Interleukin 6 Indirectly Induces Keratinocyte Migration

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IL-6-deficient transgenic mice (IL-6 KO) display significantly delayed cutaneous wound healing. To further elucidate the role of IL-6 in skin wound healing, epidermal keratinocyte and dermal fibroblast cells were isolated from neonatal IL-6 KO mice and treated with rmIL-6. It was found that rmIL-6 alone did not significantly modulate the proliferation or migration of cultured IL-6 KO keratinocytes. rmIL-6, however, significantly induced the migration of IL-6 KO keratinocytes (up to 5-fold) when co-cultured with dermal fibroblasts. Culture supernatants from IL-6-treated fibroblasts were also found to induce the migration of keratinocytes to a similar degree. Genomics analysis of treated fibroblasts indicated that rmIL-6 does not induce any known soluble keratinocyte migratory factors. rmIL-6 treatment of fibroblast, however, induced a rapid and sustained phosphorylation of STAT3 protein. These data indicate that IL-6 could influence wound healing by inducing keratinocyte migration through the production of a soluble fibroblast-derived factor, and its activity may be associated with STAT3 activation.

Key words: cytokines/epidermis/inflammation/wound healing


In the United States, over 6 million individuals develop chronic skin ulcers annually (Singer and Clark, 1999), and the restoration or augmentation of cutaneous wound healing has long been an elusive goal for health care professionals. Based on gross morphology, the skin can be divided into two major components: the epidermis and dermis. The epidermis is primarily made up of keratinocytes, which form stratified layers of increasingly differentiated cells, terminating in the outermost tough, keratin layer known as the stratum corneum. Beneath the epidermis, the dermis is predominantly composed of an elastic collagen matrix, which contains numerous cell types including fibroblasts, and immune cells such as Langerhans cells. Following trauma to the skin, these diverse cell types must interact and cooperate in an orderly sequence of events that, when simplified, includes coagulation, inflammation, formation of a provisional collagen matrix (granulation tissue), re-epithelialization, and remodeling (for a review, see Bello and Phillips, 2000). While much research has been accomplished to describe the many aspects of dermal and epidermal cell activity during wound healing, the precise mechanisms of the interplay between dermal and epidermal tissue during this complex process are not entirely known.

IL-6 is a pleiotropic cytokine that is involved in the growth and differentiation of numerous cell types, including those of dermal and epidermal origin (Sehgal, 1990). In the skin it is produced primarily by epidermal keratinocytes, while macrophages, Langerhans cells, and fibroblasts in the dermis represent other sources of the cytokine (Paquet and Pierard, 1996). Increased levels of IL-6 have been associated with a number of skin pathologies, such as psoriasis (Grossman et al., 1989), scleroderma (Koch et al., 1993), and systemic lupus erythematosus (Fugger et al., 1989). Overexpression of IL-6 in the skin of normal rats induces epidermal proliferation and inflammation (Sawamura et al., 1998), while transgenic mice overexpressing IL-6 display little more than a thickened stratum corneum (Turksen et al., 1992).

While it seems that the role of IL-6 is well established in disease conditions, relatively little is known about its role in skin repair. It has been well documented that an inflammatory response following cutaneous wounding is a prerequisite for healing, and inflammatory cytokines, such as IL-6, might be intimately involved in this process. For instance, glucocorticoids decrease the expression of proinflammatory cytokines in wounds, and interfere with wound repair (Hubner and Werner, 1996). Furthermore, elimination of macrophages, a major source of inflammatory cytokines, from a wounded site delays wound healing (Leibovich and Ross, 1975). IL-6 is readily detected in cutaneous wounds (Hubner and Werner, 1996; Kondo and Ohshima, 1996), and in the supernatant from keratinocyte cultures subjected to in vitro wounds (Sugawara et al., 2001). Streptozotocin-induced diabetic mice displayed delayed wound healing, and low levels of IL-6 in wound fluid (Fahey et al., 1991). IL-6 expression in dermal fibroblasts also decreases with age, which may contribute to delayed cutaneous wound healing in the elderly (Goodman and Stein, 1994). Most significantly, IL-6-deficient transgenic mice (IL-6 KO) display significantly delayed cutaneous wound healing compared to wild-type control animals (Gallucci et al., 2001b; Lin et al., 2003). Wounds from IL-6-deficient mice displayed multiple defects including delayed re-epithelialization, greatly decreased granulation tissue,
inhibited neovascularization, and required up to 3-fold longer to heal.

In this article, we describe a cooperative interaction between dermal and epidermal cells that sheds light on the role of IL-6 during wound healing. We detail the effects of rmIL-6 on dermal and epidermal cell proliferation and migration in an in vitro culture system utilizing skin cells from neonatal IL-6 KO mice. This model allows for the examination of the effects of this cytokine without the confounding influence of endogenously produced cytokine. We investigate the possible identity of an IL-6-induced fibroblast-derived keratinocyte migratory factor by genomic analysis, and the possible signaling pathways that may lead to its induction.

Results

IL-6 is not a mitogen for keratinocytes A mechanism by which IL-6 could affect wound healing is through increased mitogenesis of epidermal keratinocytes. To test this hypothesis, freshly isolated epidermal keratinocytes from IL-6 KO mice were incubated with various concentrations (0–500 ng per mL) of rmIL-6 or KGM for 16 h. DNA synthesis was assessed by BrdU uptake using a commercially available ELISA kit. KGM media induced significant DNA synthesis in cultured keratinocytes compared to SFKGM control (Fig 1). Treatment with rmIL-6 did not induce a significant increase in BrdU incorporation at any of the concentrations utilized in either SFKGM or KGM.

IL-6 indirectly modulates keratinocyte migration through a soluble fibroblast-derived motogenic factor

One of the primary activities of wound keratinocytes is migration across the wound bed. IL-6 could affect this process in either a direct manner, or indirectly by inducing the production of migratory factors from nearby cells in the dermis. Notably, the dermis contains numerous fibroblasts, which upon wounding proliferate and are the primary cells found in granulation tissue. To investigate this hypothesis, freshly isolated keratinocytes were seeded into tissue culture-treated transwell inserts (8 μm pore) and exposed to KGM or various concentrations of rmIL-6, with or without co-culture with fibroblasts. The cultures were allowed to incubate for 16 h, and migrating keratinocytes were determined by enumerating the cells that had migrated across the porous membrane via light microscopy. Treatment with rmIL-6 from 1 to 100 ng per mL did not induce migration of keratinocytes across the transwell inserts (data not shown). When keratinocytes from IL-6 KO mice are cocultured with dermal fibroblasts (also from IL-6 KO mice) and exposed to rmIL-6 however, the keratinocytes readily migrated across transwell inserts in the absence of collagen or other matrix proteins (Fig 2). Culture supernatants collected from overnight cultures of fibroblasts treated with rmIL-6 also induce migration of keratinocytes (Fig 3). When IL-6-treated fibroblast culture supernatants were heat treated (100 °C, 5 min), the motogenic potential of the supernatants was completely abrogated (Fig 3).

One of the stimuli that induces keratinocyte migration is contact with collagen I from damaged cell matrix in the wound (Pilcher et al., 1997). Others have shown that IL-6 alone could induce keratinocyte migration across a collagen matrix (Sano et al., 1999). To determine if FCM could influence epidermal cell migration on a collagen matrix, primary keratinocytes from IL-6 KO mice were prepared as described above, and seeded onto 100 mm collagen I-coated plates. Once confluent, the cells were serum and growth factor starved for 8 h. An in vitro “wound” was produced and the cells were incubated with 10 ng per mL rmIL-6, FCM, or SFKGM for 24 h. Cells migrating across the wound margin were enumerated in specific microscope fields that had been designated and photographed the previous day for comparison. As shown in Fig 4A and D, rmIL-6 alone induced migration to a minor, but insignificant degree when compared to serum-free media control. FCM,

![Figure 1](image)

**Figure 1**

IL-6 is not a mitogen for keratinocytes from IL-6 KO mice. Epidermal keratinocytes were isolated from IL-6 KO mice, and seeded on collagen I-coated 96-well plates. Cells were serum/growth factor starved for 4 h, treatments were applied in quadruplicate, and incubated 16–20 h at 37 °C and 5% CO₂. Following incubation, 10 μL of 0.1 mM BrdU was added to each well, and the plate incubated an additional 8 h. BrdU uptake was determined by ELISA according to the manufacturer’s instructions (Roche, Indianapolis, Indiana). Results were obtained via an ELISA plate reader, and raw data expressed as mean ± SEM (n = 4) percent negative (non-treated) control.

![Figure 2](image)

**Figure 2**

IL-6 induces keratinocyte migration indirectly through a dermal cell-produced soluble factor. Dermal cells (primary fibroblasts) and epidermal keratinocytes from IL-6 KO mice were cultured in 24-well tissue culture dishes to approximately 80% confluency. Transwell culture inserts (8 μm, tissue culture treated) were placed in the wells, and IL-6 KO keratinocytes were seeded into the transwell enclosures. Cultures were cultured in complete media (KGM), or treated with the indicated concentrations of rmIL-6 for 16–18 h in serum-free/supplement-free media (SFKGM) at 37 °C and 5% CO₂. Following incubation, transwell membranes were stained with hematoxylin, and migrating cells were enumerated by light microscopy. Data are expressed as mean +/−SEM (n = 7) number of cells counted in five random fields when viewed at ×20 magnification approximately 800 μm (∗significantly different from serum-free media control, p < 0.05).
however, induced significant migration across the wound margin (Fig 4A and E).

One mechanism by which apparent migration could occur is merely by spreading of keratinocytes as a result of proliferation. To test this hypothesis, freshly isolated epidermal keratinocytes from IL-6 KO mice were incubated with SFKGM, FCM with or without 10 ng per mL rmIL-6, or KGM media for 16 h. DNA synthesis was assessed by BrdU uptake using a commercially available ELISA kit as described above. The results of these assays showed that neither non-treated nor IL-6-treated fibroblast culture supernatants induced a significant mitogenic response from keratinocytes as determined by DNA synthesis (Fig 5).

IL-6 does not promote mitogenesis or survival of fibroblasts in culture To determine whether the apparent induction of a fibroblast-derived migratory factor was associated with the proliferation or survival of dermal fibroblasts in response to rmIL-6 treatment, cells were assayed for DNA replication and viability by BrdU uptake (ELISA) and WST (tetrazolium dye) assay respectively. As shown in Fig 6, rmIL-6 did not induce mitogenesis of dermal fibroblasts at concentrations ranging from 1 to 100 ng per mL as determined by BrdU uptake (light bars). WST assay showed a small but significant increase in cell activity at 1.0 ng per mL, but at higher concentrations (i.e., those associated with keratinocyte migration), tetrazolium dye metabolism was not significantly different from control (dark bars).

The identity of the fibroblast-derived keratinocyte migratory factor could not be determined by genomics analysis As shown in Fig 3, following heat treatment (100°C for 5 min) IL-6-treated FCM lost all motogenic activity, indicating that the migratory factor may be a protein. In an attempt to identify the mRNA of this IL-6-induced migratory factor, fibroblasts from IL-6 KO mice were incubated for 2 h in the presence of 10 ng per mL rmIL-6. This concentration of cytokine was determined as optimal from transwell migration experiments (Fig 2). Total cellular RNA was collected and gene array experiments were conducted (Clontech Atlas Mouse 5k Array, cat #7906-1, Clontech). Two identical genomics experiments were carried out, the array data from identical treatments were averaged utilizing the “average several arrays” function of Atlas Image (Clontech), and then gene expres-
negative (SFKGM) control. Light bars ≈ quadruplicate. Cells were incubated 16–20 h at 37 °C and 5% CO2. Following incubation, 10 μL of 0.1 mM BrdU was added to each well, and the cells were then incubated an additional 8 h. Medium was then aspirated from the wells and ELISA determined BrdU uptake according to the manufacturer’s instructions (Roche). Results were obtained via an ELISA plate reader, and raw data expressed as mean ± SEM (n = 4) percent negative (non-treated) control (*significantly different from SFKGM control, p < 0.05).

**Figure 5**
Dermal culture supernatants do not induce proliferation of keratinocytes. Epidermal keratinocytes from IL-6 KO mice were seeded onto collagen I-coated 96-well culture plates and allowed to adhere for 4 h. The cells were washed twice with PBS and treatments were added in quadruplicate as indicated, and incubated for 16–18 h. Following incubation, 10 μL of 0.1 mM BrdU was added to each well, and the cells were then incubated an additional 8 h. Medium was then aspirated from the wells and ELISA determined BrdU uptake according to the manufacturer’s instructions (Roche). Results were obtained via an ELISA plate reader, and raw data expressed as mean ± SEM (n = 4) percent negative (non-treated) control (*significantly different from SFKGM control, p < 0.05).

**Figure 6**
**IL-6 is not a mitogen fibroblast from IL-6 KO mice.** Dermal fibroblasts isolated from IL-6 KO mice were seeded at 1 × 104 per well on tissue culture-coated 96-well plates. The cells were serum/growth factor starved for 4 h, and treatments were applied in quadruplicate. Cells were incubated 16–20 h at 37 °C and 5% CO2. BrdU or WST reagent (Roche) was added to the wells according to the manufacturer’s instructions. The plates were incubated an additional 3 h (WST) or 8 h (BrdU), and the assays were completed according to the manufacturer’s instructions. Results were obtained via an ELISA plate reader, and raw data expressed as mean ± SEM (n = 4) percent negative (SFKGM control) light bars = BrdU, dark bars = WST1 reagent (*significantly different from SFKGM control, p < 0.05).

IL-6 induces phosphorylation of STAT3, but not ERK or Akt in dermal fibroblast from IL-6 KO mice. The signal transduction pathway of the IL-6 receptor (IL-6R) is complex and involves essentially three pathways: JAK/STAT3, ERK1/2, and the more recently described PI3K/Akt pathway. To determine which pathway might be predominately stimulated by rmIL-6 in IL-6 KO fibroblasts, dermal cells were exposed to the cytokine at various time points, and nuclear protein was isolated from fibroblast cultures. Quantitation of phospho-STAT3, ERK1/2, and Akt proteins was accomplished by western blot. From these analyses, it was found that STAT3 was phosphorylated in a time- and concentration-dependent manner, whereas the phosphorylation state of ERK1/2 and Akt did not appear to be affected by rmIL-6 treatment (Fig 7).

**Discussion**

IL-6-deficient mice display greatly impaired wound healing as evidenced by delayed wound closure, decreased inflammation, granulation tissue formation, neovascularization, and incomplete re-epithelialization (Gallucci et al., 2000; Lin et al., 2003). This impairment was noted for as long as 15 d, nearly triple the normal healing time. IL-6 KO mice express normal levels of functional IL-6 receptor (Kopf et al., 1994), and wound healing could be reconstituted by treating IL-6 KO mice with rmIL-6 (Gallucci et al., 2001a; Lin et al., 2003) or an IL-6 expression plasmid (Gallucci et al., 2001a).

While these results indicate that IL-6 is intimately involved in the wound-healing process, they do not provide
information concerning the mechanism of IL-6 during the process of re-epithelialization. IL-6 could modulate re-epithelialization by directly acting on keratinocytes, inducing mitogenesis or migration. It might also act indirectly by inducing the expression of various keratinocyte-derived factors in an autocrine or paracrine fashion. Previous studies, however, concerning the effects of IL-6 on keratinocyte proliferation offer conflicting results (Grossman et al, 1989; Elder et al, 1992; Oyama et al, 1998; Sawamura et al, 1998; Sato et al, 1999). Recent experiments utilizing normal human epidermal keratinocyte (NHEK) cultures (Sugawara et al, 2001), and those presented in this article (Fig 1), did not demonstrate that IL-6 was a keratinocyte mitogen, agreeing with studies by Elder et al (1992) and Oyama et al (1998). Thus, it seems that IL-6 may not directly induce keratinocyte mitogenesis. This does not preclude the possibility of IL-6 acting in an indirect fashion though. Indeed, IL-6 can induce KGF expression in fibroblasts (Brauchle et al, 1994; Chedid et al, 1994), and EGF receptor in keratinocytes in vitro (Oyama et al, 1998), both of which would induce keratinocyte proliferation. Herein we show that culture supernatants collected from IL-6-treated IL-6 KO fibroblasts do not induce significant proliferation of cultured epidermal cells from IL-6 KO mice (Fig 5), further indicating that this cytokine does not indirectly mediate the mitogenesis of keratinocytes.

Another possible mechanism by which IL-6 may influence re-epithelialization is through the modulation of keratinocyte migration. While both proliferation and migration of keratinocytes contribute to re-epithelialization, they appear to be two separate events. For example, in vitro keratinocyte migration occurs early, after only 2–3 h, as compared to mitogenesis, which requires at least 24 h (Cha et al, 1996; O’Ttoole et al, 1997). Additionally, TGFβ is well known to induce keratinocyte migration, while it greatly inhibits proliferation (Sarret et al, 1992; Garlick and Taichman, 1994). One of the primary observations from our preliminary experiments involving IL-6 KO mice was that while the leading edge of the wounded epithelium displayed some thickening (perhaps indicating some mitogenesis was occurring in the absence of IL-6), it also showed profoundly decreased migratory activity, which was restored by rmIL-6 treatment (Gallucci et al, 2000). Herein we show that this is also true in vitro. When keratinocytes from IL-6 KO mice are co-cultured with dermal cells (also from IL-6 KO mice) and exposed to rmIL-6, the keratinocytes readily migrated across transwell inserts in the absence of collagen or other matrix proteins (Fig 2). If either rmIL-6 is not present in the culture media, or the keratinocytes are not co-cultured with dermal cells, no migration was apparent. Paradoxically, we have thoroughly reviewed elsewhere (Heinrich et al, 2003).
Briefly, IL-6R is multimeric, and composed of two basic subunits. The α subunit does not contain signal-transducing elements, whereas the β subunit (gp130) is known to primarily mediate signal transduction, leading to the activation of at least three different signaling paths: JAK/STAT3, ERK/MAPK, and PI3/Akt. To determine which IL-6R/gp130 signal transduction pathway might be activated by IL-6 treatment of fibroblasts, the phosphorylation states of STAT3, ERK1/2, and Akt from IL-6 KO dermal cells were analyzed. As shown in Fig 7, neither ERK1/2 nor Akt were significantly modulated in primary dermal fibroblasts by exposure to rmIL-6. There was a robust activation, however, of STAT3 in fibroblasts that occurred approximately 5 min following treatment, and remained high out to 1-h post-treatment. This seems to indicate that the STAT3 pathway is associated with IL-6-induced fibroblast modulation of keratinocyte migration. Interestingly, STAT3-deficient mice also display impaired wound healing similar to IL-6 KO mice. Sano et al (1999) reported that while migration was impaired, proliferative activity of keratinocytes from STAT3-deficient mice was not affected. This group also reported that cytokines that activated STAT3, such as EGF and IL-6, could induce the migration of keratinocytes in vitro on a collagen I matrix, and that the deletion of STAT3 abrogated the mitogenic potential of these factors. We have also found that IL-6 can directly induce a moderate (although not statistically significant) amount of migration in keratinocytes from IL-6 KO mice (Figs 4a and d) on collagen I-coated tissue culture plates. We show, however, that FCM induced a significantly more robust migratory response from keratinocytes on collagen I than IL-6 alone (Figs 4a and e), and as stated above we have also found that IL-6-mediated migration of keratinocytes on tissue culture-treated plastic can only be induced upon co-culture with dermal fibroblasts (Fig 2). This indicates that, in response to IL-6 stimulus, fibroblasts produce factors that can compensate for the absence of migratory stimulus from cell matrix contact, or perhaps induce matrix formation by keratinocytes.

The physical separation of keratinocytes and fibroblasts in the transwell assay indicates that dermal cells are producing a soluble migratory mediator as a result of treatment with IL-6. A number of soluble factors have been identified, which influence keratinocyte migration in vivo and in vitro. While TGFβ is probably the most studied, and has been shown to increase keratinocyte migration in vivo, there are other growth factors and cytokines that have been shown to induce migration as well (Table I). In an attempt to identify this IL-6-induced migratory factor, fibroblasts from IL-6 KO mice were incubated with rmIL-6, and mRNA was isolated from these cells and analyzed by microarray. To rule out the possibility of false positive or negative data, the expression of known migratory factors were confirmed by RT-PCR (Table II). Unfortunately, none of the genes currently identified as a soluble keratinocyte migratory factor were modulated by IL-6 treatment (Table II). It should be taken into account, however, that the genomic data presented herein represent only one time point following IL-6 exposure. Since the keratinocyte migration assays were allowed to occur over a 16-h period, it is possible that the migratory factors are modulated at a later time point. It is also possible that IL-6 may upregulate a receptor on keratinocytes for a factor that is constitutively expressed by fibroblasts, or induce a keratinocyte-derived factor that modulates fibroblast activity or function, which ultimately results in keratinocyte migration. Thus, it may be possible to elucidate the migratory factor with further genomic analysis of IL-6-stimulated dermal and epidermal cells. Experiments are currently underway to investigate this possibility. Additionally, while the factor is most likely a protein based on its sensitivity to heat denaturation (Fig 3), the activity of the migratory factor may not be associated with its transcriptional regulation. It could be that this factor is released preformed from intracellular stores, or perhaps modified from an inactive form to an active form by an enzyme that is in turn modulated by IL-6. In these scenarios, genomics analysis would miss these processing events and proteomic analysis would be necessary to identify the factor.

One mechanism by which IL-6 might appear to be influencing the expression or activity of a migratory factor is merely by promoting the survival of the cells of origin. The net result of this would be an increase in the concentration of the migratory factor in cultures treated with the cytokine, even though the cytokine itself did not specifically affect the mRNA or protein expression level of the factor. IL-6 seems to have varying effects on cells of fibroblast lineage, which appears to be associated with the anatomical origin of the cells. For instance, IL-6 induces the angiotensin-II-induced proliferation of cardiac fibroblasts (Sano et al, 2000), while it inhibits the proliferation of synovial (Nishimoto et al, 2000) and dermal fibroblasts (Mihara et al, 1996). Our data agree with the later study, and show that IL-6 does not induce the proliferation of dermal fibroblasts as determined by BrdU uptake (Fig 6). While it does not seem to promote mitogenesis however, it may affect the differentiation or survival of these cells.

As stated above, IL-6 readily induces STAT3 activation in fibroblasts (Fig 7), and constitutively expressed STAT3 has been shown to protect fibroblasts from the apoptotic effects of UV irradiation, serum starvation, and STAT1 activity (Shen et al, 2001), and can downregulate Fas expression in fibroblast cell lines (Ivanov et al, 2001). When examined by tetrazolium dye assay however, which is a measure of energy expenditure and cell viability, IL-6 did not have a significant effect on fibroblast survival at the concentrations (>10 ng per mL), which produced appreciable keratinocyte migration (Fig 6). Interestingly, the lowest concentration (1.0 ng per mL) induced a small but significant increase in cell viability. The implications of this finding are not yet known.

In conclusion, our results indicate that IL-6 has obvious indirect effects on the modulation of keratinocyte motility. This complex mechanism appears to involve a novel interaction between dermal and epidermal cells, and the interplay of an as yet unidentified soluble fibroblast-derived factor(s). While the STAT3 signal transduction pathway seems to be activated in fibroblasts by IL-6 treatment, further studies will be necessary to determine the exact nature of the mechanism of this cytokine on fibroblast function, gene regulation, and its relation to wound healing.
Materials and Methods

Keratinocyte isolation IL-6 KO mice were housed in an AAALAC accredited facility, and all procedures involving animals were reviewed and approved by the OUHSC Institutional Animal Care and Use Committee. All operations were performed using the aseptic technique unless otherwise noted. Keratinocytes from the skin of neonatal IL-6 KO or wild-type C57 pups were isolated essentially as by the method of Hager et al. (1999). Briefly, newborn mouse pups (0–48 h old) were euthanized by decapitation, rinsed in 70% ethanol, and the skin was removed. Skins were incubated dermis down in culture dishes containing HBSS + 3.6% dispase (Sigma, St Louis, Missouri) overnight at 4°C. The epidermis was separated from the dermis and placed in a tube containing fresh 0.25% trypsin, vortexed vigorously, and incubated for 10 min at 37°C. Epidermal cells were dispersed by repeated aspiration with a pipette and filtered through a sterile gauze pad into a 50 mL conical tube. An equal volume of low calcium, complete keratinocyte growth medium (KGM, Cascade Biologies, Portland, Oregon) was added to the epidermal keratinocytes, and the cells were centrifuged at 200 × g for 10 min. Keratinocytes were resuspended in growth medium, plated at 6–10 × 10^4 per cm^2 on rat tail collagen 1-coated 100 mm culture dishes (Becton Dickinson, Franklin Lakes, New Jersey), and incubated at 37°C and 5% CO_2 and 95% room air.

Dermal fibroblast isolation The dermis was collected and separated from the epidermis as stated above. The dermis was placed in HBSS + 0.05% Type 1 collagenase and incubated at 37°C with constant agitation. The cell suspension was filtered through sterile gauze into a 50 mL conical tube. An equal volume of DMEM + 5% FBS containing, 60 IU per mL penicillin, 100 IU per mL streptomycin, and 4 mM glutamine (DMEM + 5%) was added and centrifuged at 200 × g for 10 min. Fibroblasts were resuspended in DMEM + 5%, plated at 8 × 10^5 per cm^2 in 150 mm culture dishes, and incubated at 37°C and 5% CO_2 and 95% room air. To produce IL-6-treated fibroblast-conditioned medium (FCM), fibroblast cultures were serum starved for 2 h, washed twice with HBSS, and incubated 24 h in SFKGM + 10 ng per mL rmIL-6. The resulting medium was collected, sterile filtered, and stored at −70°C until use.

Proliferation assays Dermal and epidermal cells were isolated and cultured as described above. Cells were seeded at 1 × 10^4 per well on collagen I-coated (keratinocytes) or tissue culture-treated (fibroblasts) 96-well plates. Cells were allowed to adhere to the plates overnight in complete media at 37°C and 5% CO_2 and 95% room air. The cells were serum/growth factor starved for 4 h, after which treatments were applied in quadruplicate. The cells were incubated 16–20 h at 37°C and 5% CO_2 and 95% room air, and either WST or BrdU (Roche, Indianapolis, Indiana) was added to the wells according to the manufacturer's instructions. The plates were incubated for an additional 2 (WST) or 8 h (BrdU), and the assays were completed according to the manufacturer's instructions. Results were obtained via an ELISA plate reader, and raw data were expressed as percent negative (non-treated media) control.

Transwell migration assay Primary fibroblasts from IL-6 KO mice were seeded into 24-well tissue culture plates at 4 × 10^4 per cm^2 in DMEM + 5% and allowed to adhere overnight at 37°C and 5% CO_2. The following day, medium was aspirated from the 24-well plate containing fibroblasts. The plate was washed twice in HBSS, and allowed to incubate in 0.5 mL of HBSS at 37°C and 5% CO_2 for 1 h. Primary keratinocytes from IL-6 KO mice grown to 80% confluency on collagen I-coated tissue culture plates were resuspended in serum-free/growth supplement-free keratinocyte growth media containing 1 mg per mL BSA, 60 IU per mL penicillin, and 100 IU per mL streptomycin (SFKGM). The rationale for using serum-free/supplement-free media was to minimize the effects of growth factors in the serum and supplements, which could confound the cell's response to added migratory factors. Serum-free/supplement-free medium is routinely used in keratinocyte migration assays (Matsumoto et al., 1991; Cha et al., 1996; Sano et al., 1999; Gibbs et al., 2000; Tokumaru et al., 2000). 2.0 × 10^4 keratinocytes were added to sterile microfuge tubes and centrifuged at 500 × g for 5 min. Medium was aspirated from the pellet and each was resuspended in 100 µL of either KGM, or SFKGM containing specific concentrations of rmIL-6. HBSS was aspirated from the 24-well plate, containing fibroblasts, and 500 µL of the identical treatments administered to the keratinocytes were added to specified wells. Transwell inserts (8 µm pore, 5 mm diameter) were placed in wells of the plate, and 100 µL of keratinocytes were added to each well corresponding to the correct treatments. Transwell plates were incubated for 16 h at 37°C and 5% CO_2. After incubation, cells were removed from the top of the insert membrane by gently swabbing the membrane with a sterile cotton swab. The membrane was rinsed twice with PBS, and aspirated to assure removal of cells. The transwell inserts were removed from the plate and placed in fixative (10% buffered formalin) overnight at room temperature. Inserts were washed once in saline immersed in a 1% hematoxylin solution overnight at 4°C, and then rinsed twice in saline. The membrane was removed from the transwell, dried, and mounted on a glass microscope slide. Migrating cells were visualized by light microscopy, and enumerated by counting five random fields at × 20 magnification in a blinded fashion.

In vitro wound assay Keratinocytes were prepared as described above, and 5 × 10^5 cells were added to 100 mm collagen I-coated tissue culture plates (Becton Dickinson) in KGM at 37°C and 5% CO_2. Once confluent, an in vitro wound was produced on the culture essentially as described by Mathew et al. (1997). Briefly, the edge of a sterile razor blade was pressed lightly against the confluent monolayer of cells. A sterile rubber policeman was then used to sweep away the monolayer on one side of the blade. The razor's edge not only acts as a barrier and straight edge to facilitate cell removal, but the blade produces a distinct wound border that facilitates easy enumeration of cells that migrate beyond the margin. The cultures were washed three times in PBS, and then incubated in SFKGM containing 10 ng per mL rmIL-6, FCM, or no treatment. After 24 h incubation at 37°C and 5% CO_2, migrating cells were enumerated by counting three fields at × 20 magnification that were designated as previously having a cell-free in vitro wound margin immediately after wounding.

Clontech Atlas Gene Array Dermal or epidermal cells were isolated as described above and allowed to become approximately 50–70% confluent. Cell culture was then treated with either 10 ng per mL recombinant mouse IL-6 (6-mL-6, Invitrogen, Baltimore, Maryland) or an equal volume of saline for 120 min. This dose and time period were previously determined as sufficient for gene induction (Sugawara et al., 2001). Cells were collected and total cellular RNA was extracted using the Qiagen RNeasy kit (Qiagen, Valencia, California) according to the manufacturer's procedure. An aliquot of total RNA (25–50 μg) was further purified for mRNA, reverse transcribed, and 32P labeled using the Clontech Atlas Pure RNA labeling kit (Clontech, Palo Alto, California) according to the manufacturer's instructions. Radiolabeled cDNA was then hybridized to the Atlas (Clontech Mouse 5k Array) plastic membrane for 20 h. The membrane was washed and exposed to a phosphorimager screen for 24 h. The phosphorimager screen was then scanned using a Storm 820 phosphorimaging system (Molecular Dynamics, Sunnyvale, California). Results were determined using AtlasImage software (Clontech). A 2-fold difference in gene expression was considered to be significant.

Semi-quantitative RT-PCR reaction Total cellular RNA was extracted from keratinocyte or fibroblast culture using the Qiagen RNeasy kit (Qiagen, Valencia, California) according to the manufacturer's instructions, and quantitated spectrophotometri-
cally. Total RNA was reverse transcribed into cDNA as described previously (Simeonova and Luster, 1995) and the reaction mixture was diluted with RNase-free water to 50 μL. PCR primers for G3PDH were purchased from Clontech. Primers for mouse genes were custom synthesized (Life Technologies, Bethesda, Maryland) from sequences presented in Table I. Samples of cDNA were amplified via PCR as previously described (Brucoleri et al., 1997). Amplified PCR products were electrophoresed in 1.5% agarose gel and scanned directly with a DigiDoc digital imaging system (UVP, Upland, California). The digital images were analyzed using NIH Image v1.57, and mRNA semi-quantitative values were normalized based on G3PDH expression.

Western blot analysis Fibroblasts were prepared as described above, and cultured to 90% confluency in 15 cm² culture plates. Prior to treatment, fibroblast cultures were washed twice with PBS, and culture medium was replaced with serum-free DMEM containing 1 mg per mL BSA, 60 IU per mL penicillin, 100 IU per mL streptomycin, and 4 mM glutamine. Following a 4 h incubation in serum-free media, treatments were applied to the cultures in fresh serum-free DMEM. At specific time points, two culture plates were rinsed once with ice-cold PBS, and cells were collected by the use of a cell scraper into ice-cold PBS. Cells were centrifuged at 400 x g at 4°C for 5 min, and nuclear protein was isolated from the cell pellet utilizing a Mercury Transfactor Extraction Kit (BD/Clontech) according to the manufacturer’s instructions. Nuclear protein (25 μg) was electrophoresed utilizing 10% SDS-PAGE (10 cm × 10 cm) mini-gels (Gradipore, Frenchs Forest, AUS) at 90 V for 45 min, and electrophoretically transferred onto nitrocellulose sheets (Pierce, Rockford, Illinois) at 25 V for 2.5 h. Following transfer, the blots were blocked in Tris-buffered saline containing 1% cold-water fish gelatin (Sigma), and 50 mM NaF for 1 h at RT. The blots were then exposed to rabbit anti-phospho-ERK1/2, anti-phospho-STAT3, or anti-phospho-Akt (Cell Signaling Technology, Beverly, Massachusetts), at a 1:1000 dilution in TBS containing 1% fish gelatin, 0.1% Tween 20, and 50 mM NaF overnight at 4°C. The blots were washed 3 × in TBS containing 0.1% Tween 20, and exposed to a fluorescently labeled (IRDye 800) secondary anti-rabbit polyclonal antibody (Rockland, Gilbertsville, Pennsylvania) at a 1:20,000 dilution in TBS containing 1% fish gelatin, and 0.1% Tween 20 for 1 h. Following three washes in TBS + 0.1% Tween, the blots were imaged via an Odyssey infrared imaging system (LI-Cor, Lincoln, Nebraska).

Statistical analysis All experiments were replicated and representative findings were shown. Statistical significance was determined by one-way ANOVA. When the F value was significant, the means were compared using Fisher post hoc analysis. In all statistical comparisons, a p value of less than 0.05 was used to indicate a significant difference.

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