for 12-well plates (Costar Corporation, Corning, NY). Normal human Hs68 fibroblasts were plated on the lower chambers of 12-well plastic dishes and were grown to confluence. After both the keratinocytes and fibroblasts reached confluence, the cells were quiesced in medium containing 0.1% dialyzed serum for fibroblasts and growth factor-deficient EpiLife medium for keratinocytes. The inserts containing the keratinocytes were transferred on to 12-well dishes with the fibroblasts. The keratinocytes were then stimulated with 2000 U of human IFN-γ for 24 h. Two different conditions served as controls. Inserts with keratinocytes but not treated with IFN-γ and inserts without keratinocytes but treated with IFN-γ. Cell migration assay of the fibroblasts was performed and

photographs were taken at 0 and 24 h and the relative distance traveled determined.

To determine the role of IP-9 in culture cross-attenuation, IP-9 production was abrogated by anti-sense phosphothiorated oligonucleotides against IP-9 (TTTCAGATGCCCTTTTCCAG; Tensen *et al*, 1999). Cells were seeded and cocultured as described above and quiesced. The keratinocytes in the quiescent media were treated with anti-sense for IP-9 for 48 h prior to the assay. In parallel, immunoblotting for IP-9 demonstrated that the treatment blocked IFN-induced IP-9 production.

Keratinocyte–keratinocyte cocultures Normal human neonatal foreskin keratinocytes were seeded on both Transwell clear inserts of polyester membrane with a 0.4 μm pore size and a diameter of 12 mm for well plates (Costar Corporation) and also on the lower chamber of 12-well plastic dishes and were grown to confluence. After the cells become confluent on both the chambers, the cells were quiesced. The keratinocytes in the inserts were stimulated with 2000 U of human IFN-γ for 24 h, and also in the other set the inserts were stimulated with IFN-γ for a few hours, washed, and then brought in contact with the keratinocytes in the lower chamber and left for 24 h. Cell migration assay of the keratinocytes were performed and photographs were taken at 0 and 24 h and the relative distance traveled determined.

Cell migration assay An "*in-vitro* wound" assay was performed in both fibroblasts and keratinocytes. Keratinocytes (40,000 per cm^2) and fibroblasts (30,000 per cm^2) were plated in tissue culture dishes. After they reach 80% confluence the cells were quiesced in Dulbecco minimal Eagle's medium containing 0.1% dialyzed serum for fibroblasts and growth factor deficient EpiLife medium for undifferentiated keratinocytes for 48h prior to being denuded by a rubber policeman at the center of the plate. EGF-induced cell migration was assessed by the ability of the cells to move into an acellular area in both fibroblasts and undifferentiated keratinocytes (Chen *et al*, 1994). The cells were then treated with or without EGF (1 nM), IP-9 (50 ng per ml), IP-10 (50 ng per ml), and CI-1 (5 μg per ml), and incubated at 37°C for 24 h; these concentrations were determined empirically to provide either maximum motility or inhibition without toxicity (Shiraha *et al*, 1999; Glading *et al*, 2000). Photographs were taken at 0 and between 23 and 24 h, and the relative distance traveled by the cells at the acellular front was determined by computer-assisted image analysis; markings on the plate ensured measurement of the same site for the photographs. The residual gap between the migrating fibroblasts and keratinocytes was measured and expressed as a percentage of the area of the wound remaining unfilled as normalized to EGF-induced "healing" of the wound. We examined cells at 40-fold magnification, one field (approximately 60–70 cells) with three measurements in each field, each experiment performed in duplicate or triplicate as mentioned. To confirm that cell proliferation was not confounding analyses (Chen *et al*, 1994), mitomycin C (0.5 μg per ml) was added at the time of wounding; the results of the 24 h motility assay were indistinguishable in the presence or absence of mitomycin C (data not shown). The continuous inclusion of mitomycin C (0.5 μg per ml) prevents cell proliferation in fibroblasts (Chen *et al*, 1994) and keratinocytes. For the undifferentiated keratinocytes the addition of mitomycin C to the quiescent media had no appreciable effect as there is no demonstrable increase in cell number for the 24 h period of the assay (98±6% of the cells at 48 h compared with 24 h); in complete media where the cell number increases over this period, mitomycin C reduced the increase in cell number down to 15±7% of the increased number in the absence of mitomycin C (a reduction of 85%).

Immunoblotting procedures IP-9 production was stimulated by creating multiple cross-hatched "wounds" in a monolayer of undifferentiated keratinocytes. Cells were lyzed at various times (0 h, 2 h, 6 h, 24 h, 48 h) after wounding with a rubber policeman. IFN-γ stimulated cells served as the positive control (Tensen *et al*, 1999). Cell lysates were separated on 18% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA). Blots were probed with anti-human I-TAC/IP-9 (Peprotech) and then incubated with an alkaline phosphatase secondary antibody followed by development with the NBT/BCIP substrate (Promega, Madison, WI). EGF (10 nM), platelet-derived growth factor (10 μg per ml), IP-9 (50 ng per ml), and IP-10 (50 ng per ml) were also tested for induction of IP-9 in undifferentiated and differentiated keratinocytes.

Similarly, cells treated with IFN-γ and with anti-sense oligonucleotides against IP-9 were lysed after 6 h and 24 h and were separated on 18% for IP-9 and 15% for MIG, probed for I-TAC/IP-9 and MIG. Blots were incubated and developed as described above.

Calpain activity assays The calpain activity in individual living cells was detected by using a Boc-LM-CMAC assay for *in vivo* proteolysis (Glading *et al*, 2000). In brief, keratinocytes and fibroblasts were plated on glass coverslips. The cells were quiesced when at 50% confluence for 48 h and then treated in the presence or absence of IP-9, CI-1, and/or EGF for 2 h, 1 h, 30 min, and 10 min, respectively. All cells were loaded with Boc-LM-CMAC for 20 min prior to mounting on glass slides. The treated and control cells were then observed for CMAC fluorescence using an Olympus fluorescent microscope (model: BX40, filter: Olympus M-NUA). Representative images of each slide were captured using a SPOTII CCD camera. (Nikon, Inc, New York, NY, USA). The image exposure settings were identical within each experiment (i.e., for no EGF and EGF treatment) but did vary slightly between experiments; thus, one can directly compare fluorescence intensity within an experiment but not between experiments. Images shown are representative of three or more separate experiments.

Adhesion assay Cell substratum adhesiveness of fibroblasts and undifferentiated keratinocytes were quantitated using an inverted centrifugation detachment assay. Cells were plated at the concentration of 10^5 cells per ml in a 12-well plate. Cells were quiesced for 24 h and then incubated with IP-9, CI-1, or EGF for 4 h, 30 min, or 10 min, respectively. Wells were completely filled with Dulbecco minimal Eagle's medium and EpiLife media, respectively, supplemented with 1% bovine serum albumin and 25 mM HEPES (pH 7.4), and then sealed with enzyme-linked immunosorbent assay sealing tape (Corning, Cambridge, MA) and centrifuged inverted for 10 min at $2920 \times g$ at 37°C using a Beckman CS6R plate centrifuge; (Beckmen Coulter Inc, Fullerton, CA) $2920 \times g$ was chosen empirically as the force required to detach approximately half of the EGF-treated cells. Before and after the centrifugation, the number of cells on the plates was counted by phase-contrast microscopy.

RESULTS

IP-9 is expressed in response to "wounding" a keratinocyte monolayer We hypothesize that IP-9 could be a "wound response" protein. If this is the case, then keratinocytes would be expected to produce IP-9 when wounded, although it is possible that IP-9 occurs secondary to cytokines generated during the inflammatory phase; this latter possibility would not minimize the role of IP-9 in repair but would have implications for the biology of wound repair and potential therapeutic interventions. Interestingly, IP-9 production could be stimulated by mechanical wounding of a monolayer of keratinocytes (**Fig 1**). IP-9 was not induced by EGF, platelet-derived growth factor, or IP-10 (data not shown). These findings prove that IP-9 could be stimulated secondary to mechanical wounding *per se*, or autocrine factors released from wounded keratinocytes.

IP-9 limits EGF receptor-mediated calpain activity and motility in human fibroblasts The expression of IP-9 by wounded keratinocytes prompted us to study the effect of this ELR-negative CXC chemokine on the major cell types in the epidermal and dermal compartments. Both fibroblasts and keratinocytes migrate and proliferate during dermal and

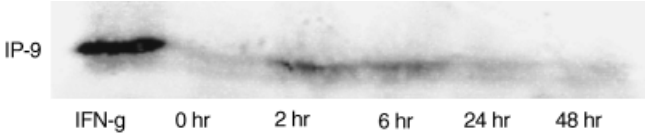


Figure 1. IP-9 is a wound response factor. IP-9 is induced by a mechanical wounding keratinocyte (undifferentiated) monolayer. Keratinocytes were grown to confluence in a six-well tissue culture plate and kept in low calcium media. Multiple cross-hatched "wounds" were created with a rubber policeman to affect a small percentage of the cells on the plate (< 5%). Cells were lyzed at different times and analyzed by immunoblot using an IP-9 specific antibody. IP-9 production *in vitro* after wounding increases by 2 h and reaching peak at about 6 h and gradually decreasing by 48 h. *Lane 1* represents the production of IP-9 after stimulation with IFN-γ. Shown is a representative immunoblot of three experiments.

epidermal regeneration. As IP-9 shares the same CXCR3 receptor as IP-10 (Farber, 1997; Baggiolini, 1998) it was not surprising that IP-9 limited EGF-induced motility (to $57 \pm 7\%$; $p < 0.05$) similarly to IP-10 (to $45 \pm 8\%$; $p < 0.05$ compared with EGF alone; p = not significant between IP-9 and IP-10 inhibition of EGF-induced motility) (**Fig 2*a***) (Shiraha *et al*, 1999). The basal, haptokinetic motility of the fibroblasts was unchanged by either chemokine. This inhibition of EGF-induced fibroblast migration was effected through a cyclic adenosine monophosphate/protein kinase A signaling pathway (Shiraha *et al*, 2002) as Rp-8Br-cyclic adenosine monophosphate blocked the inhibitory effect (**Fig 2*b***). In addition, EGF-induced fibroblast proliferation was not affected by IP-9, similar to IP-10 (Shiraha *et al*, 1999) (data not shown). IP-9 also inhibited EGF-induced calpain activity (to $25 \pm 5\%$; $p < 0.05$) (**Fig 2*c***). These data suggest that IP-9 functions similarly to IP-10 in preventing fibroblast migration (Shiraha *et al*, 1999).

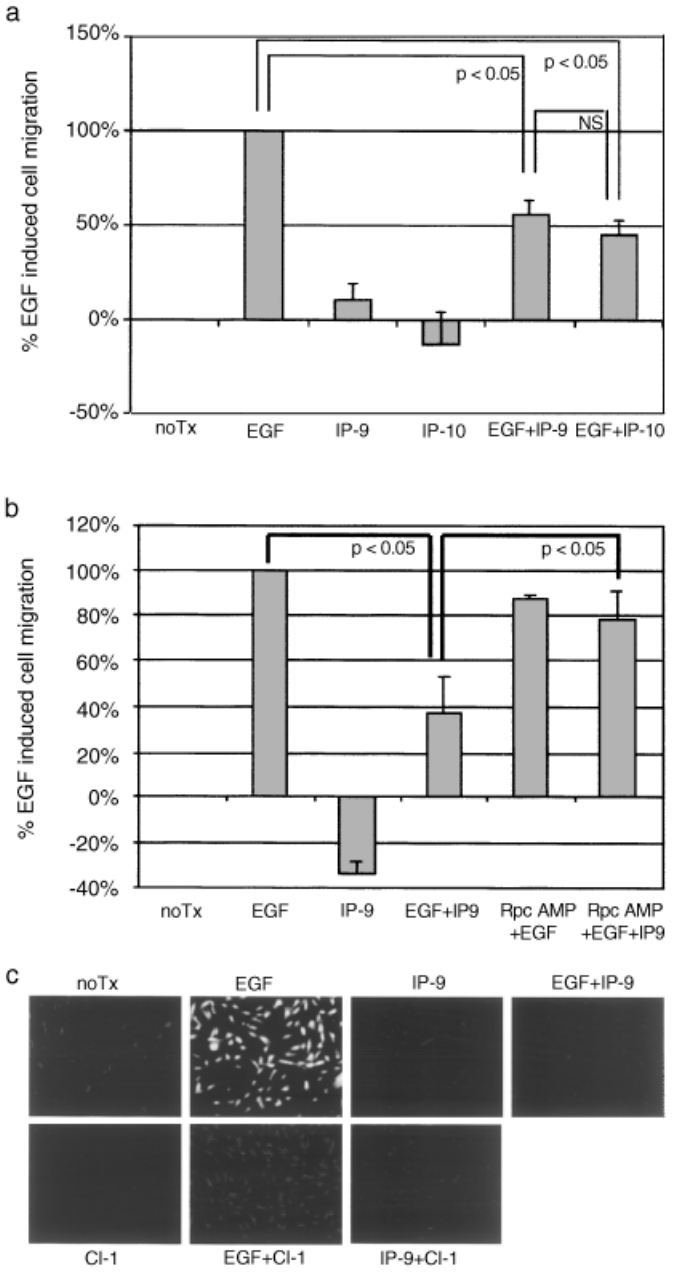


Figure 2. IP-9 and IP-10 inhibit EGF-induced cell migration (*a,b*) and calpain activation (*c*) in dermal fibroblasts. (*a,b*) Early passage human dermal fibroblasts were tested for motility in an *in vitro* wound healing assay. Cells were treated with EGF (1 nM) or IP-9 (50 ng per ml) throughout the assay; Rp-8Br-cyclic adenosine monophosphate (50 μM) was also present throughout. The values are shown as the ratio of the EGF (1 nM)-induced cell motility. (*c*) BOC fluorescence was determined after treatment with EGF (1 nM) for 10 min or IP-9 (50 ng per ml) for 120 min. The times were chosen empirically for maximal fluorescence (in the case of IP-9, this was determined based on undifferentiated keratinocytes, see below); CI-I (5 μg per ml) was added 30 min prior to ligand. The values are mean ± SEM of six independent studies each performed in triplicate for motility or three experiments for calpain activation. Statistical analysis was performed by Student's t test.

EGF-induced fibroblast migration is blocked by keratinocyte-produced IP-9 From the foregoing data we predicted that keratinocytes limit fibroblast motility secondary to IP-9 acting as a soluble paracrine factor. This was tested *in vitro* by a Transwell coculture-based wound assay. Keratinocytes in the inserts were exposed to IFN-γ (2000 U) to stimulate IP-9 production; IFN-γ was added to empty inserts as a control. EGF-induced migration of the human dermal fibroblasts was diminished in the wells in which the keratinocytes were treated with IFN-γ (**Fig 3*a***); IFN-γ alone had no effect on fibroblast migration. To ascertain that this abrogation of motility was due to IP-9 we prevented IFN-γ-induced IP-9 production using anti-sense oligonucleotides. In the face of anti-sense blockade of IP-9, IFN-γ treatment of keratinocytes had no effect on fibroblast cell motility (**Fig 3*b***). IFN-γ-induced IP-9 production was blocked by anti-sense oligonucleotides to IP-9 (**Fig 3*c***). Anti-sense to IP-9 alone did not significantly affect either basal or EGF-stimulated motility; with the sense control oligonucleotide having no effect on motility (**Fig 3*b***). The anti-sense oligonucleotide was specific for blocking IP-9 production, as production of a related IFN-γ-induced protein, Mig, was unaffected by either sense or anti-sense oligonucleotides to IP-9 (**Fig 3*d***). That Mig production had no effect on fibroblast migration is likely due to the fact that it is only weakly produced and is detectable only after extended time periods, whereas IP-9 is rapidly and robustly produced in response to IFN-γ. These findings strongly support a paracrine model of cross-attenuation of stromal cell motility by epithelial-derived factors.

IP-9 promotes cell migration and calpain activity in undifferentiated human keratinocytes The suppression of motility in fibroblasts underlying basal keratinocytes would signal a switch from wound repair to dermal remodeling; however, the adjacent keratinocytes need to migrate to re-epithelialize the wound. Therefore, we determined whether undifferentiated keratinocytes were similarly inhibited by IP-9. Surprisingly, IP-9 not only enhanced EGF-induced motility (to $137 \pm 19\%$; $p < 0.05$) and calpain activity but actually promoted motility ($51 \pm 11\%$; $p < 0.05$ compared with no treatment) and calpain activity by itself (**Fig 4*a,b***). The concentration used provided maximal motility stimulation, with half maximal being at approximately 25 ng per ml IP-9 promoting motility at $\approx$ 20% of the EGF-induced maximal level. Even at these lower IP-9 concentration, the motility effect appears additive to that of EGF (10 ng per ml IP-9 $> 112 \pm 10\%$; 25 ng per ml $> 137 \pm 17\%$; 50 ng per ml $> 155 \pm 15\%$). As expected, IP-10 acted similarly to promote motility ($61 \pm 17\%$) and enhance the EGF-induced response ($157 \pm 30\%$) ($p > 0.10$ between IP-9 and IP-10 treatments). These data suggested that the CXCR3 signaling pathways were fundamentally different between the two cell types.

IP-9 induces keratinocytes migration in a paracrine fashion To further assess whether the increase in cell migration in keratinocytes by IP-9 may be physiologically relevant we employed the coculture system. We could induce keratinocyte motility when a separate keratinocyte monolayer was treated with IFN-γ (**Fig 5**). This occurred even when the keratinocytes in the insert were exposed to IFN-γ in a distant culture dish and

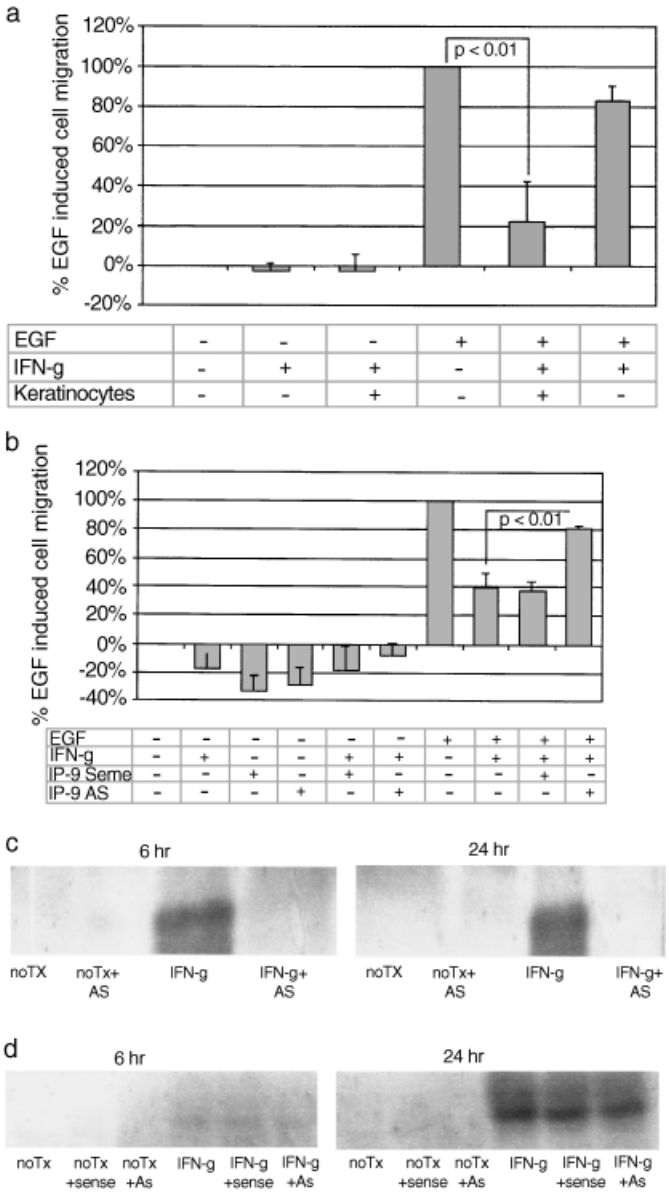


Figure 3. Keratinocyte-derived IP-9 abrogates EGF-induced fibroblast motility. (*a*) Inserts with or without keratinocytes were treated with IFN-γ (2000 U) or EGF (1 nM) for 24 h and the fibroblasts in the bottom wells were tested for motility in an *in vitro* wound healing assay. The values are shown as the ratio of the EGF-induced cell motility of three experiments each in duplicate. (*b*) Fibroblast cell motility was determined by a coculture *in vitro* wound healing assay in which the keratinocytes were exposed to anti-sense oligonucleotides against IP-9 along with sense oligonucleotides serving as controls. The values are shown as the ratio of the EGF (1 nM)-induced cell motility of three experiments each in duplicate. Statistical analyses were performed by Student's t test for both (*a*) and (*b*). (*c*) Keratinocytes were stimulated with IFN-γ (2000 U) in the presence or absence of phosphothiorated oligonucleotides (20 μM) for the designated times. Cells were then lyzed and IP-9 production determined by immunoblotting. Shown is one of two determinations. (*d*) Keratinocytes were stimulated with IFN-γ (2000 U) in the presence or absence of phosphothiorated oligonucleotides targeting IP-9 (both sense and anti-sense 20 μM). Cells were then lyzed and immunoblotted for Mig production.

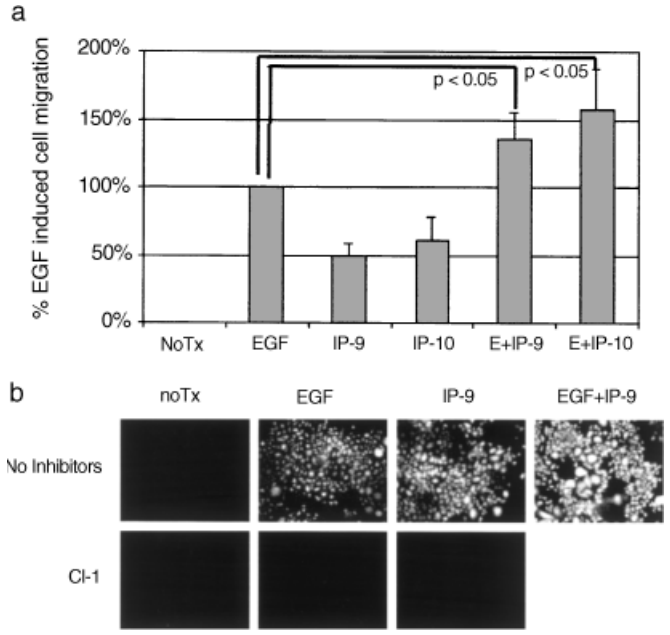


Figure 4. IP-9 and IP-10 promote cell migration and calpain activity in undifferentiated keratinocytes. (*a*) Early passage human keratinocytes were tested for induced motility in an *in vitro* wound healing assay. Cells were treated with EGF (1 nM) or IP-9 (50 ng per ml) throughout the assay. The values are shown as the normalized ratio of the EGF (1 nM)-induced cell motility of nine experiments each performed in duplicate or triplicate. (*b*) Undifferentiated keratinocytes were tested for calpain activation following stimulation by IP-9 (50 ng per ml) for 120 min or EGF (1 nM) for 10 min by the intracellular cleavage of the synthetic substrate Boc-LM-CMAC and subsequent fluorescence as shown by a representative experiment of three.

washed prior to generating the coculture (data not shown). That the motility was slightly if not statistically significantly higher in the IFN-γ-treated than the IP-9-treated cells might be due to proper presentation of ligand, avoiding the problems of receptor desensitization due to an initial bolus. Anti-sense oligonucleotide abrogation of IP-9 prevented the paracrine induction of keratinocyte motility (**Fig 5**). The anti-sense oligonucleotides actually reduced cell motility below baseline, which may reflect a basal level of IP-9 generation promoting cell motility. Whatever the reason for this, the important result is that the paracrine signaling is dependent on IP-9 production.

IP-9 induces keratinocyte motility via a calpain-dependent process The activation of calpain by IP-9 in undifferentiated keratinocytes would be predicted to increase motility (Huttenlocher *et al*, 1997; Palecek *et al*, 1998); however, this needed to be tested directly. Inhibition of calpain by CI-1 (5 μg per ml) prevented induction of motility by either EGF or IP-9 in undifferentiated keratinocytes (**Fig 6**). This inhibitor also abrogated calpain activity triggered by IP-9 (**Fig 4**).

IP-9, via calpain, controls fibroblast and keratinocyte de-adhesion Calpain activity is required for lessening the adhesion of cells to substratum to enable productive motility (Huttenlocher *et al*, 1997; Glading *et al*, 2000). If IP-9 is acting via calpain then de-adhesion should be modulated in the two major cell types. Using an inverted centrifugation assay, which enables the detection of altered adhesiveness of even tightly adherent cells (Shiraha *et al*, 1999; Glading *et al*, 2000), we found that either EGF or IP-9 reduced the adhesiveness of undifferentiated keratinocytes (**Fig 7*a***). At a force empirically determined to dislodge approximately half the cells upon EGF treatment for 30 min and IP-9 for 2 h, but few if any of untreated cells, calpain inhibitor I prevented EGF and IP-9 induced cell de-adhesion. Interestingly, IP-9-induced de-adhesion was additive with EGF ($p < 0.05$ compared with EGF or IP-9 alone). In fibroblasts IP-9 acted oppositely, abrogating EGF-induced de-adhesion (**Fig 7*b***) similar to IP-10 (Shiraha *et al*, 1999).

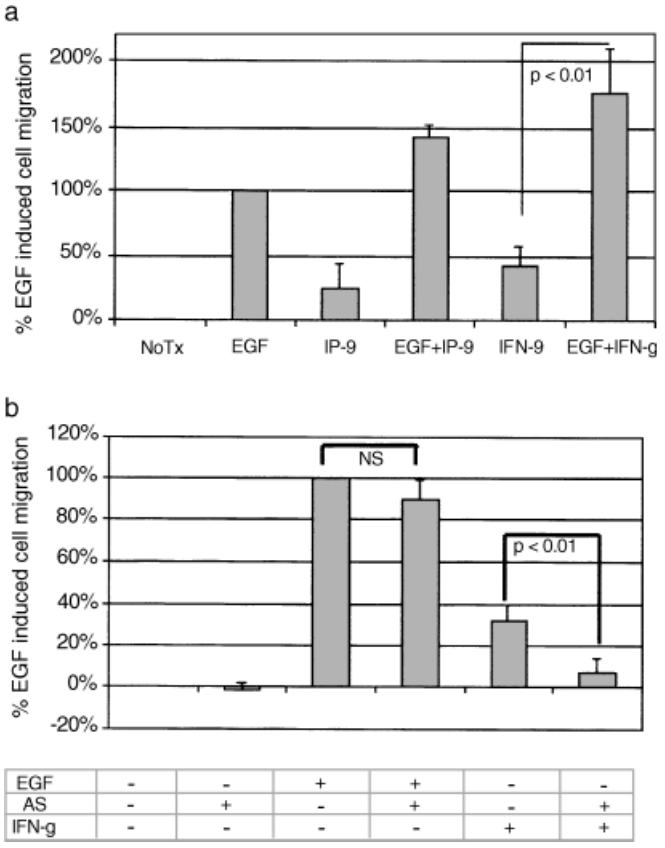


Figure 5. Keratinocytes derived IP-9-induced cell migration in undifferentiated keratinocytes . (*a*) Inserts with keratinocytes were treated with IFN-γ (2000 U), IP-9 (50 ng per ml), or EGF (1 nM) for 24 h, and the keratinocytes in the bottom wells were tested for motility in an *in vitro* wound healing assay. IFN-γ-induced motility, in the absence or presence of EGF, was statistically no different from IP-9 treated cells; however, these cells were more motile than the no treatment or EGF alone treated cells, respectively ($p < 0.05$). (*b*) Inserts with keratinocytes were treated with IFN-γ (2000 U) in the presence or absence of phosphothiorated anti-sense oligonucleotides against IP-9 for 24 h, and the keratinocytes in the bottom wells were tested for motility in an *in vitro* wound healing assay. The values are shown as the ratio of the EGF (1 nM)-induced cell motility of at least two experiments each in duplicate. Statistical analysis was performed by Student's t test.

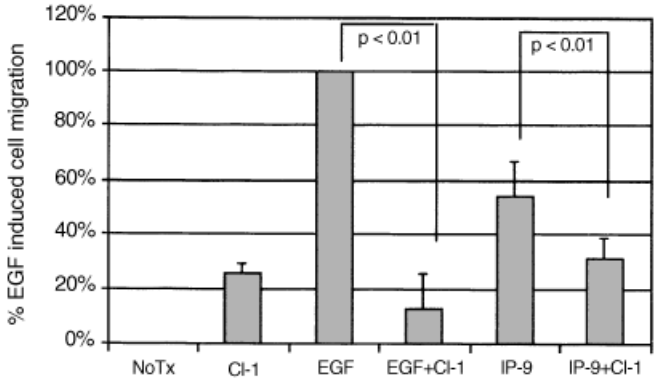


Figure 6. IP-9-induced cell migration requires calpain activation in keratinocyte. Pharmacologic inhibition of calpain by CI-1 (5 μg per ml) limited both EGF- and IP-9-induced motility in both undifferentiated keratinocytes. The values are shown as the ratio of the EGF (1 nM)-induced cell motility. The values are mean ± SEM of six independent studies each performed in triplicates. Statistical analysis was performed by Student's t test.

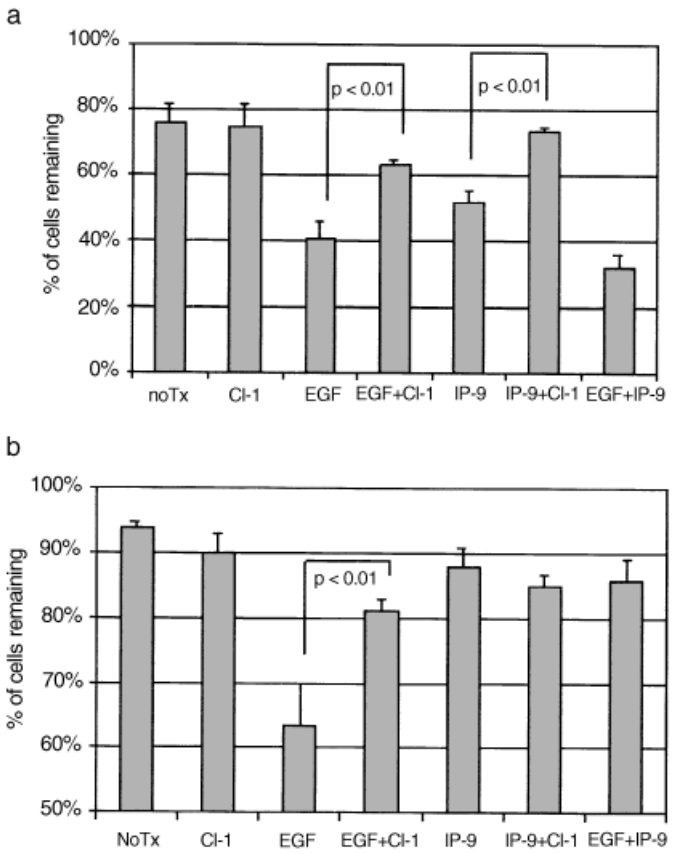


Figure 7. EGF and IP-9 promote cell de-adhesion in undifferentiated keratinocytes but IP-9 inhibits de-adhesion in fibroblasts. De-adhesion was performed on keratinocytes (*a*) or fibroblasts (*b*) after treatment with EGF (1 nM) for 30 min or IP-9 (50 ng per ml) for 120 min; CI-I (5 μg per ml) was added 30 min prior to ligand. Values are calculated as percentage of precentrifugation cells remaining adherent. The values are mean ± SEM of six independent studies each performed in triplicates. Statistical analysis was performed by Student's t test.

DISCUSSION

The aim of this study was to determine if soluble signals from keratinocytes might be the co-ordinators of dermal and epidermal events during the process of wound healing. We focused on a putative agent for this communication, the secreted soluble ELR-negative CXC chemokine IP-9, as it activates the same receptor as IP-10 that we earlier demonstrated to abrogate EGF-induced fibroblast motility. Herein, we report that IP-9 could indeed be a wound response factor as it is produced upon mechanical wounding of monolayer cultures of undifferentiated keratinocytes. Unexpectedly, this chemokine induced opposite behaviors for the different cell types of fibroblasts and keratinocytes in promoting the motility of undifferentiated keratinocytes that reflect *in vitro* the *in vivo* phenotype of the keratinocytes needed to re-epithelialize the wound bed. Intriguingly, this induction of motility occurs via positive modulation of the same calpain-dependent de-adhesion mechanism that IP-9 negatively impacts in fibroblasts. These findings provide for a model in which the same surface receptor can effect opposite behaviors in different cell types.

Our findings explore an interesting phenomenon wherein a chemokine inhibited cell migration in dermal fibroblasts while promoting migration in keratinocytes. These diametric cell responses in distinct cell lineages would be required for appropriate wound healing. Following wound injury fibroblasts from the dermis (Lawrence and Diegelmann, 1994) and keratinocytes from the epidermis enter into the wound and migrate (Singer and

Clark, 1999). It is well established that re-epithelialization is needed for proper wound repair with appropriate dermally determined tensile properties and strength. Most of this dermal reinforcement occurs during the remodeling phase for weeks after re-epithelialization. The process of re-epithelialization begins within hours of injury and eventually results in complete resurfacing. If the keratinocytes fail to re-epithelialize, the underlying dermis appears to remain in the granulation state (Jaffe *et al*, 1999); however, for larger wounds there is a mix of wound healing phases across the wound, i.e., the central portions may remain in the inflammatory and regenerative phases even when the original margins are fully healed. This is seen as the basement membrane is reconstituted and populated by differentiated basal keratinocytes just a few cell body lengths behind the advancing wound edge. Fibroblasts underlying this healed margin would functionally need to be in the remodeling phase in which migration is minimized. Inhibition of rear detachment channels the motility associated contractile forces into matrix contraction (Allen *et al*, 2002). The ELR-negative CXC chemokines would accomplish this phenotypic change, with IP-10 being produced by the neovascular endothelial cells (Luster *et al*, 1995; Strieter *et al*, 1995) and IP-9 presumably from the undifferentiated keratinocytes (**Fig 1**). That the ability of keratinocytes to modulate negatively EGF-induced cell motility in fibroblasts via a diffusible factor that is highly likely to be IP-9 (**Figs 3 and 5**) provides proof of principle that such paracrine attenuation signaling exists; however, as IP-9 is soluble and diffusible, it would reach and affect the adjacent migrating keratinocytes, which are required for re-epithelialization. Thus, one might predict that these cells would be resistant to the anti-motility effects of IP-9 mainly by lacking the receptor. Surprisingly, these keratinocytes respond to IP-9 by increased migration. Thus, one ends up with an elegant system in which one signal might synchronize the separate dermal and epidermal compartments during epidermal repair.

These findings raise a number of questions, but also provide new opportunities and tools to pursue them. Despite the caveat that these studies have been performed *ex vivo*, and thus require animal studies to confirm extrapolation to actual wound repair, a number of findings are tenable. First, to the best of our knowledge this is one of the first reports showing that calpain is contrarily modulated by the same signal, CXCL10/11 in this situation, in different cells. As the activating cascades to the ubiquitous calpain isoforms are not described, this system may provide insight. Already, our ongoing initial studies suggest that differential usage of heterotrimer G protein isoforms might lie at the heart of the dichotomous responses in the two different cell types, which would be an intriguing mode of signal channeling at the immediate postreceptor level. As the productive targets of calpain-induced de-adhesion have yet to be fully deciphered, despite numerous tantalizing hints from both *in vitro* and *in vivo* studies (Beckerle *et al*, 1987; Yamaguchi *et al*, 1994; Carragher *et al*, 1999; Glading *et al*, 2002), the convergence of proteolysis might aid in future identifications. Second, and still to be determined, is what signals prevent further keratinocyte migration and induce redifferentiation. Obviously from our data, the signal(s) is not the same as for fibroblasts. Prime candidates are matrix components/and or contact inhibition as modulated by cell density (Wahl *et al*, 1992; Polk *et al*, 1995). Our findings of opposite effects of IP-9 on the migration of keratinocytes *vs* fibroblasts highlight the pitfalls of extrapolating findings to different cell types. Lastly, our findings suggest avenues for clinical investigations. Based upon our model of IP-9 production, which limits, at least in part, the fibroblastic response, then this signaling either may be deficient in excessive scarring or may be used to limit scarring.

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