Transcriptional Signature of Epidermal Keratinocytes Subjected to in Vitro Scratch Wounding Reveals Selective Roles for ERK1/2, p38, and Phosphatidylinositol 3-Kinase Signaling Pathways

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Covering denuded dermal surfaces after injury requires migration, proliferation, and differentiation of skin keratinocytes. To clarify the major traits controlling these intermingled biological events, we surveyed the genomic modifications occurring during the course of a scratch wound closure of cultured human keratinocytes. Using a DNA microarray approach, we report the identification of 161 new markers of epidermal repair. Expression data, combined with functional analysis performed with specific inhibitors of ERK, p38MAPK and phosphatidylinositol 3-kinase (PI3K), demonstrate that kinase pathways exert very selective functions by precisely controlling the expression of specific genes. Inhibition of the ERK pathway totally blocks the wound closure and inactivates many early transcription factors and EGF-type growth factors. p38MAPK inhibition only delays “healing,” probably in line with the control of genes involved in the propagation of injury-initiated signaling. In contrast, PI3K inhibition accelerates the scratch closure and potentiates the scratch-dependent stimulation of three genes related to epithelial cell transformation, namely HAS3, HBEGF, and ETS1. Our results define in vitro human keratinocyte wound closure as a repair process resulting from a fine balance between positive signals controlled by ERK and p38MAPK and negative ones triggered by PI3K. The perturbation of any of these pathways might lead to dysfunction in the healing process, similar to those observed in pathological wounding phenotypes, such as hypertrophic scars or keloids.

Epidermal wound healing is a complex physiological process involving multiple cell features, such as proliferation, migration, and differentiation of the keratinocytes situated at the edge of the wound. All of these steps involve a well orchestrated regulation of multiple signaling pathways that control the expression of many genes endowed with diverse crucial functions (growth factors, cytokines, proteases, integrins, cell cycle genes, and extracellular matrix components). Disrupted expression of these molecules or of their signaling pathways can block or accelerate the healing process, thereby leading to chronic wounds or hypertrophic scars or keloids. Transgenic and knock out mice models have largely helped in defining some crucial genes involved in the wound healing response (1), but the great majority of the genes involved in skin wound healing remain unidentified. Recently, several global expression studies using DNA microarrays have approached this question in such models and have brought interesting insights in terms of a genomic characterization of wound healing (2–4). Obvious structural differences between murine and human skin limit notably the generalization of such data to the wound healing features occurring in human tissues. Such a biological limitation has justified the development of new experimental approaches using human models. At present, only one transcriptomical study relative to human skin wound healing has been published (5). Although in vitro approaches are relevant to embrace globally what happens during skin wound healing, the complexity provided by the coexistence of many cell types makes difficult the identification of mechanisms specific to keratinocytes. This in vitro approach allowed the analysis of events occurring immediately after injury but was unable to address specific questions about later events affecting cell migration, proliferation, or differentiation. Numerous in vitro studies have addressed these questions, quantifying various parameters associated with reepithelialization. For instance, in vitro “wound healing” has been modeled by treating skin cells with soluble factors known to be secreted in vivo after injury, such as TNFa, or by serum addition (6, 7) (i.e. conditions that are supposed to mimic the context of wound healing). Such
studies have led to the identification of interesting gene profiles. However, these procedures do not take into account either the putative signaling generated by the disruption of the extracellular matrix or the cell to cell interaction, which both play pivotal roles in the healing process.

Recently, we have developed an original “wounding” device that creates continuous long scratches within cell cultures. This device increases the number of cells participating in the in vitro “wound” closure (up to 40–50% of the cell layer) when compared with the classical scratch models and allows the detection of a wide spectrum of molecular events stimulated in response to injury (8, 9). Interestingly, this system can generate at the same time shear stress signals and soluble derived factors. It thus represents a powerful tool to investigate the global alterations of gene expression occurring throughout the “healing” process of normal human keratinocytes (NHK) in culture. Combining a DNA microarray approach with our “wounding” device, we have 1) performed an accurate analysis of the global gene expression profile in cultured human keratinocytes after scratching, 2) identified molecular signatures of the response to injury, and 3) elucidated the molecular and functional contributions of the ERK, PI3K, and p38MAPK pathways during human keratinocyte wound healing.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human keratinocytes were isolated from healthy neonatal foreskin as described (10). Cell culture keratinocytes were seeded on γ-irradiated 3T3-J2 fibroblast feeder layers (2 × 10⁵ cells/cm²) and grown in Green’s medium (½ Dulbecco’s minimal Eagle’s medium, ½ Ham’s F-12 medium, 20 mM HEPES, 1000 units/ml penicillin, 1000 units/ml streptomycin, 5 μg/ml insulin, 0.4 mg/ml hydrocortisone, 10 μM cholera toxin, 10 μM recombinant human EGF, 2 μM triiodothyronine, and 18 μM adenine.

**Antibodies and Western Blotting**—Antibodies against phosphorylated forms of ERK1/2, ATF, and AKT were from Cell Signaling Technology and were used at a 1:6000 dilution. After migration on PAGE and transfer onto Immobilon-P membrane (Millipore), the specific proteins were revealed using the Millipore ECL detection system.

**Scratching Protocol**—The cell scratching was performed using a “scarificator,” previously described by Turchi et al. (8). In classical conditions, 40–50% of the cell culture was scratched, the width between the “wound edges” being about 300–400 μm. The cultures were stopped by washing with phosphate-buffered saline, followed by a quick freezing to −80 °C, at different times after wounding (1, 3, 6, 15, and 24 h). For each time of the kinetic series, nonscratched cells were used as controls. In experiments performed with U0126, SB203580, and LY294002, the inhibitors were preincubated with the cells, 1 h before scratching at a respective concentration of 10, 20, and 15 μM.

**RNA Extraction**—5–10 × 10⁵ cells were quickly homogenized in 10 ml of a 4 M guanidium thiocyanate solution, 25 mM sodium citrate, pH 7.0, 100 mM β-mercaptoethanol, 0.5% N-laurylsarcosine, followed by the addition of 1 ml of 2 M sodium acetate, pH 4.0, 8 ml of freshly water-equilibrated phenol, and 2 ml of chloroform. After 15 min on ice, samples were centrifuged for 20 min at 7500 × g. The RNA of the upper aqueous phase was precipitated with one volume of isopropyl alcohol. The sample was incubated 1 h on ice, after which the RNA was pelleted by centrifugation for 20 min at 7500 × g. The pellet was washed twice with 70% ethanol and then resuspended in RNase-free water.

**cDNA Microarrays**—The DNA microarray analysis concerning the identification of the genes altered in response to cell scratching was performed on three independent experiments, derived from three distinct cell cultures. Results were generated after two independent microarray analyses, meaning that each time point was the result of six independent analyses. The data relative to the effect of U0126, SB203580, and LY294002 on the transcriptomical healing response were obtained from two independent experiments performed using two distinct cell cultures. Results were generated after two independent microarray analyses.

The cDNA microarray contained 4200 distinct cDNA probes. It has been previously described in Moreilhon et al. (11). Gene selection was based on relevance to inflammation, infection, differentiation, ion transport, cell signaling, cell migration, proliferation, etc. A large fraction of the probes also corresponded to transcripts encoding membrane proteins.

The list of the 4200 probes is available on the World Wide Web at www.microarray.fr/IPMC/cDNA_microarray5k.html, and the microarray is archived in GEO under reference GSE1853. The cDNA probes were PCR-amplified from cDNA derived from Universal Human Reference RNA (Stratagene) by reverse transcription. Probes had the following properties: 1) they have a normalized length of 250 ± 19 bp; 2) they have a normalized GC content of 52 ± 8%; 3) they were specific for a unique human gene; and 4) they were controlled by DNA sequencing. PCR products were purified by using a QIAquick 96 PCR purification kit (Qiagen), resuspended in 3SSC with an average spotting concentration of 200 ng/μl. Microarrays were printed with an SDDC-2 microarrayer (Bio-Rad) on homemade aldehyde-coated glass microscope slides. Data presented in the present work only refer to sequence-verified probes. Each PCR product was spotted four times on each slide (two independent clusters of two spots spatially separated) to reduce positional bias of the fluorescence readout.

**Cy3- and Cy5-labeled cDNA, Postprocessing, and Hybridization**—The CyDye-labeled first-strand cDNAs were generated with the CyScribe First-Strand cDNA labeling kit (RPN 2600; Amersham Biosciences), as described by Moreilhon et al. (11), using 10 μg of total RNA as template. Unincorporated CyDye nucleotides were removed using the nucleotide removal kit (Qiagen).

**Data Collection and Analysis**—Microarrays were scanned either on GenePix4000B or on a ScanArray5000 microarray scanner (PerkinElmier Life Sciences). Both machines provided similar results (not shown). 16-bit TIF images were quantified with the corresponding software (GenePix Pro 5.0 program (Axon Instruments) for the GenePix and Quantarray for the ScanArray). Intensities (either total or background values) were defined as average intensities for each spot. Negative controls were spotted on each slide. They corresponded to nonmammalian mRNA sequences with no significant identity with any...
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human sequences. Such spots were used as controls. Data were normalized using a dye swap method as described by Moreillon et al. (11). We found that this method, although requiring duplication of experiments, improves the reproducibility of the quantification.

Scratched and nonscratched RNA samples were reverse transcribed in the presence of known amounts of the corresponding nonmammalian “control” RNAs, namely DmdNaC and DgNaC (12, 13), added at a respective scratched/nonscratched ratio of 5 and 0.2. Only experiments showing a good correlation between the experimental and true ratios for these controls were kept for analysis. Results are expressed as average ratios of intensities in “scratched cells” over intensities in “nonscratched cells.”

Data were then analyzed and/or visualized with MEV (14), or with the stand alone program GeneANOVA (15), and the Significance Analysis of Microarrays (SAM) Excel TM plug-in (16). Lists of genes significantly down- and up-regulated under the different experimental conditions were selected by analysis of variance or using SAM. For the analysis of variance, the signal was modeled as proposed by Kerr and Churchill (17) according to Equation 1,

\[ y_{ijkg} = \mu + A_i + D_j + T_k + G_g + (TG)_{kg} + (AG)_{ig} + (DG)_{ig} + \epsilon_{ijkg} \quad (Eq. 1) \]

where \( y_{ijkg} \) is the fluorescent intensity of array \( i \) and dye \( j \) representing treatment \( k \) and gene \( g \). \( \mu, A_i, D_j, T_k, \) and \( G_g \) are factors representing average signals among the whole experiment, one array, one dye, one treatment, or one gene, respectively. \((TG)_{kg}\), \((AG)_{ig}\), and \((DG)_{ig}\) are two-factor interactions between treatment and gene, array and gene (spot effects), and dye and gene, respectively. \( \epsilon_{ijkg} \) represents a residual noise component, modeled as a Gaussian distribution with mean 0 and variance \( \sigma^2 \).

\((TG)_{kg}\) denotes differences in expression for particular treatment and gene combinations that are not explained by the average effects on these treatments and genes. They represent the effects of interest, which were modeled using GeneANOVA. SAM analysis was performed as described by Tusher et al. (16). Ontologies attached to each gene were then used to classify altered genes according to main biological themes, as described in Ref. 18. Annotation of our probes was also accessible through MEDIANTE, our own microarray data base (19), which stores annotations derived from public data bases. Additional statistical analyses, including principal component analysis, were performed with GeneANOVA. The set of data is accessible in the GEO data base under the reference GSE6820.

Quantitative Real Time One-step Reverse Transcription-PCR and Western Blot—RNAs were extracted as described above. mRNA expression levels were quantified by real time one-step reverse transcription-PCR using SYBR-Green PCR Master Mix (Applied Biosystems). The results are the average of three separate experiments. The oligonucleotide sequences corresponding to the different genes used for real time quantitative PCR are available upon request.

RESULTS AND DISCUSSION

Identification of the Transcriptomal Profile Associated with Keratinocyte Response to in Vitro Injury—This study was performed in order to identify genes from confluent NHK, the expression of which was altered after a mechanical scratching (i.e. an in vitro system used to recapitulate some aspects of skin wound reepithelialization). For that purpose, we used a device that creates calibrated long scratches, allowing a subsequent detection and quantification of overall molecular events (8). We observed that 5–6 h after scratching, cells began to migrate into the denuded space resulting from the tearing of the keratinocyte sheet. This space was totally filled after 25–30 h (not shown).

Microarray Data Mining—To identify the genes accompanying the migration and proliferation of NHK in our scratch assay, we used a ~4200 sequence-verified cDNA microarray, already described in Ref. 11 and further detailed under “Experimental Procedures.”

NHK were scratched and harvested after 1, 3, 6, 15, and 24 h. Total RNAs were isolated and reverse transcribed into fluorescent cDNAs according to standard protocols (see “Experimental Procedures” for microarray procedures). Fig. 1 shows a typical MA plot (20) of our experiments. It represents the ratio of scratched (S) cells and nonscratched (NS) signals according to the average intensity of the signal 6 h after scratching. Although the expression of most genes was not modified (\( M = 0 \), several probes were decided up- or down-regulated in a significant and reproducible way (corresponding to the points located over and below \( M = 0 \) in Fig. 1). Variation was corroborated by the use of exogenous nonhuman controls (namely DmdNaC and DgNaC). Their presence at defined concentrations in the RNA samples of nonscratched and scratched cells helped to validate each experiment (see “Experimental Procedures”).

Since the results presented here correspond to experiments performed at least three times on three or more distinct cell cultures, selection of genes was performed using an analysis of variance as described by Didier et al. (15). Cut-offs were defined as follows. The \( p \) value was chosen below \( 10^{-4} \), and statistical...
variance was above 40%. Significant transcripts were also identified using the SAM software, according to Ref. 16. The cut-off for discriminating differential expression was set at 2-/H11006/0.75-fold for the up-regulated and down-regulated genes. These statistical analyses allowed us to select 118 up-regulated and 43 down-regulated genes during the healing time course in wounded cells (Fig. 2).

An overview of the major gene pattern is summarized in Fig. 2. This analysis clearly revealed the existence of five clusters of transcripts, depending on their distinct time courses of variation. Cluster 1 included 34 transcripts associated with early genes. They were transiently altered between 0 and 3 h after injury. Cluster 2 (intermediate genes) included 19 transcripts, the expression of which was modified between 3 and 6 h after injury. Cluster 3 (late genes) included 56 transcripts that were altered between 6 and 24 h after injury. Cluster 4 (sustained) included 46 transcripts that were rapidly modified after "injury" but remained up-regulated or down-regulated for at least an additional 15 h. Finally, cluster 5 corresponded to three up-regulated transcripts exhibiting a "biphasic" pattern, with a first burst of stimulation between 1 and 3 h after injury, followed by a second burst after 15 h.

To draw a clearer picture of the functional relevance of these genes, we used the Ease program (18) to further classify the selected transcripts into seven functional families according to the biological properties of the proteins that they encode (Fig. 3). These seven families corresponded to the most significant families identified by the EASE algorithm. The F1 family grouped together the regulators of transcription; the F2 family grouped together signal transducers; the F3 family grouped together growth factors and cytokines; the F4 family grouped together transcripts associated with ECM component/remodeling and cell/cell interaction; the F5 family grouped together transcripts associated with cell proliferation and apoptosis; and the F6 family grouped together transcripts associated with metabolism and transport. The F7 family ("miscellaneous genes") grouped together transcripts, the function of which remains unclear (Fig. 3). It is interesting to note some striking relationships between the five distinct clusters and the seven families. They are developed below.
FIGURE 3. "Heat map" representing the genes altered in response to scratching and classified in function of their biological activity by using the Ease program. The legend at the top of the plot indicates the position of the genes that we have classified as "early," "intermediate," and "late" genes.
Transcription Factors and Signal Transducers—45% of the transcripts belonging to the “early genes” cluster also belonged to the transcription factor (F1) family. Most of them, such as ATF3, EGR1, JUN, JUNB, fos, gata3, ets1, KLF10, TNFAIP3, ZFP36, and NR4A2, were early transient genes stimulated between 1 and 3 h after scratching. fos, JUN, JUNB, EGR1, and ATF3 (Table 1) have previously been reported in wound healing studies (21–23). Such observations validate our technical approach and suggest that it is relevant to dissect the transcription-inducing activities of TGFβ (27). Its stimulation indicates that, besides their capability to induce apoptosis in response to stress signals, TGFβ family members also probably trigger “pure” antimitotic or differentiation signals intervening in proliferation arrest when cells attain confluence. Parallel to proapoptotic TGFβ signals, stimulation of the NF-κB pathway was noticed to occur rapidly after scratching. Due to well known antiapoptotic properties of NF-κB, it is tempting to speculate that NF-κB activation participates in the stress response by promoting the production of survival signals. These observations also indicate that pro- and antipapoptotic signals can be detected simultaneously immediately after injury. The fact that NHK maintain their ability to migrate and to proliferate a long time after scratching seems to indicate that antipapoptotic signals remain prominent during the whole process of scratch closure.

The stimulation of IRF6, an interferon-regulated factor, shows that besides TGFβ-dependent transcription factors, other signaling pathways are involved in the control of the proliferation/differentiation balance in the disrupted keratinocyte sheet (28). Among the early transcription factors, some are potential regulators of cell migration. This is, for instance, the case for KLF6, a Krüppel-like family member shown to repress MMP9 transcription in endothelial cells (29). Since MMP9 is up-regulated some time after scratching, the stimulation of KLF6 might prevent a premature activation of MMP9.

The “signal transducer” family (F2) included up-regulated transcripts (RGS2, SGK, RND3, TRIB1, EPHA2, PIM-1, and BCAR3) as well as down-regulated transcripts (P2RY2, P2RY1, P2Y5, RASGRF1, and VAV3). The altered “signal transducer” members belonged mainly to the intermediate cluster; 53% of them were detected within 6 h following “injury,” suggesting that they play a role in the propagation of adaptive signals rather than in the triggering of the early response. Such transcripts encode distinct functional proteins, such as serine/threonine kinases (PIM-1, MAP4K4, SGK, and PINK1), receptor tyrosine kinases (EPHA2), phosphatases (PTPN12 and PTPrN), molecular adaptors (DAPP1, AKAPl2, BCAR3, and TNIp1), GTPases or GTPase-related proteins (RND3, RGS2, ARHGAP29, RASA1, and RRAD), and growth factors/cytokine receptors (TGFBR2, CD40, and TNFRSF8). Among these signal transducers, we observed that 1) RND3, RRAD, ARHGAP29, PTPN12, RASA1, and ARHE (30–35) are antagonists of the small GTPase-related proteins Rho, Rac, and Ras; 2) TNIp1 and TNFAIP3 inhibit NF-κB signaling without sensitizing cells to apoptosis (36–39), and 3) SKIL is an antagonist of the TGFβ pathway that interacts with smad2 and smad3 and represses their transcriptional activity (40). Rho GTPases, NF-κB, and TGFβ signaling are rapidly activated in response to “injury” and play a role in cell migration, proliferation, and survival. Our data suggest that in addition to these signals, negative feedback mechanisms are delivered to limit 1) an excessive or uncontrolled migration or adhesion through the regulation of ARHE, RND3, RRAD, ARHGAP29, RASA1, or PTPN12, 2) the damaging action of proinflammatory cytokines, such as TNFα, through the stimulation of TNIp1 and TNFAIP3, or 3) the effects of TGFβ through SKIL.

The induction of BRCA3 and MAP4K4 is also in agreement with the promigratory signaling program launched by scratching. Indeed, BCAR3 encodes a protein interacting with EGRF

### Table 1

| Symbol | Locus.Link | Molecular function (Ease) | Reference |
|--------|------------|---------------------------|-----------|
| V-EGF  | 7422       | Growth factor activity    | 49        |
| V-EGFC | 7424       | Growth factor activity    | 49        |
| TGFα   | 7039       | Growth factor activity    | 49        |
| AREG   | 374        | Growth factor activity    | 49        |
| EREG   | 2069       | Growth factor activity    | 49        |
| HBBEGF | 1839       | Growth factor activity    | 49        |
| ATF3   | 467        | Transcription factor      | 23        |
| IL8    | 3576       | Chemokine activity        | 49        |
| IL18   | 3606       | Chemokine activity        | 79        |
| JUN    | 3458       | Cytokine activity         | 80        |
| PDGFB  | 5155       | Growth factor activity    | 49        |
| TGFβ1  | 7040       | Regulator of proliferation| 49        |
| FST    | 10468      | Inhibitor of TGFβ superfamily | 81    |
| INHBA  | 3624       | TGFβ receptor binding protein | 81    |
| PTHHL  | 5744       | Growth regulator          | 82        |
| FOS    | 2353       | Transcription factor      | 22        |
| JUN    | 3725       | Transcription factor      | 83        |
| PPARD  | 5467       | Transcription factor      | 84        |
| EGR1   | 1958       | Transcription factor      | 85        |
| LAMA3  | 3909       | Cell adhesion             | 70        |
| LAMB3  | 3914       | Cell adhesion             | 70        |
| LAMC2  | 3918       | Cell adhesion             | 70        |
| PLA1U  | 5328       | Trypsin/chymotrypsin activity | 86    |
| SERPINB2| 5055      | Endopeptidase inhibitor   | 87        |
| SERPINE1| 5054      | Endopeptidase inhibitor   | 88        |
| ITGB4  | 3691       | Cell-matrix adhesion      | 89        |
| HAS1   | 3036       | Cell adhesion             | 90        |
| HAS2   | 3037       | Cell adhesion             | 90        |
| HAS3   | 3038       | Cell adhesion             | 90        |
| THBS1  | 7057       | Adhesion molecule activity| 91        |
| PTGS2  | 5743       | Prostaglandin biosynthesis| 92        |
| KRT6B  | 3854       | Cell structure            | 93        |
| KRT16  | 3868       | Cell structure            | 93        |
| MMP9   | 4318       | Cell matrix remodeling    | 94        |
that sustains its activation (41). Moreover, BCA2 enhances cell migration, in synergy with its carboxyl-terminal binding partner Cas (42). MAP4K4 plays a role in cell migration and invasion, since its inactivation prevents tumor cell migration in carcinoma cells (43, 44).

We observed that NHK scratching strongly decreases the expression of the VAV3 oncogene. Such a down-regulation is typical of the activations of TGFβ and EGF pathways (45). It has been shown that VAV3 is important for cell migration and cytoskeletal organization (46, 47) and that high levels of VAV3 perturb cytokinesis and lead to the appearance of multinucleated cells (48). The down-regulation of VAV3, which occurs some time after scratching, could contribute to slow down cell migration when cell culture approaches confluence but could be also a regulatory cell division signal, provided to prevent abnormal cytokinesis.

**Growth Factors, Cytokines, and Receptors**—The majority of soluble factors identified in our study included regulators of cell proliferation and/or migration, such as members of the EGF family (HBEGF, EREG, AREG, VEGF, TGFA, PDGF, INHBA, FST, IFNG, and PTHLH) and also proinflammatory cytokines or chemokines, such as ILSRA, IL8, IL18, and LIF. Several of them have been previously associated with wound healing using in vivo and in vitro models (49, 50).

Accumulating evidence indicates that the Wnt pathway is activated in response to mechanical injury (51–53) and that excessive Wnt activation leads to hypertrophic scars or keloids (54). Here we show that scratched NHK expressed strong levels of DKK1, an inhibitor of the canonical Wnt/β-catenin pathway (55). From that perspective, DKK1 may interfere with the Wnt pathway in finely regulating its amplitude and/or duration along the scratch closure in order to avoid excessive cell migration and/or proliferation. In line with this concept, NHK tearing also stimulated the expression of IL1RN, an inhibitor of IL-1 signaling. We have previously shown that IL-1α and -β are rapidly released from intracellular pools after NHK scratching (8) and are most likely involved in NHK proliferation (56). The rapid up-regulation of IL1RN could constitute a way to regulate the extracellular IL1 concentration and to limit its hyperproliferative effect.

NHK scratching also decreased the expression of transcripts associated with NHK proliferation or migration, such as FGFR2/3. FGFR2 is the specific receptor of the keratinocyte growth factor (KGF and FGF7), and its inactivation has been shown to abrogate the reepithelialization process (57). Therefore, FGFR2 down-regulation is likely to constitute a mechanism to stop the action of KGF and to prevent proliferative signals upon confluence.

**Genes Involved in ECM Remodeling, Cytoskeleton, and Migration**—Keratinocyte migration into the scratch zone requires major modification of the ECM composition of the cell to cell interactions and of the cytoskeletal organization. Such processes are clearly highlighted in our analysis (see F4 in Fig. 3), with the up-regulation of transcripts such as ITGB4, LAMA3, B3, C2, PLAU, SERPINB2/E1, THBS1, MMP9, HAS1/2/3, ITGA5, etc. (extracellular matrix remodeling molecules) as well as KRT6 and KRT16 (cytoskeleton reorganization) (Table 1). Such a signature is consistent with a pattern of migrating and proliferating cells (see Table 1 for references).

In addition, we also identified other transcripts (CRYAB, CLDN1, CLDN9, ITP2, ADAMTS1, PCDH1, VIL3, MAP4, SEMA3C, and 7A). We observed a transient up-regulation of CLDN1/9 claudin-1/9 and ITP2 (tight junction protein 2), three molecules involved in cell cohesion, suggesting that keratinocyte cohesion probably increased after scratching. This observation is in line with a video microscopy analysis showing that NHK do not dissociate from each other in order to migrate individually into the scratch “bed” but rather slide together due to high cell cohesion.

We originally found that scratching stimulates the expression of SEMA3A family members, such as SEMA3C. SEMA3C has been reported to play a key role in axonal growth and migration of endothelial cells, but no role of SEMA3C in keratinocyte migration has ever been evoked. A recent study reports that SEMA3A, a member of the SEMA3 family, decreases keratinocyte migration (58). From this perspective, SEMA3C induction could represent a brake to counterregulate positive promigratory signals generated by released soluble factors when cells approach confluence.

Several transcripts, also involved in cell-to-cell and cell-ECM interaction, were down-regulated. Two of them, ITGA2B (α2 integrin) and KRT10 (keratin 10), have captured our attention. ITGA2 has been reported to mediate attachment, spreading, and migration on collagen (59). Our data show that ITGA2 is down-regulated when cells attain confluence, suggesting that the inhibition of ITGA2-associated signaling is probably an important part of the message to slow down migration and can be considered as a marker of the final phases of the repair process.

KRT10 is one of the major structural proteins of differentiated suprabasal keratinocytes, which belongs to the large family of intermediate filament-regulating proteins. We observed that KRT10 is expressed at a high level in confluent keratinocytes, indicating that, although this culture mostly consists of basal keratinocytes, it also contains a subpopulation of suprabasal differentiated ones. KRT10 is down-regulated in tumor cells and in keratinocytes after wounding, but the functional consequences of these adaptations are not yet completely understood (60). Some data have indicated that keratin 10 filaments have intrinsic bundling properties that confer rigid cell architecture and limit keratinocyte dynamic properties (61, 62). It has been also reported that down-regulation of KRT10 is important for keratinocyte proliferation. Indeed, ectopic expression of KRT10 in the basal epidermal layer of transgenic mice leads to a strong decrease in proliferation and reduced tumorigenesis (63). Recently, it has also been demonstrated that the knock-out of KRT10 in mice is accompanied by a significant epidermal hyperproliferation, associated with an activation of the mitogen-activated protein kinase (MAPK) pathway. Moreover, the concomitant up-regulation of KRT6 and KRT16 in KRT10 null epidermis provokes increased keratinocyte motility (64). Our NHK scratch assay, obtained by using primary human cells, recapitulates this situation (i.e. KRT10 decrease and KRT6/16 stimulation). We can imagine that a decrease in KRT10 is required to activate the migration and proliferation programs
in order to ensure the closure of the scratch zone. By analogy with what has been reported in vivo, the scratch-promoted down-regulation of KRT10 suggests that the differentiated KRT10-positive NHK also participate in the repair process.

**Cell Proliferation and Apoptosis**—As expected, NHK scratching induces a variety of proproliferative transcripts (F5 family), highlighting the fact that a burst of proliferation is in fact triggered by disrupting the integrity of the hyperconfluent sheet. Among these identified genes, CCNE1, CCNF, and CCNG2 belong to the cyclin family members and appear transiently up-regulated between 3 and 6 h after injury. CCNE1 has been shown to be up-regulated in cells entering the G1 phase. It is important to note that the induction of CCNE1 can be detected in our experimental system, indicative of a massive synchronous entry into the cell cycle, and a burst of proliferation induced in response to scratching. The fact that CCNF is also up-regulated indicates that cells not only enter G1 but also reach the S/G2 phase (65). The strong decrease of GADD45B expression, a gene that has been involved in G1 arrest (66), indicates that scratching can abolish signals that counteract cell cycle stimuli. Interestingly, GADD45 down-regulation is in line with the inactivation of NFYA, a transcriptional factor that has clearly been shown to be involved in p53-independent GADD45 regulation (67).

Interestingly, scratching also strongly stimulated the expression of the antiapoptotic gene MCL1, and, in agreement with this, a decrease of the proapoptotic gene TNFSF10 was also detected. This suggests that NHK develop mechanisms of self-protection against the proapoptotic agents potentially released after injury.

**Genes of Miscellaneous Functions**—The last family of genes endowed with a well-defined biological function (F6) corresponds to proteins involved in metabolic processes (PTGS2, ODCA, TXNRD1, IMPA2, DHR53, SUCGLI, HK1, and COMT). Transporters of glucose, neurotransmitters, amino acids, chloride, sulfate, and zinc (SLC2A1, SLC6A2, SLC7A5, SLC6A14, GABRD, SLC26A4, and SLC39A9) were regulated. We also noted the presence of SLC25A16, a transporter located in the inner mitochondrial membrane that facilitates the rapid transport and exchange of molecules between the cytosol and the mitochondrial matrix space. In particular, we note the up-regulation of PTGS2 and ODCA, genes shown in in vivo wound healing models to control NHK migration and NHK activation, respectively (68).

**Validation of Our DNA Array Results**—Two quantitative PCR approaches were performed in order to confirm our DNA microarray data. First, we randomly selected 24 transcripts out of 118 and analyzed their expression in scratched versus non-scratched keratinocytes by real time quantitative PCR at the time point corresponding to their maximum stimulation determined by the DNA array. The data showed an excellent correlation with the microarray data (Fig. 4, A and B; coefficient of correlation equal to 0.888 between the gene induction factors measured by microarray and quantitative PCR). Second, we chose a sample of four up-regulated and two down-regulated genes, and we analyzed them by real time quantitative PCR, during the scratch closure kinetics, namely at 3, 6, 15, and 24 h after injury. The results confirmed that a very good correlation existed between the DNA array data and the results obtained by real time PCR (Table 2).

**Role of the ERK, p38MAPK, and PI3K Signaling Pathways in in Vitro Scratch Closure**—In previous work, we have shown that scratch wounding at a keratinocyte layer strongly activates the p38MAPK, ERK1/2 (8), and PI3K pathways, but further studies dissecting the downstream effects of these pathways on “scratch-induced” keratinocyte migration and proliferation have never been undertaken. To investigate the respective role of these signaling pathways and to identify the expression profiles associated with each pathway, we used the well known ERK cascade inhibitor U0126, the p38MAPK inhibitor SB203580, and the PI3K inhibitor LY294002 in in vitro scratching experiments.

![Graph](image)

**FIGURE 4.** A, real time quantitative PCR analysis to analyze the expression of the genes identified in our DNA array analysis. 24 of the genes shown altered in the DNA array analysis were randomly selected, and their expression ratios existed between the DNA array data and the results obtained by real time PCR (Table 2).

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Confluent keratinocytes were torn in the absence or in the presence of each inhibitor, and the kinetics of the scratch closure was analyzed by light microscopy. In the absence of inhibitors, the migration of cells located at the scratch edges started 5–6 h after injury and ended about 30 h (Fig. 5, A and B, Ct). When cells were torn in the presence of U0126, SB203580, or LY294002, the kinetics of “bed” closure was strongly altered. U0126 totally prevented the closure even after 44 h (Fig. 5, A and B; U0126). This inhibition resulted in a complete inhibition of both cell migration and proliferation as assessed respectively by time lapse experiments and bromodeoxyuridine incorporation (not shown). The p38MAPK inhibitor, SB203580, did not abolish the closure but strongly delayed it (Fig. 5, A and B; SB203580). Indeed, we observed that the “bed” remained “opened” 44 h after scratching, whereas it was completely closed in control conditions. We verified that these inhibitors effectively blocked their respective target kinases (Fig. 5C) and that none of them had toxic effects when added to scratched cells (not shown). In contrast, LY294002 did not inhibit cell migration and proliferation but rather accelerated these processes, thereby leading to a total “bed” closure 19 h after scratching (Fig. 5, A and B; LY294002).

Effects of ERK, p38MAPK, and PI3K Inhibitors on the NHK Transcriptome Response to Scratching—We next analyzed the effect of U0126, SB203580, and LY294002 on the gene expression profile of scratched keratinocytes (Fig. 6; supplemental data). Cells were incubated with or without these inhibitors and then scratched. Cells were then harvested after different times, and the expression profile analysis was performed as described above. We observed that 64% of the up-regulated genes were inhibited (by more than 70%) by one of the three tested inhibitors, 32 by...
FIGURE 6. *“Heat map” representing the genes controlled by ERK, p38MAPK, and PI3K pathways in response to scratching.* Confluent NHK were torn in the presence of U0126, SB203580, and LY294002. Cells were collected after the indicated times. Total RNAs were isolated, fluorescently labeled, and used in DNA array hybridization assays as described under “Experimental Procedures.” A–C, genes inhibited by U0126 (A), SB203580 (B), and LY294002 (C) after scratching. D, genes that are potentiated when NHK were scratched in the presence of LY294002. The numeric values corresponding to these experiments are presented in the supplemental data. The color scale is representative of log 2 of the ratio of scratched cells versus nonscratched ones. E, Venn diagram of the number of genes whose expression was inhibited by U0126 (U0), SB203580 (SB), and LY294002 (LY). The values relative to each time point result from two independent experiments, derived from two distinct cell cultures. Results were generated after two independent microarray analyses (each time point represents the mean of four independent analyses).
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U0126, 26 by SB203580, and 14 by LY294002 (Fig. 6, A–C, respectively). Among these 72 genes, 16 genes (22%) were co-mitantly controlled by two signaling pathways, eight genes (11%) controlled by both ERK and p38MAPK, five genes (7%) by ERK and p38, and two genes (3%) by p38 and p38K. It is interesting that only the VEGF gene was controlled by all three signaling pathways (Fig. 6E). The fact that 36% of the genes were insensitive to the three inhibitors clearly indicates that other signaling pathways also play an active role in the scratch closure.

The analysis of the genes altered after scratching in U0126-treated cells reveals that the ERK pathway controls a majority of early genes (cluster 1), suggesting that ERK probably participates in the very first steps of the healing process. These results would explain the dramatic inhibitory effect of U0126 on scratch closure. Accordingly, we observed that U0126 inhibited a majority of transcription factors, including FOS, EGR1, ETS1, ZFP36, KLF10, JUNB, PPAR8, and PRDM1, which represent 40% of the transcription factor up-regulated after scratching. A promoter analysis using the Genomatix database (available on the World Wide Web at www.genomatix.de) reveals that about 60% of the up-regulated genes, inhibited by U0126, contain EGR1, FOS (AP-1), or ETS1 binding sites in their promoter sequences (not shown), suggesting that among all of the up-regulated transcription factors, EGR1, FOS, and ETS1 play a major role in the control of keratinocyte “bed” closure.

A second remarkable effect of U0126 corresponds to its inhibition of many of the EGF family members. The fundamental role of these molecules in keratinocyte migration and proliferation (49) explains the dramatic consequences of U0126 on the scratch closure. Little information is available concerning the molecular regulation of EGF family members in response to an “injury type” stimulus. Our study demonstrates that many EGF family members are concomitant downstream events of a unique pathway involving the ERK/p38. Promoter analysis also reveals the presence of consensus EGR1, Ets1, and c-Fos sites in the promoter regions of AREG, EREG, HBEGF, VEGF, TGFα, probably indicating that their U0126-dependent repression might account for the inhibition of the expression of EGF family members. EGR1, Ets1, and c-Fos (AP-1) sites are often co-occi in PubMed (69) associated with other U0126-inhibited genes, such as cytokines (IL6 and IL8) or with genes involved in cell migration (THBS1, PTGS2, and SERPINE1) thus reinforcing the participation of these transcription factors to close signaling networks cooperating in keratinocytes submitted to injury. It is interesting to see that U0126 also abrogates the KLF10 stimulation and the down-regulation of VAV3, two targets of TGFβ, suggesting that ERK signaling also controls the early TGF pathway.

In contrast to ERK, p38MAPK does not directly “integrate” the immediate scratch-generated signaling but is probably involved in the propagation of events immediately induced after scratching (Fig. 6B). Indeed, p38MAPK is neither an upstream event of EGR1, ETS1, or FOS stimulation nor involved in the regulation of the EGF family members, which could explain the weaker effect of SB203580 on scratch closure. In contrast, p38MAPK mostly participates in the stimulation of intermediate and late genes (clusters 2 and 3) that could significantly influence cell migration and proliferation. Indeed, p38MAPK inhibition impairs the stimulation of genes involved in cytoskeleton reorganization, such as RASA1, RARα, and PTPN12, but also blocks the expression of genes involved in cell adhesion/migration, such as LAMA3 and ITGB4 (70), and the stimulation of the promigratory kinase MAPK4 (43). The weaker effect of SB203580 could also be explained by its modest inhibitory effect on the expression of EGF family members. Indeed only TGFA is inhibited by SB203580. Although TGFA probably plays an important role in keratinocyte migration and proliferation during wound healing (49, 50), we can imagine that other SB203580-insensitive EGF family member genes, such as HBEGF, can compensate for TGFA inhibition.

The ERK and p38MAPK pathways also appear to be involved in the molecular cascade responsible for scratch-mediated gene repression (supplemental data). Indeed, we found that U0126 inhibits the down-regulation of 8 of the 43 genes that are repressed, after taming, notably KRT10, VAV3, GADD45B, DDIT4, CNDP2, IMPA2, P2RY1, and NPY, and that SB203580 impairs the down-regulation of GADD45B, KRT10, P2Y5, and TFAP2A. We noted that the scratch-mediated repression of KRT10 and GADD45B was inhibited both by SB203580 and U0126, indicating that these genes are controlled both by the ERK and p38MAPK pathways (supplemental data). The role of KRT10 in keratinocyte migration, proliferation, and cell plasticity is discussed above and highlights the possible importance of its down-regulation to achieve a complete closure. Here we show that the KRT10 decrease is totally abolished by U0126 and SB203580, which might account for the scratch closure inhibition observed in the presence of these inhibitors. Elsewhere, GADD45 is also probably involved in the inhibition of cell proliferation when inhibiting ERK and p38 signaling.

As a highly novel finding, we observed that inhibition of PI3K enhanced the scratch-stimulated up-regulation of ETS1, HBEGF, and HAS3 (Fig. 6D). This LY294002-dependent potentiation corresponded to a 2-fold increase of the expression level of these genes associated with their prolonged activation. Because PI3K inhibition increased the rate of scratch closure (Fig. 5), we hypothesize that, under normal healing conditions, PI3K signaling exerts global negative regulatory effects that might limit the amplitude of stimulation of several “injury”-activated signals, notably HB-EGF, ETS1, and HAS3.

Additional observations suggest that PI3K inhibition of these three genes is physiologically relevant. Indeed, the exogenous addition of HBEGF strongly accelerates the scratch closure, and Ets1 inhibition by siRNA silencing totally inhibits the process (not shown). The fact that HAS3, HBEGF, and ETS1 are inhibited by U0126 further supports the idea that these genes are indeed key regulators of NHK scratch closure. It is interesting that when looking at pathological situations, it has been reported that HAS3, Ets1, and HBEGF are up-regulated in carcinoma cells (colon and oral) and contribute to epithelial tumor growth and metastasis (71–74). The involvement of these genes in cancer processes supports the classical concept linking wound healing to tumorigenesis (75) and opens new molecular clues to diseases such as chronic wounds or dystrophic recessive epidermolysis bullosa (76).
To date, most of the studies related to PI3K have demonstrated its positive action on cell proliferation and survival. However, recent studies have also shown that PI3K can be a negative regulator of biological processes. Indeed, it has been clearly reported that the RNA interference-mediated down-regulation of the PI3K target, AKT, increases epithelial breast cancer cell migration and invasion through the inhibition of NFAT degradation (77). Along this line, our study shows that PI3K signaling also exerts an inhibitory effect on cell migration and proliferation during in vitro reepithelialization, a “nontumoral” situation presenting many analogies with cellular invasion.

Conclusions—This work identifies the modification of the transcriptional profile of human NHK in culture after mechanical scratching as an in vitro attempt to mimic aspects of the reepithelialization process. A significant number of the modified genes have been previously reported in multiple in vivo reepithelialization studies (see Table 1), conferring to our system ad hoc properties for a powerful tool to approach the regulation of human keratinocyte wound healing. The analysis of the functions of the altered genes draws an interesting picture of the regulation of the process and shows that scratching generates both stimulatory (promigratory, proliferative, and antiapoptotic) and inhibitory (antiangiogenic, proliferative, and proapoptotic) signals that serve as a counterbalance to finely regulate the closure of the keratinocyte sheet. A second aim of this work was to define the specific respective roles of the relevant signaling pathways (ERK1/2, p38MAPK, and PI3K) in NHK migration and proliferation in this particular “wound” closure context. Our results indicate that in vitro scratch closure results from a fine balance between “pro-closure” signals controlled by ERK and p38MAPK and “anti-closure” ones involving the PI3K pathway. Any deregulation of the equilibrium between these signals can lead to pathological phenotypes. Our results offer several new clues for future clinical research. For instance, it has recently been proposed that MAPK inhibitors can be used as anti-inflammatory therapeutics to treat pathologies such as rheumatoid arthritis (78), indicating that in some situations, inhibitors of classical signaling pathways can be used in the clinic. Due to the observed properties of LY294002 in our in vitro context, the local application of this drug might constitute a new approach to treat pathological conditions with delayed healing, such as leg ulcers or chronic wounds.

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