Keloid fibroblast responsiveness to epidermal growth factor and activation of downstream intracellular signaling pathways

LATHA SATISH, PhD[a]; MARY BABU, PhD[b]; KIEN T. TRAN, PhD[a]; PATRICIA A. HEBDA, PhD[c,d]; ALAN WELLS, MD, DMSc[a]

Keloids, which overgrow the boundaries of the original injury, represent aberrations in the fundamental process of wound healing that include over-abundant cell in-migration, cell proliferation, and inflammation, as well as increased extracellular matrix synthesis and defective remodeling. To understand the key events that result in the formation of these abnormal scars would open new avenues for better understanding of excessive repair, and might provide new therapeutic options. We examined epidermal growth factor receptor (EGFR)-induced cell motility in keloid fibroblasts, as this receptor initiates cell migration during normal wound repair. We show that keloid fibroblasts respond to EGF-induced cell migration but the response is somewhat diminished compared to normal adult fibroblasts (~30% reduced); the mitogenic response was similarly blunted (~5% reduced). Keloid fibroblasts express near normal levels of EGFR (82%), but show a much more attenuated activation of EGFR itself and the motility-associated phospholipase C-γ. This was reflected in part by rapid loss of EGFR upon exposure to EGF. Interestingly, while extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK-MAPK) activation was relatively robust in keloid fibroblasts, the downstream triggering of the motility-associated calpain activity was blunted. This was reflected by high cell–substratum adhesiveness in the keloid fibroblasts. Thus, the blunted migratory response to EGF noted in keloid fibroblasts appears due to limited activation of two important biochemical switches for cell motility.**(WOUND REP REG 2004;12:183–192)**

Keloids are neoplastic growths resulting from excessive response to cutaneous injury. Keloids are raised pathological scars that, over extended time periods, overgrow the margins of the original wound and cause damage to the healthy dermal tissue.[1,2] Keloids persist for years and cause pain, persistent itching, disfiguration, and limit function; unfortunately, they rarely regress. These disorders represent aberrations in the fundamental processes of wound healing, which include cell migration, cell proliferation, inflammation, increased synthesis of cytokines and extracellular matrix proteins, and defective remodeling. Probing the intracellular and molecular signaling events of cells derived from the keloid lesions would open new avenues for better understanding of excessive repair, which might provide new therapeutic considerations.

BSA	Bovine serum albumin
DMEM	Dulbecco's Modified Eagle's medium
EGF	Epidermal growth factor
EGFR	EGF receptor
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
PLC-γ	Phospholipase-Cγ
TGF	Transforming growth factor

Several hypotheses have been put forward regarding the biochemical, immunological, and molecular

From the Departments of Pathology[a], Otolaryngology[c] and Cell Biology & Physiology[d], University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; and Biomaterials Division[b], Central Leather Research Institute, Adyar, Chennai, India.

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Reprint requests: Alan Wells, MD, DMSc, Department of Pathology, 713 Scaife Hall, University of Pittsburgh, Pittsburgh, PA 15261. Fax: (412) 647-8567; Email: wellsa@msx.upmc.edu

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basis for the occurrence of keloids. Histologically, keloids appear to consist of massively increased dermal tissue[3] underlying relatively increased expression of proliferation-associated keratins 5/14 in the basal layers of keratinocytes.[4] Fibroblasts isolated from keloids produce two to three times more collagen than fibroblasts from normal skin,[5] due to increased collagen synthesis and decreased collagen breakdown in the keloid fibroblasts.[3,6,7] The loss in collagen balance is reflected by changes in the matrix metalloproteinases (MMPs) during abnormal wound healing.[8] Similarly, other matrix proteins are altered, e.g., fibronectin and tenascin levels are elevated.[9–12] Thus the exuberant overproduction of matrix forms the keloid, but the root cause must involve the dermal fibroblasts that produce these proteins.

Apart from changes in the extracellular matrix proteins, keloid fibroblasts also show altered responses to various growth factors, although there is disagreement as to the extent of altered response to epidermal growth factor (EGF), platelet derived growth factor, insulin-like growth factor-1, and transforming growth factor (TGF)-β.[13–17] The healing wound contains a wide array of growth factors exerting different functions during the overlapping temporal processes of healing. These growth factors profoundly influence multiple processes including cell migration, cell proliferation, matrix production and degradation. These influences all promote matrix accumulation.[18] Ligands for the EGF receptor (EGFR) are present throughout wound repair;[19] TGFα is liberated from platelets and is made in paracrine and autocrine fashion by fibroblasts, and heparin binding-EGF is produced by macrophages and myocytes. Thus, alterations in keloid fibroblast responsiveness to EGFR activation would have significant implications for keloid development.

Previous reports have been conflicting as to the response of keloid fibroblasts to EGF. An early study reported that keloid fibroblasts, while expressing normal EGFR numbers, responded to EGF only half as well as normal fibroblasts.[14] Such lack of enhanced responsiveness to EGF was confirmed by another investigation.[15] More recently, it was reported that keloid fibroblasts present greater phosphorylation and increased expression of EGFR, although responsiveness to EGFR ligands was not reported.[20] However, none of these reports have examined key intracellular signaling pathways downstream from EGFR activation and their concomitant cellular responses.

In the present investigation, we focused our interest on EGF and its downstream signaling cascade events. Dermal fibroblast motility and proliferation are regulated by numerous growth factors;[21] among those that are most robust are factors that activate EGFR. We chose EGF as our activating ligand because in vitro there appears to be little if any difference in cellular responses to the major soluble EGFR ligands.[19] EGFR signaling elicits two pathways that are key in wound repair, mitogenesis and migration, and the latter has not been well studied in fibroblasts derived from keloid lesions. Full EGF-induced cell migration requires phospholipase-C γ (PLC- γ) signaling.[22] Inhibition of PLC-γ signaling specifically abrogates cell migration but not cell proliferation,[22,23] indicating that this pathway is required for EGFR-induced cell locomotion.

Another major signaling pathway from EGFR is via a mitogen-activated protein kinase (MAPK) signaling pathway, which is required for both cell mitogenesis and cell migration,[24] with migration requiring extracellular signal-regulated kinase (ERK)-induced m-calpain activation.[25] In the present study we decipher the role of these two intracellular effectors, PLCγ and ERK-MAPK during EGF-induced signal transduction mechanism in keloid fibroblasts. The present study shows that keloids possess a real, although limited, response to EGF; this was due to relatively weak activation of downstream motility-signaling pathways, likely due to rapid degradation of EGF receptor.

MATERIALS AND METHODS

Human recombinant EGF was obtained from Collaborative Biomedical Products (Bedford, MA). Two keloid fibroblast and three normal adult fibroblast specimens were randomly selected from a bank of human cells maintained by one of the investigators (PAH). One specimen was derived from an earlobe keloid surgically removed from an 8-year-old male, the second was derived from keloid tissue excised from the lumbar region of a 57-year-old male, and the third keloid fibroblast cell line, purchased from ATCC (CRL-1762, Manassas, VA), was from a 35-year-old female. The normal fibroblast lines were derived from breast skin surgically removed from 20-year-old and 22-year-old females and from an eyelid of a 66-year-old male. The race of the individuals from whom these samples were obtained was not recorded routinely. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Both normal and keloid tissues were de-identified and classified as excess pathological tissue, exempt from requiring informed consent, as determined by the University of Pittsburgh or the Children's Hospital of Pittsburgh institutional review boards (Pittsburgh, PA). In all experiments at least two independent normal adult and keloid fibroblasts were assessed with quantitatively similar results.

Keloid and normal adult fibroblast cultures

Both keloid and adult normal adult fibroblasts were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 percent fetal bovine

serum (FBS), 1 × antibiotics (penicillin and streptomycin), 1 × nonessential amino acids, and 1 × sodium pyruvate (all from Life Technologies, Rockville, MD).[26] Cells were quiesced by culturing for 48 hours in DMEM containing 0.1 percent dialyzed FBS when the cultures had reached 80 percent confluence; this reduces cell proliferation by >90 percent while maintaining cell viability.[27] All cultures were used at passage levels that allowed for more than seven more passages (>20 population doublings) prior to senescence; at these lower passage numbers, the EGF responsiveness of fibroblasts is found to be unaffected by cellular aging.[26]

Cell surface EGF receptor number

Normal adult and keloid fibroblasts were plated onto 12-well Falcon tissue culture plates and grown until 80 percent confluence. Cells were placed in serum-starved media for 48 hours and surface EGFR number was determined by a previously determined assay.[26,28] Briefly, cells were washed twice with ice-cold WHIPS buffer (20 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml polyvinylpyrrolidone) and incubated with binding buffer (DMEM containing 25 mM HEPES, 0.5 percent bovine serum albumin [BSA]) containing saturating levels of ^{125}I-EGF (ICN, Costa Mesa, CA) for 3 hours on ice. After the incubation, cells were washed six times with 2 ml ice-cold WHIPS buffer followed by a wash with an acid stripping buffer containing urea (50 mM-glycine-HCl, 100 mM NaCl, 2 mg/ml polyvinylpyrolidone, 2 M urea, pH 3.0) to remove surface-bound radioactivity. Surface-bound radioactivity is a direct indication of cell receptor number.[29] Cell number per well was determined in tandem via counting with a hemocytometer in triplicate for each condition. Competition with excess of cold ligand was used to determine nonspecific binding.

In vitro cell migration assay

EGF-induced cell migration was assessed by the ability of the cells to move into an acellular area.[30] Cells were quiesced for 48 hours in DMEM with 0.1 percent dialyzed FBS prior to being denuded by a rubber policeman. The cells were then treated with or without EGF (1, 2, 5, or 10 nM) and incubated at 37°C for 24 hours. Photographs of the same three locations at the edge of each wound were taken at 0 and 24 hours, and the relative distance traveled by the cells at the acellular front was determined.

Cell proliferation assay

Growth factor–induced proliferation was determined by incorporation of [^{3}H]-thymidine at defined time points previously determined to cover the initial S-phase.[23] Cells were grown to confluence in 12-well plates and quiesced for 48 hours in DMEM with 0.1 percent dialyzed FBS and then incubated with the indicated concentration of growth factors for 16 hours. [H^3]-Thymidine purchased from Amersham Bioscience Corp. Piscataway, NJ (5μCi/well) was added, and cells were incubated for a further 10 hours. Cells were treated with 5 percent trichloroacetic acid for 30 minutes at 4 °C and incorporated label solubilized by 1 N NaOH. Samples were analyzed by liquid scintillation counting (Beckman, Fullerton, CA).

Adhesion assay

Cell substratum adhesiveness of normal and keloid fibroblasts was quantitated using an inverted centrifugation detachment assay.[25] Cells were plated at the concentration of 10^5 cells/well in a 12-well plate. Cells were quiesced for 24 hours and then incubated with or without EGF (1 nM) for 30 minutes. Wells were completely filled with DMEM and supplemented with 1 percent BSA and 25 mM HEPES (pH 7.4), then sealed with enzyme-linked immunosorbent assay sealing tape (Corning, Cambridge, MA) and centrifuged inverted for 10 minutes at different speeds viz., 2,000 (910 × g), 2,500 (1420 × g), 2,800 (1782 × g), and 3,200 (2329 × g) r.p.m. at 37 °C using a Beckman CS6R plate centrifuge. Before and after the centrifugation, the number of cells on the plates was counted under phase-contrast microscopy.

Calpain activity assay

The calpain activity in individual living cells was detected by using a Boc-LM-CMAC assay for in vivo proteolysis.[25] In brief, normal adult and keloid fibroblasts were plated onto glass coverslips. The cells were quiesced when at 50 percent confluence for 48 hours and then treated in the presence or absence of EGF (1 nM [10 minutes] or 10 nM [5 minutes]), respectively. All cells were loaded with Boc-LM-CMAC (*t*-BOC-Leu-Met-chloromethylaminocoumarin, Molecular Probes, Eugene, OR) for 20 minutes 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. 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 two or more separate experiments.

Immunoblotting

Immunoblotting was employed to detect protein and phosphorylated protein levels of EGFR, PLCγ, and

ERK. Keloid and normal adult fibroblasts were grown to confluence and then quiesced for 48 hours in DMEM containing 0.1 percent dialyzed serum. Cells were treated with EGF (1 nM [10 minutes] or 10 nM [5 minutes]) and also for varying time intervals with EGF 10 nM as indicated in the figure legends. Cell lysates with an equal amount of protein as determined using Bio-Rad assay kit were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane Immobilon-P (Millipore, Cambridge, MA). Blots were probed using the following antibodies: EGFR (catalog # 28–0005, Zymed, San Francisco, CA), phosphotyrosine (PY20, # P11120, Transduction Laboratories, San Diego, CA), pan ERK (# E17120, Transduction Laboratories) and dual phosphorylated ERK (#9101, Cell Signaling Technology, Beverly, MA), PLCγ1 mixed monoclonals (# 05–163, Upstate Biotechnology, Lake Placid, NY), and phosphorylated PLCγ (p783; # 2821, Cell Signaling Technology). To confirm equal loading of proteins, immunoblots were probed against anti-α-actin (#A 2066, Sigma, St. Louis, MO). Further, densitometric analysis for the phosphorylated proteins was performed using NIH image 1.62.

RESULTS

EGF induces motility and proliferation in keloid fibroblasts

Keloid fibroblasts have been reported as responding to growth factors, although the relative extent of response has been debated.[13–15] We report here that keloid fibroblasts do respond to EGF in terms of increased motility (Figure 1), although to a lesser extent than that noted in normal adult fibroblasts. The reduced responsiveness was not limited to migration, as EGF-induced proliferation was similarly diminished (Figure 1b). This diminished responsiveness is not due to a higher basal level of cell activity, as the migration and thymidine incorporation in the absence of EGF was similar between the keloid and normal fibroblasts. Furthermore, an autocrine EGFR-stimulatory loop, which would mask exogenously added EGF, is not functioning in the keloid fibroblasts, as a specific inhibitor of the EGFR, PD153035, had no effect on basal cell activities (data not shown). Lastly, this diminution in responsiveness does not appear to be preferential for EGFR, as the proliferative response to platelet derived growth factor also appears to be blunted in keloid fibroblasts when compared to normal adult fibroblasts (data not shown).

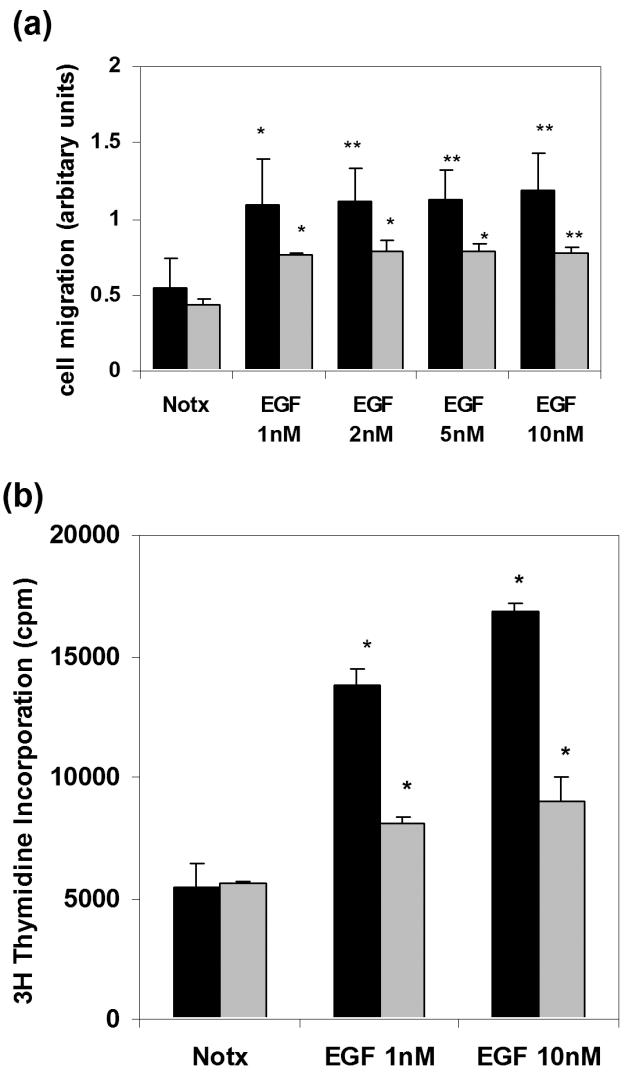

FIGURE 1. EGF induces cell migration and proliferation in keloid fibroblasts. (a) Normal human adult fibroblasts (dark bars) and keloid fibroblasts (gray bars) were tested for motility in an in vitro wound healing assay. Cells were treated with increasing concentrations of EGF. The values are normalized and shown as EGF-induced motility at each concentration. (b) The different concentrations of EGF (1 nM-10 nM) were tested for the ability to induce proliferation in normal adult fibroblasts and in keloid fibroblasts by a thymidine incorporation assay. The values are mean $\pm$ SEM of six independent studies each performed in triplicate for motility/proliferation and three independent studies each performed in triplicate for cell de-adhesion. Statistical analysis was performed by Student's *t*-test. *$p < 0.05$ compared to paired no EGF treatment, **$p < 0.01$.

Keloid fibroblasts rapidly attenuate EGFR signaling

As the two cell responses to EGF appeared blunted, we asked whether EGFR signaling was intact. Before studying the intracellular signaling cascade events we determined the EGFR receptor levels in normal and keloid fibroblasts. We did not find significantly lower levels of surface EGFR prior to exposure to EGF because keloid cells express 81.7 ± 2.3 percent of the EGFR/cell compared to normal fibroblasts (Figure 2), in agreement with previous findings.[14]

Upon ligand binding, EGFR rapidly autophosphorylates and then undergoes internalization with both dephosphorylation and degradation serving to attenuate

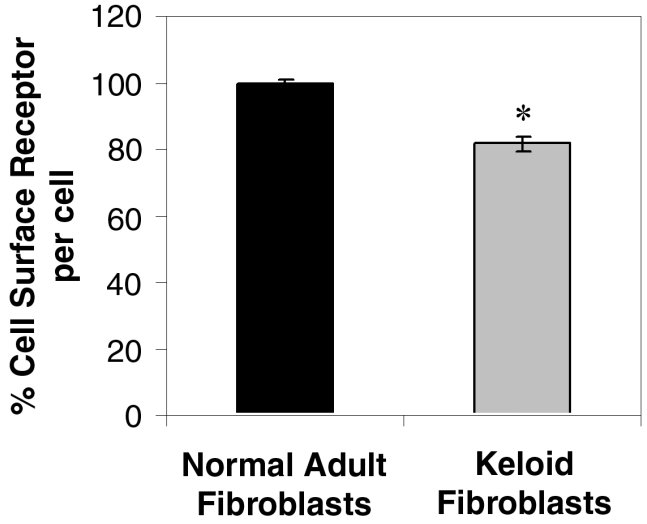

FIGURE 2. EGFR surface presentation is relatively intact. EGFR receptor numbers were determined by standard scatchard analyses. The number of binding sites was calculated by Scatchard plot using linear regression. Shown are representative values of two independent studies each done in duplicate. Statistical analyses were performed using Student's *t*-test. *$p < 0.05$.

this signaling.[31] Upon EGF exposure, one notes phosphorylation of EGFR in normal fibroblasts (Figure 3a). This is limited in the keloid fibroblasts with significantly decreased phosphorylation at saturating doses of EGF (10 nM; Figure 3b). This lack of phosphorylation reflects a rapid loss of EGFR upon ligand exposure. Whereas the total level of EGFR in normal fibroblasts remains relatively constant over the brief 10-minute exposure, there is a marked loss of keloid EGFR. Interestingly, total cellular EGFR in keloid cells is less than half that in normal cells (Figure 4), when normalized to either cell number or actin. This would suggest that there is less "reserve" EGFR for replacement upon ligand-induced internalization.[32] In concordance with this suggestion, we note rapid loss of total EGFR upon exposure to EGF, although the rate of decrement appears similar to normal fibroblasts (Figure 4a, b).

Rapid attenuation of EGFR signaling might limit downstream signals, resulting in a blunted response to EGF. We examined two pathways that diverge at the receptor level. The mito- and moto-genic pathway leading to ERK MAPK was relatively intact when examined acutely (Figure 5a) and over an extended period of EGF exposure (Figure 5b). Even at diminishing doses of EGF, the pERK levels were maintained in the keloid fibroblasts. However, the PLCγ signaling pathway, required for induced cell migration, was attenuated compared to normal fibroblasts, after both short-term and extended ligand exposure (Figure 6). That ERK is less dramatically affected is not unexpected, as the phosphorylation of this molecule results after a four-step amplification cascade that is more sensitive to low-level EGFR signaling.[33] These data suggest that while keloid fibroblasts are capable of responding to EGFR ligands, the rapid degradation of the receptor blunts the cellular responses.

Keloid fibroblasts show reduced calpain activity and increased substratum adhesiveness. To enable rear de-adhesion during EGFR-mediated motility, the plasma membrane-associated ERK fraction activates m-calpain.[34] We found that despite relatively normal ERK activation (Figure 5), the level of calpain activation was diminished (Figure 7). As such, we would expect that EGFR-mediated cell de-adhesion would be absent. This was evident when we quantified cell–substratum adhesiveness (Figure 8), wherein the maximum achievable g force (3200 r.p.m. or 2329 g) failed to dislodge the keloid fibroblasts.

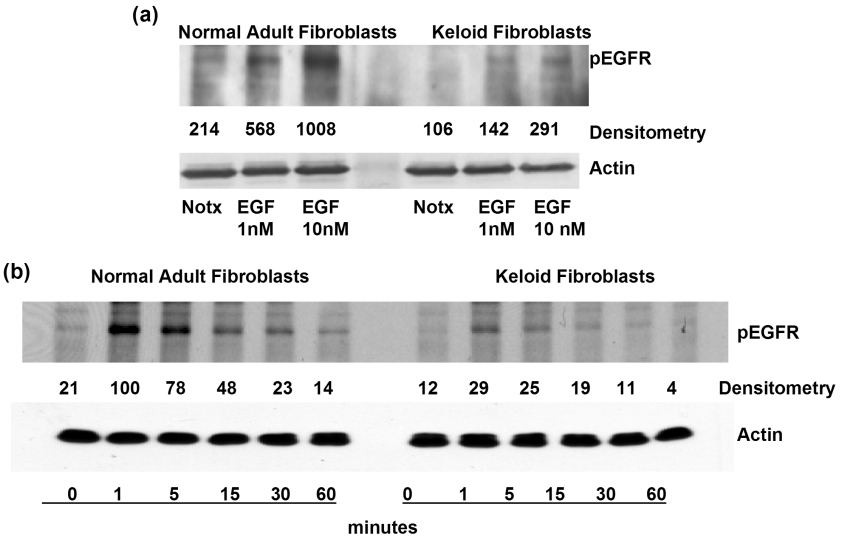

FIGURE 3. Phosphorylation of EGFR is relatively diminished in keloid fibroblasts. Normal adult fibroblasts and keloid fibroblasts were grown to confluence and then quiesced. (a) Cells were either induced/uninduced with EGF 1 nM (10 minutes) or EGF 10 nM (5 minutes) and cell lysates were collected. (b) Alternatively, cells were treated with a saturating dose of EGF (10 nM), and cell lysates were collected at varying time intervals. Cell lysates were resolved on a 7.5 percent gel and phosphorylated proteins detected by antibodies specific for phospho-tyrosine (PY 20) or α-actin, serving as a loading control. Shown is a representative immunoblot of three independent experiments. Numbers below the immunoblots represent densitometry analysis as determined utilizing National Institutes of Health image 1.62.

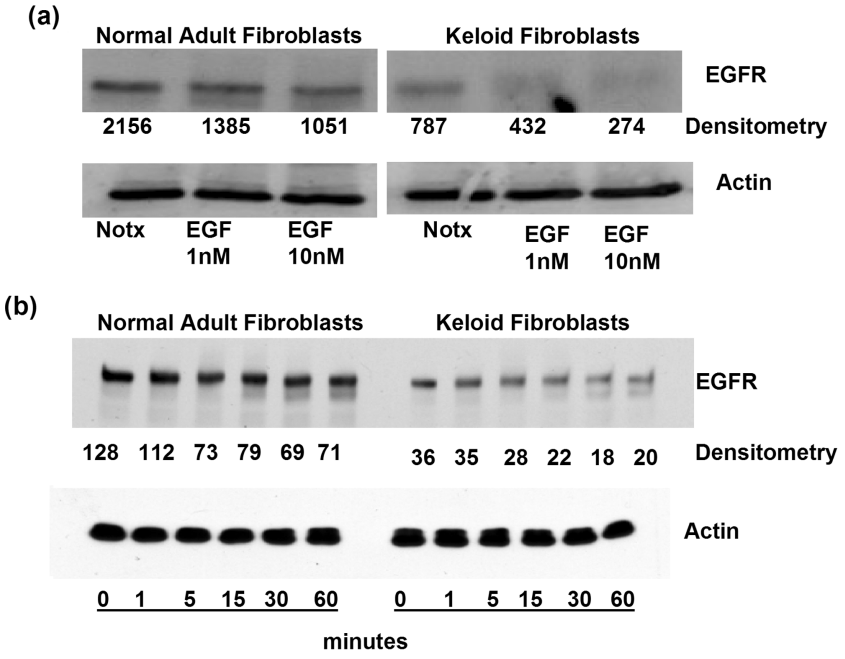

FIGURE 4. Keloid fibroblasts rapidly down-regulate EGFR. Normal adult fibroblasts and keloid fibroblasts were grown to confluence and then quiesced. (a) Cells were either induced/uninduced with EGF 1 nM (10 minutes) or EGF 10 nM (5 minutes) and cell lysates were collected. (b) Alternatively, cells were treated with a saturating dose of EGF (10 nM), and cell lysates were collected at varying time intervals. Cell lysates were resolved on 7.5 percent gels and phosphorylated proteins detected by antibodies specific for EGFR or α-actin, serving as a loading control. Shown is a representative immunoblot of three independent experiments. Numbers below immunoblots represent densitometry analysis as determined utilizing National Institutes of Health image 1.62.

DISCUSSION

Keloids are characterized by the exuberantly excessive accumulation of dermal material in response to a wound. Over time, these growths extend beyond the margins of the original, often minor, wound. To achieve such a mass, keloids require the proliferation and in-migration of fibroblasts as well as their subsequent production of extracellular matrix. To understand the molecular bases for keloid development, investigators have focused their attention on signals that promote these processes in normal wound healing, including growth factors and their implications during scar formation.[16,35–37] Although previous studies have reported on the responses of keloid cells to growth factors,

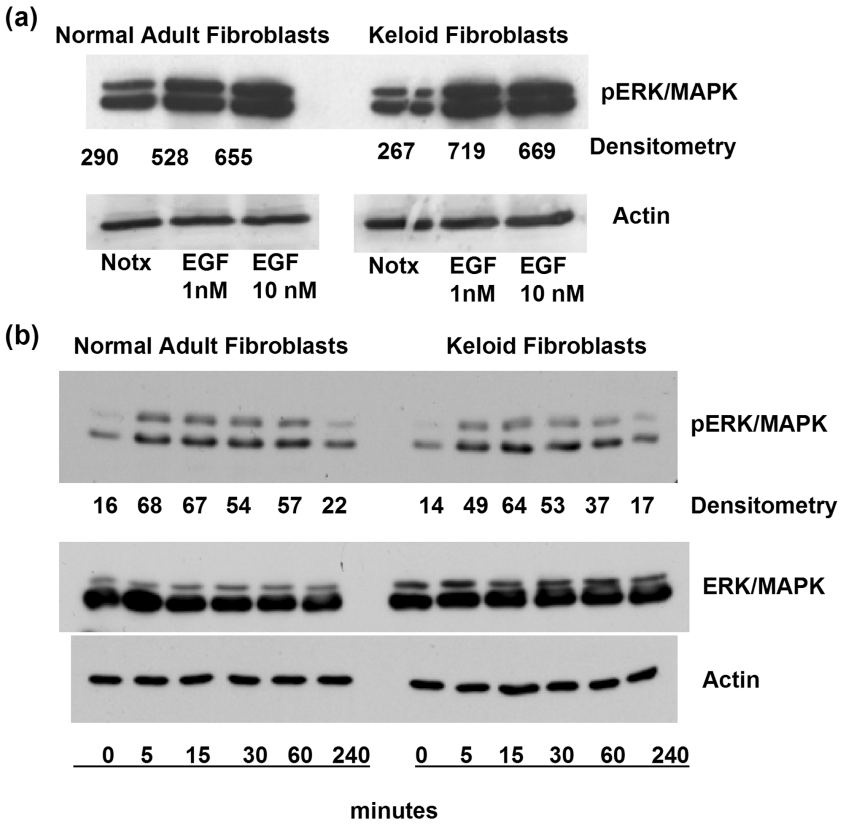

FIGURE 5. ERK phosphorylation is retained in keloid fibroblasts. Normal adult fibroblasts and keloid fibroblasts were grown to confluence and then quiesced. (a) Cells were either induced/uninduced with EGF 1 nM (10 minutes) or EGF 10 nM (5 minutes) and cell lysates were collected. (b) Alternatively, cells were treated with a saturating dose of EGF (10 nM), and cell lysates were collected at varying time intervals. Cell lysates were resolved on a 7.5 percent gel and phosphorylated proteins detected by antibodies specific for ERK, dual phosphorylated ERK, or α-actin, serving as a loading control. Shown is a representative immunoblot of three independent experiments. Numbers below immunoblots represent densitometry analysis as determined utilizing National Institutes of Health image 1.62.

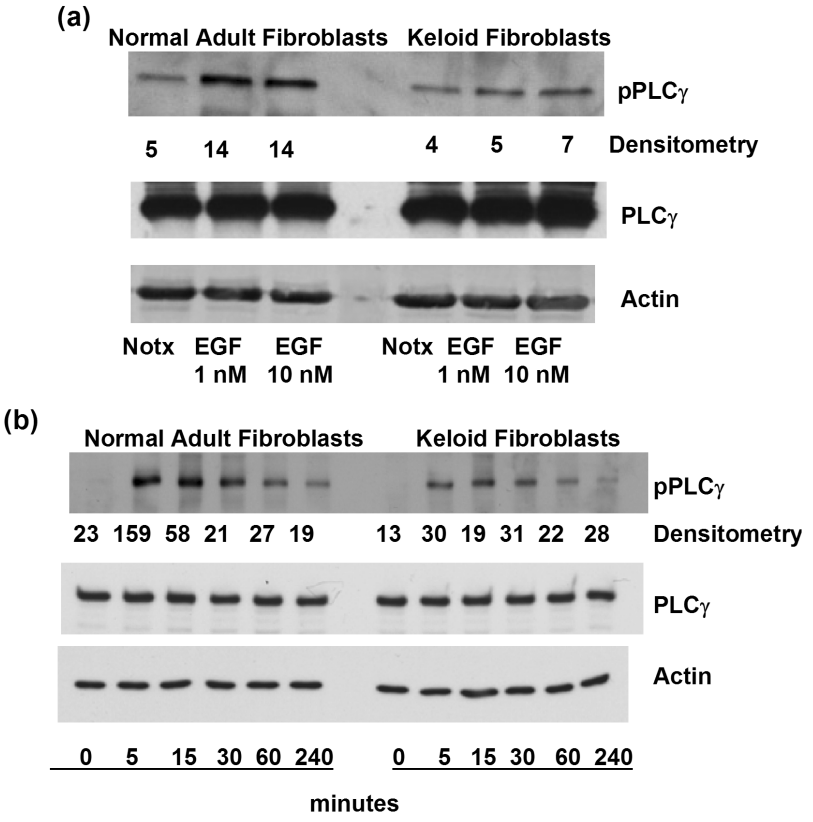

FIGURE 6. Phosphorlyation of PLCγ is limited in keloid fibroblasts. Normal adult fibroblasts and keloid fibroblasts were grown to confluence in 6-well tissue culture plates and quiesced. (a) Cells were either induced/uninduced with EGF 1 nM (10 minutes) or EGF 10 nM (5 minutes) and cell lysates were collected. (b) Alternatively, cells were treated with a saturating dose of EGF (10 nM), and cell lysates were collected at varying time intervals. Cell lysates were resolved on a 7.5 percent gel and phosphorylated proteins detected by antibodies specific for PLCγ, phosphorylated PLCγ, or α-actin, serving as a loading control. Shown is a representative immunoblot of three independent experiments. Numbers below immunoblots represent densitometry analysis as determined utilizing National Institutes of Health image 1.62.

intracellular signaling cascades have remained relatively unexplored. As ligands to EGFR are present throughout multiple stages of wound repair, we investigated the effects of EGF on keloid fibroblast migration.

The EGFR is expressed on many types of cells, including fibroblasts, keratinocytes, and vascular endothelial cells.[38,39] Ligands for EGFR, EGF, TGFα, heparin binding-EGF and amphiregulin in particular, have been implicated in the healing of a variety of wounds[40] including EGF itself, because it is released during the initial platelet plug and then produced in the wound by macrophages, fibroblasts, smooth muscle cells, and even keratinocytes. Therefore, it is present even in the later stages of wound repair, the ones during which, in normal healing, the repopulation and matrix production are halted and the dermis matured.[18] This "stop phase" is likely to be the stage that is not fully functional in keloid development, and thus signals present during this stage are of interest.

We found that EGFRs were present on keloid fibroblasts to a level not dissimilar from normal fibroblasts, even though the total EGFR content of the fibroblasts was significantly reduced. This deficit in total EGFR was reflected by reduced EGFR phosphorylation and limited downstream signaling. The major signaling pathways that have been identified for EGF-induced cell migration are the PLCγ signaling and the ras-raf-MEK-ERK/MAP kinase to m-calpain signaling cascade.[25,27,41] PLCγ signaling is necessary for induced chemokinetic but not for haptokinetic motility.[22,42] PLCγ levels were similar in both normal adult fibroblasts and in keloid fibroblasts, but activation of PLCγ was decreased. ERK MAPK activation status, as reflected by dual phosphorylation of p42/p44 ERK,

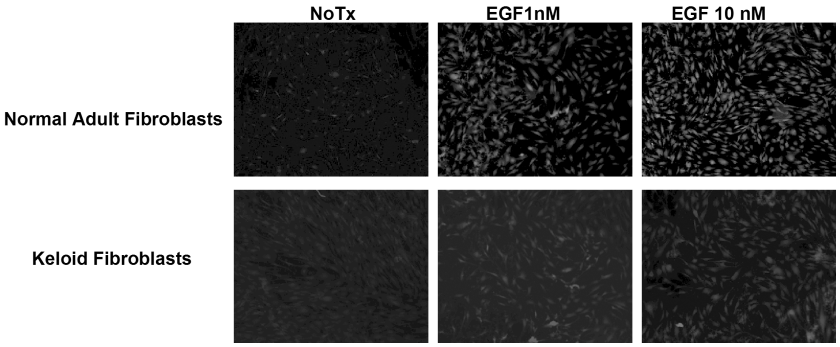

FIGURE 7. EGF did not induce significant calpain activity in keloid fibroblasts. Calpain activity was determined by BOC fluorescence in normal adult and keloid fibroblasts after treatment with EGF (1 nM) for 10 minutes and calpain cleavage of the synthetic substrate Boc-LM-CMAC was observed. The subsequent fluorescence is shown by a representative experiment of three independent studies. The time was chosen empirically for maximal fluorescence.

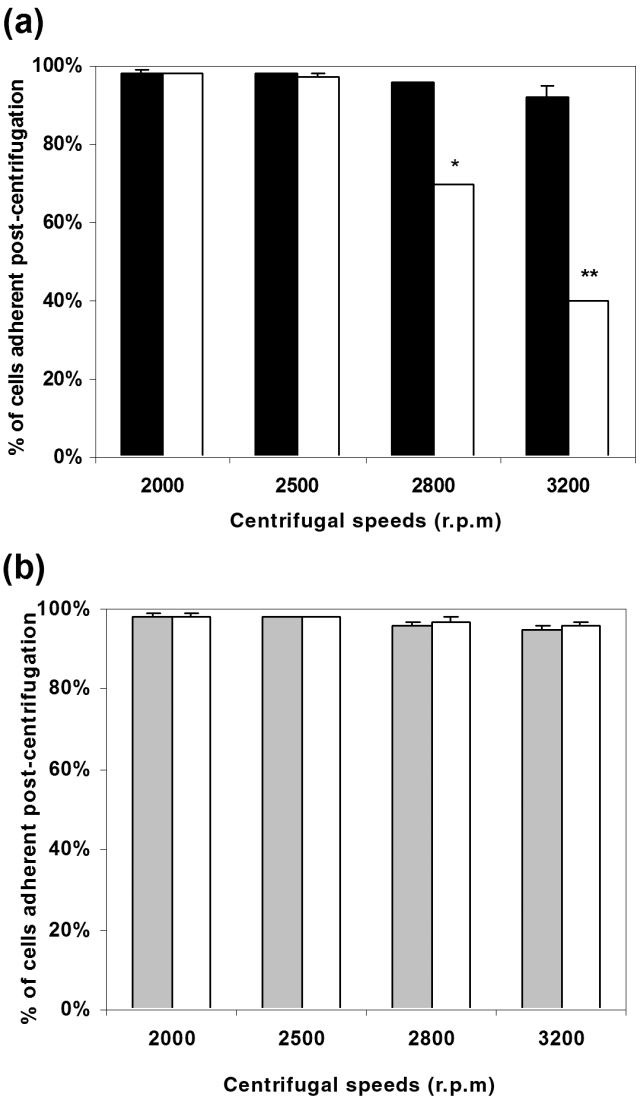

FIGURE 8. Keloid fibroblasts are more adherent to substratum even in the face of EGF. Cell substratum adhesiveness of (a) normal adult fibroblasts and (b) keloid fibroblasts at different centrifugal speeds was studied using the inverted centriguation assay. Cells were plated on to 12-well plates and were quiesced after confluence. Cells were treated without (filled bars) or with (open bars) EGF (1 nM) for 30 minutes after and before centrifugation the cells in the plates were counted by phase contrast microscopy. Values are calculated as percentage of precentrifugation cells remaining adherent. The values are mean ± SEM of three independent studies each performed in triplicate. Statistical analysis was performed by Student's *t*-test. $*p < 0.05$ compared to paired no EGF treatment, $**p < 0.01.46$.

was relatively intact, but this was not unexpected due to the amplification cascade that appears to maintain signaling even from reduced EGFR activation levels (Tran K, Personal Communication). However, the downstream activation of m-calpain, dependent on the small fraction of plasma membrane-associated ERK,[34] was similarly reduced. Thus, the EGFR signaling cascade, while intact and functional, appears diminished in keloid fibroblasts.

EGF induced both cell migration and proliferation of keloid fibroblasts, even if the responses were somewhat diminished compared to normal adult fibroblasts. In addition, keloid cells appeared resistant to EGF-induced de-adhesion, although this might reflect both reduced EGFR signaling and altered matrix production by keloid cells. Still, the diminutions in the cell responses were in line with similar decrements in EGFR autophosphorylation and activation status of PLCγ and m-calpain, two critical molecular switches for motility. One possible, and uninformative, explanation for this would be that the keloid fibroblasts were "aged" compared to the "aged" dermal fibroblasts, as "aged" dermal fibroblasts showed reduced EGFR levels and reduced responsiveness.[26] This is not likely the cause, as the pERK levels were retained, and even increased, in the keloid fibroblasts whereas they are reduced in parallel in the "aged" fibroblasts. Interestingly, despite ERK being robustly activated, the proliferative response was reduced to an even greater degree than the motility response. While ERK is required for EGFR-mediated proliferation,[43] it is not sufficient;[31] therefore, we would assume that the diminished EGFR signaling leads to reduced activation of the still-to-be-determined other mitogenic signaling cascades.[44] A second question arises of whether this diminution in cell responses is global or specific to EGFR. An initial inquiry suggests specificity as the response of our keloid cells to platelet-derived growth factor was similar to that of the normal adult fibroblasts. It was for this reason that we focused on signaling events downstream of EGFR activation and found the correlation between reduced receptor signaling and cell responsiveness. While others have noted the reduced responses to growth factors in keloid fibroblasts, we are the first to provide evidence of similarly reduced intracellular signaling cascades.

Our finding of decreased EGFR signaling and subsequent cell responses appear to contradict the medical situation of an overabundant dermal layer. However, this contradiction might be false because keloid development appears to result from a failure to "stop" the healing process and mature the nascent wound dermal layer.[18] As such, any continued in-migration and expansion of dermal fibroblasts would, over time, result in an enlarging dermal compartment. Thus, future studies need to focus on whether keloid fibroblasts respond to stop signals as much as on how they respond to regenerative and repopulation signals.

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REFERENCES

1. Rudolph R. Widespread scars, hypertrophic scars and keloids. Clin Plast Surg 1987;14:253–60.
2. Tredget EE, Nedelec B, Scott PG, Ghahary A. Hypertrophic scars, keloids and contractures: the cellular and molecular basis for therapy. Surg Clin North Am 1997;77:701–30.
3. Ladin DA, Garner WL, Smith DS Jr. Excessive scarring as a consequence of healing. Wound Rep Reg 1999;3:6–14.
4. Prathiba V, Rao KS, Gupta PD. Altered expression of keratins during abnormal wound healing in human skin. Cytobios 2001;104:43–51.
5. Diegelmann RF, Cohen IK, McCoy BJ. Growth kinetics and collagen synthesis of normal skin, normal scar and keloid fibroblasts in vitro. J Cell Physiol 1979;98:341–6.
6. Cohen IK, Peacock EE. Keloids and hypertrophic scars. In: McCarthy JG, May JW, Littler JW, editors. Plastic surgery. Philadelphia: Saunders, 1990:732–47.
7. Uitto J, Perejda AJ, Abergel RP, Chu ML, Ramirez F. Altered steady-state ratio of typeI/type III procollagen mRNAs correlates with selectively increased collagen type I procollagen biosynthesis in cultured keloids fibroblasts. Proc Natl Acad Sci USA 1985;82:5935–9.
8. Neely AN, Clendening CE, Gardner J, Greenhalgh DG, Warden GD. Gelatinase activity in keloids and hypertrophic scars. Wound Rep Reg 1999;7:166–71.
9. Babu M, Diegelmann R, Oliver N. Fibronectin is overproduced by keloid fibroblasts during abnormal wound healing. Mol Cell Biol 1989;9:1642–50.
10. Oliver N, Babu M, Diegelmann R. Fibronectin gene transcription is enhanced in abnormal wound healing. J Invest Dermatol 1992;99:579–86.
11. Sible JC, Rettig WJ, Eriksson E, Smith SP, Oliver N. Gene expression of tenascin is altered in normal scars and keloids. Wound Rep Reg 1995;3:37–48.
12. Dalkowski A, Schuppan D, Orfanos CE, Zouboulis CC. Increased expression of tenascin C by keloids in vivo and in vitro. Br J Dermatol 1999;141:50–6.
13. Kikuchi K, Kadono T, Takehara K. Effects of various growth factors and histamine on cultured keloid fibroblasts. Dermatology 1995;190:4–8.
14. Harper RA. Keloid fibroblasts in culture: abnormal growth behaviour and altered response to the epidermal growth factor. Cell Biol Int Rep 1989;13:325–35.
15. Haisa M, Okochi H, Grotendorst GR. Elevated levels of PDGFα receptors in keloid fibroblasts contribute to an enhanced response to PDGF. J Invest Dermatol 1994;103:560–3.
16. Yoshimoto H, Ishihara H, Ohtsuru A, Akino K, Murakami R, Kuroda H, Namba H, Ito M, Fujii T, Yamashita S. Overexpression of insulin-like growth factor-1 (IGF-I) receptor and the invasiveness of cultured keloid fibroblasts. Am J Pathol 1999;154:883–9.
17. Babu M, Diegelmann R, Oliver N. Keloid fibroblasts exhibit an altered response to TGF-β. J Invest Dermatol 1992;99:650–5.
18. Babu M, Wells A. Dermal-epidermal communication in wounds. Wounds 2001;13:183–9.
19. Wells A. The EGF receptor signaling system—a model for growth factor receptor signaling. In: Goffin V, Kelly PA, editors. Hormone signaling. Norwell, MA: Kluwer Academic Publishers, 2002:57–80.
20. Chin GS, Liu W, Steinbrech D, Hsu M, Levinson H, Longaker MT. Cellular signaling by tyrosine phosphorylation in keloid and normal human dermal fibroblasts. Plast Reconstr Surg 2000; 106:1532–40.
21. Lawrence WT, Diegelmann RF. Growth factor in wound healing. Clin Dermatol 1994;12:157–69.
22. Chen P, Xie H, Sekar MC, Gupta KB, Wells A. Epidermal growth factor receptor-mediated cell motility: phospholipase C activity is required, but MAP kinase activity is not sufficient for induced cell movement. J Cell Biol 1994;127:847–57.
23. Chen P, Xie H, Wells A. Mitogenic signaling from the EGF receptor is attenuated by a phospholipase C-γ/protein kinase C feed back mechanism. Mol Biol Cell 1996;7:871–81.
24. Xie H, Pallero MA, Gupta D, Chang P, Ware MF, Witke W, Kwiatkowski DJ, Lauffenburger DA, Murphy-Ulrich JE, Wells A. EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLC signaling pathway. J Cell Sci 1998;111:615–24.
25. Glading A, Chang P, Lauffenburger DA, Wells A. Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. J Biol Chem 2000;275:2390–8.
26. Shiraha H, Gupta K, Drabik K, Wells A. Aging fibroblasts present reduced epidermal growth factor (EGF) responsiveness due to preferential loss of EGF receptors. J Biol Chem 2000;275: 19343–51.
27. Shiraha H, Gupta K, Glading A, Wells A. Chemokine transmodulation of EGF receptor signaling: IP-10 inhibits motility by decreasing EGF-induced calpain activity. J Cell Biol 1999; 146:243–53.
28. Reddy CC, Niyogi SK, Wells A, Wiley HS, Lauffenburger DA. Engineering epidermal growth factor for enhanced mitogenic potency. Nat Biotech 1996;14:1696–9.
29. Wiley HS, Cunningham DD. Epidermal growth factor stimulates fluid phase endocytosisin human fibroblasts through a signal generated at the cell surface. J Cell Biochem 1982;19:383–94.
30. Chen P, Gupta K, Wells A. Cell movement elicited by epidermal growth factor receptor requires kinase and autophosphorylation but is separable from mitogenesis. J Cell Biol 1994;124: 547–55.
31. Wells A. EGF receptor. Int J Biochem Cell Biol 1999;31:637–43.
32. Gladhaug IP, Christoffersen T. Rapid constitutive internalization and externalization of epidermal growth factor receptors in isolated rat hepatocytes: monensin inhibits receptor externalization and reduces the capacity for continued endocytosis of epidermal growth factor. J Biol Chem 1988;263:12199–203.
33. Swindle CS, Tran KT, Johnson TD, Banerjee P, Mayes AM, Griffith L, Wells A. Epidermal growth factor (EGF)-like repeats of human tenascin-C as ligands for EGF receptor. J Cell Biol 2001; 154:459–68.
34. Glading A, Uberall F, Keyse SM, Lauffenburger DA, Wells A. Membrane proximal ERK signaling is required for M-calpain activation downstream of EGF receptor signaling. J Biol Chem 2001;276:23341–8.
35. Younai S, Nichter LS, Wellisz T, Reinisch J, Nimni ME, Tuan TL. Modulation of collagen synthesis by transforming growth factor-beta in keloid and hypertrophic scar fibroblasts. Ann Plast Surg 1994;33:148–51.
36. Bettinger DA, Yager DR, Diegelmann RF, Cohen IK. The effect of TGF-β on keloid fibroblast proliferation and collagen synthesis. Plast Reconstr Surg 1996;98:827–33.
37. Polo M, Smith PD, Kim YJ, Wang X, Ko F, Robson MC. Effects of TGF-β2 on proliferative scar fibroblasts cell kinetics. Ann Plast Surg 1999;43:185–90.
38. Nanney LB, McKanna JA, Stoscheck CM, Carpenter G, King LE. Visualization of epidermal growth factor receptors in human epidermis. J Invest Dermatol 1984;82:165–9.
39. Nanney LB, Stoscheck CM, King LE Jr, Underwood RA, Holbrook KA. Immunolocalisation of epidermal growth factor

receptors in normal developing human skin. J Invest Dermatol 1990;94:742–8.
40. Nanney LB. Epidermal and dermal effects of epidermal growth factors during wound repair. J Invest Dermatol 1990;94:624–9.
41. Wells A, Gupta K, Chang P, Swindle S, Glading A, Shiraha H. Epidermal growth factor receptor-mediated motility in fibroblasts. Microsc Res Tech 1998;43:395–411.
42. Carloni V, Romanelli RG, Pinzani M, Laffi G, Gentilini P. Focal adhesion kinase and phospholipase C-γ involvement in adhesion and migration of human hepatic stellate cells. Gastroenterology 1997;112:522–31.
43. Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouyssegur J. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J 1999;18:664–74.
44. Jones SM, Kazlauskas A. Growth-factor-dependent mitogenesis requires two distinct phases of signaling. Nature Cell Biol 2001;3:165–72.