Inhibition of JNK Promotes Differentiation of Epidermal Keratinocytes*

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In inflamed tissue, normal signal transduction pathways are altered by extracellular signals. For example, the JNK pathway is activated in psoriatic skin, which makes it an attractive target for treatment. To define comprehensively the JNK-regulated genes in human epidermal keratinocytes, we compared the transcriptional profiles of control and JNK inhibitor-treated keratinocytes, using DNA microarrays. We identified the differentially expressed genes 1, 4, 24, and 48 h after the treatment with SP600125. Surprisingly, the inhibition of JNK in keratinocyte cultures in vitro induces virtually all aspects of epidermal differentiation in vivo: transcription of cornification markers, inhibition of motility, withdrawal from the cell cycle, stratification, and even production of cornified envelopes. The inhibition of JNK also induces the production of enzymes of lipid and steroid metabolism, proteins of the diacylglycerol and inositol phosphate pathways, mitochondrial proteins, histones, and DNA repair enzymes, which have not been associated with differentiation previously. Simultaneously, basal cell markers, including integrins, hemidesmosome and extracellular matrix components, are suppressed. Promoter analysis of regulated genes finds that the binding sites for the forkhead family of transcription factors are over-represented in the SP600125-induced genes and c-Fos sites in the suppressed genes. The JNK-induced proliferation appears to be secondary to inhibition of differentiation. The results indicate that the inhibition of JNK in epidermal keratinocytes is sufficient to initiate their differentiation program and suggest that augmenting JNK activity could be used to delay cornification and enhance wound healing, whereas attenuating it could be a differentiation therapy-based approach for treating psoriasis.

Inflamed tissue cannot efficiently perform its natural function because the inflammatory signals usurp the standard signal transduction pathways. For example, normal epidermal differentiation is disrupted in psoriasis and other inflammatory dermatoses, where hyperproliferation and active migration of epidermal keratinocytes replace their restrained and orderly progression from the basal to the cornified layer (1–4). The balance between keratinocyte differentiation and activation is affected by the stress signals from the environment, the infiltrating lymphocytes, or the underlying mesenchymal tissue (5, 6). These signals result in specific patterns of gene expression, produced in the nucleus either by the differentiation-specific transcription factors, e.g. KLF4 and Grhl3 (7, 8), or by activation-specific factors, most importantly the AP1 family (9, 10). The JNK stress-activated protein kinases stand at the fulcrum of signal transduction pathways that activate the AP1 proteins (5), and therefore we decided to explore the role of JNKs in controlling the keratinocyte cell fate.

JNK1, also known as MAPK8 or SAPK1, is essential for epidermal morphogenesis during development (11). Targeted disruption of either JNK1 or JNK2 (MAPK9) results in a mild, restricted phenotype, because their roles are largely redundant (12, 13), but when both are disrupted the effects are lethal (14). Despite their overlapping function, the two JNK isoforms have different roles in skin cancer: whereas JNK1−/− mice are more tumor-sensitive, JNK2−/− mice are more tumor-resistant than the wild type (15). JNK is not active in healthy human epidermis, but its activity is elevated in psoriasis (16); tumor necrosis factor α and UV light exert their pro-inflammatory effects in part via JNK activation (17, 18), and JNK is activated in keratinocytes with mutations in epidermal keratin genes that increase the cell sensitivity to stress (19).

In culture, epidermal keratinocytes resemble more the activated, psoriatic and wound-healing keratinocytes than the normal, differentiating ones (20). Low JNK activity in cultured keratinocytes can be further activated e.g. by UV light (21–24). Active JNK is associated with keratinocyte proliferation (25) but does not play a role in keratinocyte motility (26–29). Certain promoters of epidermal differentiation, e.g. vitamin D3, exert their effects in part through inhibition of JNK (30, 31), others, such as Ca2+, do not involve the JNK pathway (32). Interferon-γ, an inhibitor of epidermal differentiation, does not affect the JNK pathway (33).

From the large and unconnected set of facts listed above it is clear that the role of JNK in the epidermis is important and complex, however it has not been systematically explored until now. To define comprehensively the effects of JNK activation in human epidermal keratinocytes, we used large DNA microar-

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2 The abbreviations used are: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ERK, extracellular signal-regulated kinase.
rays to determine the transcriptional profiles in cells treated with SP600126, a specific JNK inhibitor (34). This drug is a reversible ATP-competitive inhibitor specific for the three isoforms of JNK and effective in human epidermal keratinocytes (33). It has been widely used in studies of the cellular stress response via the JNK pathway. We followed the SP600126-caused changes in gene expression over 48 h. We find that the most prominent transcriptional change is the induction of epidermal differentiation markers. Indeed, the inhibition of JNK stimulates many aspects of epidermal differentiation in vitro, including stratification, inhibition of proliferation and migration, and formation of cornified envelopes. We conclude that the activation of JNK in skin serves to inhibit epidermal differentiation in the processes that involve activated keratinocytes such as psoriasis and wound healing.

**EXPERIMENTAL PROCEDURES**

Human Keratinocyte Cultures, SP600125 Treatment, and Preparation of Labeled cRNA—Human neonatal foreskin epidermal keratinocytes were first grown in a defined keratinocyte growth medium (KGM) supplemented with 2.5 ng/ml epidermal growth factor and 0.05 mg/ml bovine pituitary extract (keratinocyte-SFM, Invitrogen), as described (35, 33). Third-passage keratinocytes were used at 50–70% confluence, at which point the cells were switched to KBM, the same medium as KGM but without supplements. Keratinocytes were treated 24 h later with 5 nm SP600125 (Calbiochem), stored in Me2SO. The SP600125-treated and control keratinocytes were observed under the microscope (Zeiss, Axiophot), and images were captured with a digital camera (Sony, DKC-5000). For microarray analyses, the cells were harvested 1, 4, 24, and 48 h after the SP600125 treatment. At each time point we harvested the treated and a corresponding, matched, untreated control sample. We isolated total RNA from the cells using RNeasy kits (Qiagen) according to the manufacturer’s instructions. ~5–8 μg of total RNA was reverse transcribed, amplified, and labeled as described (35, 33).

GeneChip Hybridization and Array Data Analysis—Labeled probe, 15 μg, was hybridized to HGU95Av2 arrays (Affymetrix). Arrays were washed, stained with anti-biotin streptavidin-phycocerythrin-labeled antibody, and scanned using the Agilent GeneArray scanner system (Hewlett-Packard). We used Affymetrix Microsuite 5.0 for data extraction, as before (33, 36). To compare data from multiple arrays, the signal of each probe array was scaled to the same target intensity value of 500 arbitrary units. To improve reliability, we checked individually the absolute expression levels and p values at all four time points. We included in the analysis only those genes determined by the algorithm to be present in at least one sample, and with the signal value of at least 100 units on at least one microarray. To reduce false-positive-regulated genes, we eliminated the genes that show a “zigzag” pattern of changes during the time course studied. Differential expressions of transcripts at each time point were determined in two ways: by calculating the -fold change, where genes were considered regulated if the expression levels differed by >2-fold relative to untreated control and by the standard Microsuite 5.0 discrimination evalua-

**JNK Inhibits Cornification**

The transcription factor binding sites in the regulated genes were identified using the oPOSSUM program (38). We first calibrated the parameters of the program using a set of identified NFκB-regulated genes (39) to obtain the optimal statistical p values in the one-tailed Fisher exact probability analysis. We then used the same parameters for the SP600125-regulated genes.

Western Blot Analyses—For preparation of the whole cell lysates, cells were washed with cold phosphate-buffered saline and lysed in radioimmune precipitation assay buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The lysates were centrifuged at 16,000 × g, 10 min at 4 °C. To analyze secreted proteins, we collected and concentrated the cell culture supernatants at each time point using Centriprep and Centricron concentrators in series (Millipore). The protein concentration of each sample was determined with Bio-Rad Protein assay reagent. 20 μg of protein for whole cell lysates, and 60 μg for secreted proteins, was loaded on 10% SDS-polyacrylamide gels or Tris-Tricine ready-Gel from Bio-Rad, transferred to polyvinyldene difluoride membrane (Immobilon-P) using a semi-dry transfer cell (Bio-Rad), and blocked in 5% evaporated milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20). The membranes were incubated with primary antibody 1 h at room temperature in 5% evaporated milk in TBST and washed extensively with TBST, then incubated with 1:10,000 anti-chicken or anti-mouse horseradish peroxidase-conjugated secondary antibodies and visualized with the Super Signal West Pico Chemiluminescent substrate (Pierce). The equivalent loading of proteins in each well was ascertained using Ponceau staining of the membrane, or re-probing with an actin-specific antibody.

**In Vitro Wounding Scratch Assay**—For wounding scratch assay, cells were grown in a 12-well dish in 1 ml of KGM. At 80% confluency, the medium was changed to KBM. After 24 h, the medium was aspirated, and the cells were treated with mitomycin C (8 μg/ml) in KBM, for 1 h in dark at 37 °C. The mitomycin solution was removed, and the cells were washed three times with KBM for 5 min each time. The medium (1 ml) was added, and the cell monolayer were scratched using a 200-μl pipette tip. The inhibitor was then added. Fresh medium with or without the

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3 M. Blumenberg and T. Banno, unpublished data.
| 1h | 4h | 24h | 48h | SYMBOL | Function | Max | Min | K |
|----|----|-----|-----|--------|---------|-----|-----|---|
| -1.5 | -0.8 | 0.6 | 0.3 | KOLK1 | Adhesion | 0.6 | -1.5 | B |
| -0.2 | 0.1 | -1.0 | 0.3 | TNFC | Adhesion | 2.2 | -1.9 | B |
| -0.1 | 0.0 | -0.1 | 0.3 | LIFCAM | Adhesion | 0.0 | -1.1 | S |
| -0.1 | -0.1 | 0.1 | 0.3 | CD99 | Adhesion | 0.1 | -1.1 | S |
| -1.5 | 0.0 | 0.0 | 0.0 | DSG1 | Adhesion, desmosomal | 2.6 | -1.6 | S |
| 0.1 | 0.2 | 0.1 | 0.1 | DSG2 | Adhesion, desmosomal | 1.2 | 0.0 | S |
| -0.2 | -0.6 | 0.1 | 0.1 | DSPC | Adhesion, desmosomal | 1.1 | 0.1 | S |
| -0.3 | -0.5 | 0.0 | 0.0 | ITGA6 | Adhesion, integrin | 0.6 | -1.0 | B |
| 0.1 | -1.2 | 0.0 | 0.0 | ITGAV | Adhesion, integrin | 0.1 | -1.5 | B |
| -0.2 | 0.2 | -0.1 | 0.0 | ITGA3 | Adhesion, integrin | 0.1 | -1.5 | B |
| -0.4 | 0.0 | -0.2 | 0.0 | ITGB4 | Adhesion, integrin | 0.1 | -1.1 | B |
| -0.4 | -1.3 | 0.0 | 0.0 | ITGAV | Adhesion, integrin | 0.1 | -1.3 | B |
| 0.1 | 0.1 | 0.0 | 0.3 | OCLN | Adhesion, junctional | 1.9 | -1.2 | S |
| 0.1 | 0.2 | 0.1 | 0.1 | GJA1 | Adhesion, junctional | 1.0 | 0.0 | S |
| -0.2 | 0.0 | 0.0 | 0.0 | ZYX | Adhesion, junctional | 0.2 | 1.0 | B |
| 0.1 | 0.0 | 0.1 | 0.3 | GLRX | Antioxidant | 1.5 | -0.2 | B |
| -0.5 | 0.4 | 0.1 | 0.1 | CBS | Antioxidant | 1.4 | -0.5 | S |
| 0.5 | 0.1 | 0.0 | 0.0 | NIP1 | Antioxidant | 1.1 | -0.8 | S |
| 0.1 | 0.1 | 0.0 | 0.0 | PCH | Antioxidant | 1.0 | -0.1 | S |
| 0.2 | 0.0 | 0.0 | 0.1 | GPX1 | Antioxidant | 0.5 | -1.1 | B |
| -0.4 | 0.0 | 0.0 | 0.0 | PHDLA2 | Apoptosis | 0.0 | -1.9 | B |
| 0.2 | -0.7 | 0.0 | 0.0 | TNFALP3 | Apoptosis inhibitor | 1.7 | -0.7 | B |
| 0.2 | 0.1 | 0.0 | 0.2 | BIN3 | Apoptosis inhibitor | 1.1 | -0.7 | S |
| -1.0 | 0.0 | -0.1 | 0.0 | BIRC2 | Apoptosis inhibitor | 0.1 | -1.5 | B |
| -1.0 | -1.0 | 0.0 | 0.0 | BIRC3 | Apoptosis inhibitor | 0.1 | -1.5 | B |
| 0.1 | 0.3 | 0.0 | 0.0 | FLJ34888 | Ca binding | 1.7 | -0.2 | S |
| 0.4 | 0.0 | -0.1 | 0.2 | CCNC | Cell cycle | 1.5 | -0.2 | B |
| 0.0 | -1.0 | 0.0 | 0.0 | MPP3 | Cell cycle | 1.0 | -2.2 | S |
| 0.2 | -0.4 | 0.0 | -0.7 | BUB1 | Cell cycle | 0.2 | -3.7 | S |
| 0.1 | 0.0 | 0.0 | -0.5 | KIF2 | Cell cycle | 0.1 | -2.1 | S |
| -0.1 | -1.1 | 0.0 | -0.1 | BEAR3 | Cell cycle | 0.1 | -1.1 | S |
| -0.3 | -1.3 | 0.0 | -0.3 | GOS2 | Cell cycle | 0.1 | -1.3 | S |
| 2.1 | 0.2 | 0.0 | -0.6 | CAS1 | Cell cycle inhibitor | 2.1 | 0.2 | B |
| 0.1 | -1.1 | 0.0 | -1.1 | CDKN1C | Cell cycle inhibitor | 0.1 | 1.3 | S |
| 0.2 | 0.1 | 0.0 | -1.1 | BTC2 | Cell cycle inhibitor | 0.1 | 1.3 | S |
| -0.2 | 0.3 | 0.0 | 0.0 | QSCN6 | Cell cycle inhibitor | 0.1 | 1.0 | S |
| 0.1 | -0.2 | 0.0 | -1.0 | CRY61 | Cell growth, proliferation | 1.0 | -0.6 | S |
| 0.0 | 0.0 | -0.1 | -1.1 | PYH1 | Cell growth, proliferation | 0.1 | -1.1 | B |
| 0.0 | 0.0 | -0.1 | -1.1 | TDP52L1 | Cell growth, proliferation | 0.1 | -1.0 | B |
| 0.0 | 0.0 | 0.0 | 0.0 | CYPIA1 | Cytochrome | 3.2 | -0.4 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | CYPIA2 | Cytochrome | 1.8 | 0.5 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | CYRIAB | Cytochrome | 1.1 | 0.0 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | CORO2A | Cytochrome | 0.8 | -0.2 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | CORO2B | Cytochrome | 0.6 | -0.2 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | DMTN | Cytochrome, actin | -0.2 | 0.0 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | DPCC1 | Cytochrome, actin | 0.4 | 0.0 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | KRT1 | Cytochrome, keratin | 0.2 | -0.1 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | KRT16 | Cytochrome, keratin | 1.9 | -0.4 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | KRT17 | Cytochrome, keratin | 1.3 | -0.1 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | KRT19 | Cytochrome, keratin | 1.3 | -0.1 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | KRT6E | Cytochrome, keratin | 1.3 | -0.4 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | UPK19 | Cytochrome, keratin, membrane | 1.1 | 0.0 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | ARHGEF2 | Cytochrome, Rho, Cdc42 | 1.2 | -0.4 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | UVN1 | Cytochrome, tropinin | 3.9 | 0.0 | B |
| 0.0 | 0.0 | 0.0 | 0.0 | MAP2 | Cytochrome, tubulin | 1.1 | 0.5 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | UGT1A10 | Detoxification | 1.0 | -0.3 | B |
| 0.0 | 0.0 | 0.0 | 0.0 | TSN | DNA binding | 2.0 | -0.3 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | SCML2 | DNA binding | 1.0 | -0.4 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | MUC2 | DNA binding | 0.1 | -0.1 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | HIST1H2BF | DNA binding, histone | 1.6 | 0.5 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | HIST2H2AA | DNA binding, histone | 1.4 | 0.1 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | HIST1H2BH | DNA binding, histone | 1.3 | 0.3 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | HIST1H2BE | DNA binding, histone | 1.2 | 0.0 | S |
| 0.0 | 0.0 | 0.0 | 0.0 | HIST2H2BE | DNA binding, histone | 1.1 | -0.4 | S |
| 1h | 4h | 24h | 48h | Symbol | Function | Max | Min | K |
|---|---|---|---|---|---|---|---|---|
| -0.5 | 1.4 | 1.0 | 0.5 | JNK3 | DNA binding, histone | 1.0 | 0.0 | 2.5 |
| -2.2 | 7.9 | 1.5 | 2.1 | JNK4 | DNA repair, synthesis | 2.1 | 0.7 | 2.5 |
| -0.1 | 0.1 | 1.0 | 0.0 | JNK5 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.4 | 0.6 | 0.0 | 0.2 | JNK6 | DNA repair, synthesis | 0.0 | 0.0 | 2.5 |
| -0.3 | 0.4 | 0.0 | 1.0 | JNK7 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK8 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK9 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.4 | 0.8 | 1.0 | 1.0 | JNK10 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.3 | 0.6 | 0.0 | 1.0 | JNK11 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK12 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK13 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK14 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK15 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK16 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK17 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK18 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK19 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK20 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK21 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK22 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK23 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK24 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK25 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK26 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK27 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK28 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK29 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK30 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK31 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK32 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK33 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK34 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK35 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK36 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK37 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK38 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK39 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK40 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK41 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK42 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK43 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK44 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK45 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK46 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK47 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK48 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK49 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK50 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK51 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK52 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK53 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| -0.1 | 0.5 | 0.0 | 1.0 | JNK54 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |
| 0.2 | 1.5 | 1.0 | 1.0 | JNK55 | DNA repair, synthesis | 1.0 | 0.0 | 2.5 |

**JNK Inhibits Cornification**
| TIME | SYMBOL | FUNCTION | MAX | MIN |
|------|--------|----------|-----|-----|
| 0.7  | DUSP6  | Phosphatase | 1.1 | -1.6|
| 0.6  | PPM1B  | Phosphatase   | 1.0 | 0.1 |
| 0.6  | DUSP1  | Phosphatase   | 0.9 | -2.2|
| 0.0  | SGI    | Protein kinase | 1.6 | 0.0 |
| -0.1 | MAPK2  | Protein kinase | 1.0 | 0.0 |
| 0.7  | 1.0    | Protein kinase | 0.7 | 1.0 |
| -0.7 | SGK1   | Protein kinase | 0.4 | -1.1|
| -0.3 | PLAU   | Protein kinase | 0.2 | -1.4|
| 0.1  | SNRK   | Protein kinase | 0.2 | -1.5|
| -0.1 | GCH    | Protein kinase | 1.7 | -0.7|
| -0.6 | PTPTE2| Protein kinase | 1.2 | -0.6|
| 0.2  | F3     | Protease      | 0.7 | 1.1 |
| 0.1  | GSK    | Protease      | 0.1 | 0.0 |
| 3.3  | SERPBN3| Phosphatase inhibitor | 3.3 | -0.1 |
| -0.5 | SERPN4| Phosphatase inhibitor | 2.9 | -1.6 |
| 0.2  | SLPI   | Protease inhibitor | 1.4 | 0.2 |
| 0.1  | CSTA   | Protease inhibitor | 1.4 | 0.1 |
| 0.5  | PI3    | Protease inhibitor | 1.1 | 0.1 |
| -0.1 | SERPN7| Protease inhibitor | 1.1 | -0.1|
| 0.6  | SERPN2| Protease inhibitor | 0.7 | -2.0|
| -0.6 | SERPN1| Protease inhibitor | 0.2 | -1.1|
| -0.5 | TIMP1   | Protease, extracellular | 1.0 | -0.5|
| 0.1  | PSM5   | Protease, ubiquitin | 1.1 | 0.1|
| 0.3  | SMURF2 | Protease, ubiquitin | 0.2 | 1.6|
| -0.2 | UBE2C  | Protease, ubiquitin | -0.2 | -1.8|
| 0.0  | UBE2S  | Protease, ubiquitin | -0.5 | -1.3|
| 0.0  | LOC169611 | Receptor | 1.0 | 0.2|
| 0.3  | MTG1S  | Receptor | 1.0 | -0.4|
| -0.3 | TTRC   | Receptor | 0.3 | -1.1|
| 0.2  | PTGER5| Receptor | 0.2 | -1.0|
| -0.6 | LRPP   | Receptor | 0.2 | -1.2|
| 0.2  | CMKOR1 | Receptor, cyto-, chemokine | 2.3 | 0.5|
| -0.3 | IFNGR1| Receptor, cyto-, chemokine | 1.0 | -0.3|
| 0.1  | IL6R   | Receptor, cyto-, chemokine | 1.1 | 0.1|
| 0.2  | EPRA4  | Receptor, ephrin | 1.9 | 0.2|
| 1.5  | EFNB1  | Receptor, ephrin | 1.6 | 0.6|
| 0.3  | DTR    | Receptor, growth factor | 1.5 | 0.5|
| 0.2  | CKFZD5| Receptor, growth factor | 1.0 | -1.0|
| -0.6 | PTPRZ1 | Receptor, growth factor | 1.3 | -0.6|
| 0.1  | BLNK   | Receptor | 1.0 | -0.1|
| -0.2 | TNKS1D1| Regulator | 0.1 | -1.1|
| 1.0  | GCH1   | Regulator, cAMP,cGMP | 1.4 | 0.6|
| 1.6  | SGP1L1| Regulator, DAG pathway | 1.5 | -0.3|
| 0.3  | SMAD3  | Regulator, DAG pathway | 1.1 | 0.3|
| 1.6  | PIK3CD | Regulator, DAG pathway | 1.6 | -0.7|
| -0.5 | PLCB3  | Regulator, IP pathway | 1.2 | -0.2|
| -0.3 | INPP4B | Regulator, IP pathway | 0.6 | -1.2|
| -0.7 | DUSP6  | Regulator, JNK,ERK,p38 | 1.1 | -1.1|
| -0.4 | PPP2R1B| Regulator, JNK,ERK,p38 | 1.0 | -1.0|
| -0.4 | DUSP4  | Regulator, JNK,ERK,p38 | 0.4 | -2.5|
| -0.2 | DUSP7  | Regulator, JNK,ERK,p38 | 0.1 | -1.1|
| 0.5  | MYC    | Regulator, Myc pathway | 1.4 | -0.6|
| -0.2 | NFKB1A | Regulator, NFkB pathway | 1.1 | -0.7|
| 0.0  | PTGS2  | Regulator, prostaglandin | 1.6 | 1.0|
| 0.1  | ALOX12B| Regulator, prostaglandin | 1.4 | 0.5|
| 0.1  | RB1    | Regulator, Ras pathway | 0.1 | 1.0|
| 0.0  | KNTC2  | Regulator, Ras pathway | 0.1 | -2.7|
| 1.4  | DAI    | Regulator, WNT pathway | 1.4 | -2.5|
| -0.2 | WNT7A  | Regulator, WNT pathway | 0.1 | -2.0|
| 0.5  | NTSC2  | RNA metabolism | 1.9 | -0.2|
| 0.0  | SFRS2P | RNA metabolism | 1.5 | 0.0|
| 0.2  | SF5    | RNA metabolism | 1.2 | -0.2|
| 0.1  | RBMS3 | RNA metabolism | 1.1 | 0.1|
| 1.1  | CLK1   | RNA metabolism | 1.1 | -0.1|
| 0.0  | POP5   | RNA metabolism | 1.1 | -0.1|
| 0.2  | HNRPH1 | RNA metabolism | 1.1 | 0.1|
| -0.2 | NT5E   | RNA metabolism | 0.3 | -1.1|
| -0.5 | SFRS3  | RNA metabolism | 0.3 | -1.0|
| -0.2 | RBM5S | RNA metabolism | -0.1 | -1.0|
| 0.4  | ITPR1  | Secreted | 4.4 | 0.6|
| 0.4  | IFGBP5 | Secreted | 3.4 | -1.3|
| 0.5  | ITGB7 | Secreted | 2.8 | 0.5|
| -0.1 | ITGB1 | Secreted | 2.7 | -3.4|
| 1.2  | VEGF  | Secreted | 1.7 | -0.2|
| 1.3  | CXCL2 | Secreted | 1.3 | -1.9|
| -0.1 | CCL2  | Secreted | 1.0 | -1.0|
| 1.0  | CCL12 | Secreted | 1.0 | 1.0|
| 0.7  | CTGF  | Secreted | 0.7 | -2.1|
| 0.7  | CXL1  | Secreted | 0.7 | -2.7|
| 0.3  | PTHLH | Secreted | 0.3 | -2.2|
inhibitor was added every 24 h, and the photographs were taken immediately after and 24 and 48 h after the scratching.

RESULTS
To identify the genes transcriptionally regulated by the JNK pathway in human epidermal keratinocytes, we compared the expression profiles of matched SP600125-treated and untreated, control cells 1, 4, 24, and 48 h after the addition of the inhibitor. Among the identified and characterized genes, a total of 303 were unambiguously regulated by the JNK inhibitor SP600125; these are listed in Table 1. We have confirmed the induction of RGS2, IGFBP3, and involucrin by SP600125 in keratinocytes using Western blotting, with actin as a loading control (Fig. 1) and additional ones at the mRNA level, using Northern blots (data not shown). In these tested cases, the regulation at the mRNA level observed in the microarrays was confirmed at the RNA and protein level.

Because the JNK pathway has been implicated in the proliferation of epidermal cells (25), we first focused on the cell cycle and DNA synthesis functional groups, expecting the SP600125 addition to suppress the expression of a large number of genes in these functional categories. Surprisingly, we found only a handful of regulated genes in these two categories (Table 1). The suppressed cell-cycle genes, e.g. MPHOSPH9, BUB1, and

| 1h  | 4h  | 24h | 48h | SYMBOL  | Function | Max | Min | K |
|-----|-----|-----|-----|---------|----------|-----|-----|---|
| -0.5| 0.2 | 0.0 | -1.4| D       | IGFBP6   | 0.2 | -1.4| f |
| 1.4 | 2.8 | 1.1 | 0.4 | TC1806  | Secreted | 0.1 | 1.3| D |
| 0.0 | -1.1| -1.0| -0.4| D       | STC2     | 2.8 | -0.3| f |
| -0.1| -0.2| 1.1 | -0.5| D       | WSB2     | 0.4 | -1.1| f |
| -1.3| 2.3 | -1.0| -1.2| D       | VAV1     | 1.1 | -0.5| f |
| 0.1 | -3.5| 2.4 | -0.3| D       | BUB1     | 1.0 | -0.3| f |
| 0.0 | 0.4 | 1.0 | 1.0 | D       | SMARCA5  | 1.0 | -0.4| f |
| 1.0 | -1.6| D   | 0.0 | D       | COPEB1   | 1.0 | -1.6| f |
| 0.1 | 0.4 | 1.0 | 0.0 | D       | SP600125 | 0.1 | 1.0| f |
| 0.1 | 2.1 | -0.7| -0.8| D       | MAFA     | 2.5 | -0.8| f |
| 0.3 | 1.8 | 0.0 | 0.0 | D       | MAFA     | 1.8 | 0.0| f |
| 1.8 | -1.7| D   | 0.3 | D       | JUN      | 1.8 | -1.7| f |
| -0.2| 1.6 | 0.2 | 0.3 | D       | SIM2     | 1.6 | -0.2| f |
| 1.5 | -0.4| 0.0 | -0.2| D       | TGFbeta3 | 1.5 | -0.4| f |
| 0.2 | 1.2 | 0.5 | 1.0 | D       | KLF4     | 1.2 | 0.1| f |
| 0.2 | 0.8 | -0.2| 1.2 | D       | VGL1     | 1.2 | 0.3| f |
| -0.3| 0.2 | 0.6 | 1.2 | D       | TRPM16   | 1.2 | -0.3| f |
| -0.4| 0.0 | 1.2 | -0.2| D       | PTPR                | 1.2 | -0.4| f |
| -0.1| -0.3| 0.3 | 1.2 | D       | VGL1     | 1.2 | -0.3| f |
| 1.1 | 0.4 | 0.2 | 1.2 | D       | DKK2/DKK3 | 1.1 | 0.4| f |
| 0.9 | 0.8 | 0.3 | 1.0 | D       | IGFBP6   | 0.9 | 1.0| f |
| 0.3 | -2.0| 0.2 | 0.7 | D       | MAFA     | 0.3 | 2.0| f |
| 0.1 | -1.2| 0.7 | 0.2 | D       | TAZ      | 0.2 | 1.2| f |
| 0.0 | -0.1| 0.1 | -1.0| D       | ILF1     | 0.1 | 0.1| f |
| 0.1 | -1.1| 0.3 | 1.2 | D       | KLK7     | 0.1 | 1.1| f |
| 0.1 | 0.4 | 1.5 | 1.0 | D       | HOP       | 1.5 | 0.1| f |
| -0.6| -1.6| D   | -3.0| D       | DLL4     | 1.0 | -1.6| f |
| 0.2 | 0.8 | 0.0 | 1.9 | D       | ZNF165   | 1.9 | -0.7| f |
| -0.1| 0.7 | 0.5 | 1.9 | D       | ZNF145   | 1.9 | -0.7| f |
| 0.6 | -0.3| 1.0 | 1.0 | D       | ZNF238   | 1.2 | -0.3| f |
| 1.2 | -0.6| 0.1 | 1.0 | D       | ZNF645   | 1.1 | -0.6| f |
| -0.4| -1.1| 0.1 | 0.2 | D       | BECL1    | 1.1 | 0.4| f |
| 1.0 | 1.0 | 0.3 | 0.4 | D       | MIZF     | 1.0 | 0.3| f |
| 0.1 | 0.2 | 0.4 | 1.0 | D       | ZNF91    | 1.0 | 0.1| f |
| 0.5 | 0.3 | 0.4 | 1.0 | D       | ZNF62    | 0.5 | 1.0| f |
| -0.4| 0.1 | -0.2| 1.0 | D       | CRIP2    | 0.2 | 1.0| f |
| 0.5 | -0.4| 0.2 | 1.0 | D       | IGF2R    | 0.2 | 1.0| f |
| 0.1 | 1.5 | -0.3| 1.0 | D       | ATP3     | 0.1 | 0.1| f |
| -1.1| 0.2 | -0.2| 0.1 | D       | TCF4     | 0.1 | 1.1| f |
| 0.2 | 0.3 | 0.1 | 1.5 | D       | EIF4G3   | 1.5 | 0.1| f |
| 0.2 | 1.1 | -0.2| 0.4 | D       | EIF3     | 1.1 | -0.2| f |
| 0.1 | 0.0 | 0.0 | 1.0 | D       | EEF1A1   | 0.0 | 0.0| f |
| 0.2 | 1.2 | 0.0 | -0.4| D       | ATP2A1   | 1.1 | -0.4| f |
| -0.9| 0.5 | -0.1| 1.0 | D       | SLC3A4   | 1.0 | 0.0| f |
| 0.0 | -0.3| 0.6 | 1.0 | D       | AQP7     | 1.0 | -0.3| f |
| -1.7| -2.3| 0.3 | 1.0 | D       | PTPA1    | 0.9 | -2.3| f |
| -0.2| 0.0 | 0.4 | -0.1| D       | PTPA1    | 0.0 | 0.4| f |
| -0.2| -1.1| 0.2 | 0.1 | D       | SLC20A1  | 0.2 | 1.1| f |
| 0.0 | 0.2 | 0.3 | 1.2 | D       | ESP51    | 1.2 | 0.0| f |
| 0.0 | -0.5| 0.1 | -0.1| D       | DOPA     | -0.1 | 1.0| f |

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KIF2C are associated with mitosis, whereas the induced cell-cycle inhibitors, GAS1 and BTG2, specifically act at the beginning of the S phase of the cycle. The association of several of the additional regulated genes with the cell cycle, e.g. BCAR3 or FH1L1, is putative or tenuous. Furthermore, the DNA replication proteins, which are usually co-regulated with the cell-cycle proteins (33), were also rare among the SP600125-regulated genes. These data suggest that JNK does not directly inhibit the proliferation of keratinocytes.

If SP600125 does not directly regulate many cell-cycle and proliferation genes, its inhibition of proliferation may be indirect, i.e. a consequence of keratinocyte differentiation. Indeed, the treatment with SP600125 induced the expression of an extraordinary number of known epidermal differentiation markers indicated with 'S,' for suprabasal, in Table 1. They include epidermal transglutaminase, involucrin, small proline-rich proteins, scelin and kallikrein-7, as well as keratins K1 and K10. The results suggest that inhibition of JNK promotes epidermal differentiation. If so, the SP600125 addition should suppress the expression of genes specific for the undifferentiated, basal layer. We marked the known basal layer-specific genes with 'B' in Table 1. Indeed, we find that all five regulated integrins, markers of basal cells, are suppressed by the SP600125 treatment, as are most proteins of the extracellular matrix, which are expected to be secreted by the basal cells, again suggesting that JNK specifically induces basal cell markers. In general, the known basal cell adhesion proteins are suppressed by SP600125, e.g. DSG2, whereas the known suprabasal adhesion proteins are induced, such as the desmosomal proteins DSC1, DSC3, DSG1, and L16D (40–42). We conclude from the above results that active JNK prevents epidermal differentiation and promotes the basal layer phenotype.

If the inhibition of JNK with SP600125 promotes epidermal differentiation, we would expect to find among the induced genes additional, novel differentiation-associated genes. For example, we find that SP600126 induces the expression of the regulators of the inositol phosphate signal transduction pathway (PIK3CD and PLCB3) and of the diacylglycerol pathway (SGPL1 and SMPDL3A); these pathways have been implicated in the regulation of epidermal differentiation (43). Moreover, SP600125 suppresses INPP4B, a negative regulator of the inositol phosphate pathway ('d' in Table 1). On the other hand, the members of the JNK/ERK/p38 pathway are suppressed by SP600125. The suprabasal, differentiating phenotype is, therefore, associated with the induction of inositol phosphate and diacylglycerol pathway proteins, whereas the basal phenotype is associated with the elevated expression of the JNK/ERK/p38 pathway proteins. Importantly, the lipid and the steroid metabolism enzymes are also induced by SP600125; these processes are also associated with epidermal differentiation (44–46). In comparison, the amino acid metabolic enzymes are not similarly regulated, indicating that JNK inhibition does not generally regulate cell metabolism but specifically enhances the metabolisms of lipids and steroids. Interestingly, several

### TABLE 2

Over-represented binding sites for transcription factors in SP600125-regulated genes

| Induced genes | B non-hits | T non-hits | Fisher P-value |
|---------------|------------|------------|----------------|
| TF Class IC B hits T hits Z-score |
| FH3 FORKHEAD | 132 | 251 8455 | 17 92 | 12.07 1.6e-04 |
| HLF bZIP | 11.1 | 129 8577 | 10 196 | 11.33 1.6e-04 |
| Nix HOME | 8.3 | 881 7272 | 47 162 | 10.12 4.0e-06 |
| FARE2 FORKHEAD | 14.8 | 48 8660 | 4 205 | 8.78 2.9e-06 |
| CREB bZIP | 12.6 | 481 8225 | 20 189 | 8.71 1.4e-06 |
| Gi1 ZN-FINGER C2H2 | 9.5 | 795 7911 | 34 175 | 8.35 4.8e-04 |
| SRY HOME | 9.2 | 569 8137 | 28 181 | 7.99 3.2e-04 |
| E4BP4 bZIP | 14.1 | 78 8628 | 5 204 | 7.97 4.5e-06 |
| SOKO HOME | 9.1 | 379 8207 | 19 190 | 7.69 2.5e-06 |
| MRPS MAS | 18.0 | 32 8674 | 3 206 | 7.09 4.8e-02 |
| krt1 TRP-CLUSTER | 16.0 | 99 8607 | 6 203 | 6.32 3.7e-02 |
| Sox5 HOME | 10.8 | 387 8319 | 17 192 | 5.20 1.4e-02 |
| E2F Unknown | 13.8 | 354 8352 | 13 196 | 5.16 9.0e-02 |
| FARE4 FORKHEAD | 11.9 | 416 8290 | 18 191 | 4.97 1.3e-02 |
| c-REL REL | 10.5 | 411 8296 | 17 192 | 4.80 2.3e-02 |
| SOX17 HOME | 10.5 | 765 7941 | 25 184 | 4.70 7.5e-02 |
| eCEP bZIP | 9.2 | 215 8491 | 9 200 | 3.72 8.1e-02 |
| AM6 RANT | 10.9 | 408 8298 | 16 193 | 2.96 4.1e-02 |
| S8 HOME | 9.1 | 719 7987 | 24 185 | 1.12 6.7e-02 |

One-tailed Fisher Exact Probability Analysis

**Selected Parameters:**
- Conservation level: Top 30% (conservation > 60%)
- Matrix match score: 0.85
- Upstream sequence length: 350
- Downstream sequence length: 50
- Number of genes included: I/S = 209/32

**Fisher P-value**
mitochondrial proteins are induced by the SP600125 treatment, including electron transporters and gluconeogenic and lipidolytic enzymes; these have not been previously identified as specific differentiation markers. Thus, increased mitochondrial metabolism may be a component of the epidermal differentiation.

Several secreted signaling proteins are induced by the SP600125 treatment, potentially to transduce signals from the differentiating keratinocytes to the nearby cell types. These include CCL20 (MIP3), a strong chemoattractant for Langerhans cells (47); CXCL12, which affects T cells; and vascular epidermal growth factor, which plays a role in angiogenesis, cancer, and wound healing (48). In contrast, PTHLH, whose expression is specific for basal cells, is suppressed by SP600125 (49).

In contrast to pro-inflammatory and immunomodulatory signals, which activate many matrix metalloproteases (36, 33), SP600125 treatment induced only kallikrein 10 among the extracellular proteases; the function of KLK10 may be related to the desquamation, another differentiation-specific process (50).

In several cases we found that, after the SP600125 addition, one member of the gene family was replaced by another family member with the same or similar function (several examples of this phenomenon are marked as ‘a,’ ‘b,’ ‘e,’ ‘f,’ and ‘g’ in Table 1). In the case of desmogleins, DSG1 is induced, whereas DSG2 is suppressed, confirming their association with the suprabasal and basal keratinocytes, respectively (40). Similarly, GJA1 (connexin 43) is induced, whereas GJB3 (connexin 31) is suppressed (51, 52). GJB3 is expressed in the basal layer during wound healing, although in normal epidermis, the antibody preferentially stains the granular layer (53); their expression in differentiating human skin seems reverse from that in calcium-induced murine differentiation (54). IGFBP5 and IGFBP3 are induced, whereas IGFBP6 and CTGF (also known as IGFBP8) are suppressed. IGFBPs are antiproliferative, and the induced and the suppressed genes apparently have the same function (55). Serine protease inhibitors SERPINB3, -B4, and -B7 are induced, whereas SERPINB2 and -E1 are suppressed. Similarly, VEGF is induced by the SP600125 treatment, vascular epidermal growth factor C is suppressed, and both are angiogenic; CXCL2 (Gro-α) is induced, CXCL1 (Gro-β) is suppressed, and both are pro-inflammatory and activate melanocytes; RGS2 is induced, whereas RGS3 is suppressed, and both are GTPase-activating inhibitors of G-protein signaling. The expression and differential function of these sets of genes in epidermis are currently unexplored.

A similar yin-yang switch seems to operate in the Kruppel transcription factor family. KLF4 is required for normal epidermal growth factor, which plays a role in angiogenesis, cancer, and wound healing (48). In contrast, PTHLH, whose expression is specific for basal cells, is suppressed by SP600125 (49).

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dermal differentiation, and it specifically accelerates epidermal barrier formation (7, 56); its enforced expression in basal cells causes squamous dysplasia (57). As expected for a differentiation-specific transcription factor, KLF4 is induced by the inhibition of JNK. At the same time, the expression of KLF7, the ubiquitous Kruppel-like factor, is suppressed (‘i’ in Table 1). KLF transcription factors and the related Sp proteins can, depending on the circumstances, either induce or repress the expression of regulated genes (58). They bind with identical or overlapping specificity to GC-rich DNA elements. Intriguingly, COPEB (also known as KLF6) is induced immediately after the treatment with SP600125, in the 1-h time point, suppressed at 4 h, to revert to background levels at 24 and 48 h post-treatment. The function of the KLF proteins in epidermal differentiation seems more complex than originally thought.

Homeobox transcription factor DLX3, a regulator of epidermal differentiation (59), is not represented on the Affymetrix microarrays. However, a highly related family member, DLX2, which has not been studied in the context of epidermal differentiation, is induced by SP600125 (‘k’ in Table 1). It may be that DLX3 and DLX2 have similar or overlapping functions that induce transcripts associated with differentiation in epidermal keratinocytes. The nuclear receptor co-activator NCOA2 (GRIP1) is induced by SP600125, whereas the co-repressor NCOR2 (SMRT) is suppressed (60) (‘b’ in Table 1). This switch would tend to enhance the induction of all genes positively regulated by the nuclear receptors in differentiating keratinocytes and to attenuate the suppression of those genes that are negatively regulated, including keratin genes (61).

To identify the transcription factors responsible for the effects of SP600125 in keratinocytes, we used the oPOSSUM program, which identifies statistically overrepresented transcription factor binding sites in the regulatory regions of submitted genes (38). oPOSSUM uses weighted recognition site matrices of transcription factor binding sites to analyze the homologous sequences of the human and murine genomes (62). The most common transcription factor binding site in the SP600125-suppressed genes is the canonical partner of Jun, c-FOS (Table 2). This datum is exactly as expected, given the molecular mechanism of SP600125 action. Interestingly, the sites for the forkhead family of transcription factors are over-represented in the SP600125-induced genes; forkhead proteins have been implicated in epidermal differentiation and may be regulated by JNK (63). The recognition sites for the Nkx homeobox transcription factors and CREB are over-represented as well; these proteins have also been implicated in promoting differentiation in various tissues, although not in the epidermis (64, 65).

Because the inhibition of JNK induces many differentiation markers, we decided to examine the physiological manifestations of epidermal differentiation in the SP600125-treated cultures of keratinocytes. As expected of an inducer of differentiation, SP600125 inhibited keratinocyte colony formation (Fig. 2A). The SP600125-treated cells could attach to the substrate after trypsinization but did not divide or form colonies. In contrast, the mock treated cells not only attached to the substrate but could cycle and form two to four cell colonies in less than 48 h. Furthermore, the SP600125-treated cells become immobile. In a scratch
assay the control cells moved to fill in the empty space and heal the “wound,” whereas the SP600125-treated cells failed to do so (Fig. 2B). Identical results were obtained with proliferating and non-proliferating, mitomycin C-treated cultures: even when cell division is prevented the untreated, control cells can repopulate the scratch, whereas the JNK-inhibited ones cannot, which means that the SP600125 directly inhibits cell motility.

The ultimate goal of epidermal differentiation is the production of cornified envelopes, cross-linked proteinaceous cell ghosts insoluble in boiling detergent solutions (66). Cornified envelopes are a product of epidermal transglutaminase, which cross-links epidermal differentiation markers (see Table 1). The transglutaminase requires Ca\(^{2+}\) for its enzymatic activity. We found that the SP600125 treatment, in the presence of Ca\(^{2+}\), indeed causes formation of cell ghosts, i.e. empty, detergent-insoluble, cross-linked proteinaceous remnants of living cells (Fig. 2C).

Finally, when we cultured keratinocytes for extended periods in the presence of SP600125, we observed that the long term SP600125 treatment can cause stratification and cornification of keratinocytes (Fig. 3). Almost immediately upon the addition of SP600125, the keratinocyte colonies retract all cellular processes and develop smooth, rounded edges, with indistinct cell-cell boundaries. Although the control cultures continue to thrive and grow, in the treated cultures the cells piled on top of one another, and in time the suprabasal cells appeared cornified. This process is similar to the formation of the stratum corneum of the healthy epidermis \textit{in vivo}.

**DISCUSSION**

The most significant conclusion from our results is that JNK activation specifically inhibits differentiation of epidermal keratinocytes. This finding is important for our understanding both of the normal epidermal differentiation and of its perturbations during pathological conditions. It may also be relevant clinically, because augmenting the JNK activity could be used to delay cornification and enhance wound healing, whereas attenuating it could become a differentiation therapy-based approach for psoriasis.

The inhibition of JNK is sufficient to bring about all relevant aspects of keratinocyte differentiation: withdrawal from the cell cycle, transcription of the differentiation markers, including lipid and steroid biosynthetic machinery, stratification, inhibition of motility, cross-linking of structural proteins, and even production of cornified envelopes, the ultimate goal of epidermal differentiation. Moreover, the inhibition of JNK suppresses the expression of the basal cell markers. It has been difficult to reproduce the differentiation of human keratinocytes in monolayer cultures \textit{in vitro}, apparently because of the activated JNK proteins. This suggests that the JNK activation is the crucial fulcrum regulating the inhibition of differentiation in inflammatory epidermal processes.

Importantly, our \textit{in vitro} studies confirm many of the concepts originated \textit{in silico}. Starting from the microarray results, we have demonstrated that the inhibition of JNK \textit{in vitro} recapitulates many aspects of epidermal differentiation \textit{in vivo}. The results illustrate the hypothesis-generating power of the microarray studies.

The comprehensive microarray data allow us to expand significantly the spectrum of identified differentiation-associated processes, adding to those previously known. For example, the inhibition of JNK induces the enzymes of lipid and steroid metabolism, which produce the hydrophobic components of the stratum corneum. Further, the inhibition of JNK induces the production of mitochondrial proteins, presumably to provide additional energy for the production of stratum corneum. Regulatory proteins belonging to the diacylglycerol and inositol-phosphate pathways are also in the induced category, suggesting that two signaling pathways are important for keratinocyte differentiation. Unexpectedly, the inhibition of JNK induces the synthesis of histones, the proteins that protect the DNA from damage, as well as several DNA repair enzymes. Given the non-replicating fate of DNA in the terminally differentiating keratinocytes, the need for such protection is unclear.

The inhibition of JNK results in suppression of expression of basal cell markers, including all regulated integrins and hemidesmosome components, most extracellular matrix components, and other known basal markers, \textit{e.g.} plectin. Unexpectedly, several other functional categories of genes are also suppressed, including the heat shock proteins and ubiquitin ligases; these have not been associated with the basal cell phenotype until now. Similarly, 5 dual-specificity phosphatases, \textit{DUSP1}, -4, -5, -6, and -7, are suppressed by JNK inhibition. These attenuate the signal transduction through the JNK/ERK/p38 pathway. Therefore, the elevated expression of the JNK/ERK/p38 regulatory pathway proteins seems associated with the basal phenotype, whereas the inositol phosphate and diacylglycerol pathway proteins are associated with the suprabasal, differentiating phenotype.

Importantly, the inhibition of JNK induces the expression of Jun and Fos (‘h’ in Table 1), the canonical AP1 transcription factors that play a role in induction of several epidermal differentiation markers (65, 67–70). This may resolve the following conundrum: AP1 binding sites have been found in many differentiation-specific genes, AP1 is activated by JNK, leading to the expectations that the activation of JNK induces the expression of differentiation markers, whereas, in contrast, we find that the activation of JNK inhibits differentiation. According to our model, the inhibition of JNK increases the production of AP1 proteins, which can then be activated by other, JNK-independent pathways (71) to induce the synthesis of differentiation markers.

The results also suggest that the proliferative effects of JNK are indirect, \textit{i.e.} by steering the cells away from differentiation and not by directly regulating the cell cycle and DNA replication genes. Apparently, the proliferation of keratinocytes by activated JNK is a by-product of the inhibition of differentiation. We note that the microarray analysis is eminently capable of identifying the genes directly associated with proliferation. For example, we have shown previously that the interferon-γ treatment inhibited keratinocyte proliferation by directly suppressing close to 100 genes in the cell cycle and DNA synthesis functional categories (33). Interestingly, the few SP600125-regulated genes in the DNA repair/synthesis functional category, \textit{e.g.} \textit{ERCC2} and \textit{SSBP2}, are all associated with DNA repair, not replication.
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The mechanisms that bring about the SP600125-directed differentiation are unknown at present. The AP1 transcription factor binding sites are over-represented in the SP600125-suppressed genes, as would be expected for targets of JNK. On the other hand, the preponderance of binding sites for the forkhead family of transcription factors in the induced genes suggests a role for one or more members of this family in epidermal differentiation. Evidently, this role merits additional focused research efforts.

In summary, we find that the inhibition of JNK in epidermal keratinocytes is sufficient to initiate their differentiation program and conclude that JNK is a specific and accessible target for modulating the cornification process in human skin.

REFERENCES

1. Nickoloff, B. J., and Nestle, F. O. (2004) J. Clin. Invest. 113, 1664–1675
2. Bernerd, F., Magnaldo, T., and Darmon, M. (1992) J. Invest. Dermatol. 98, 902–910
3. Fuchs, E., and Raghavan, S. (2002) Nat. Rev. Genet. 3, 199–209
4. Dotto, G. P. (1999) Crit. Rev. Oral Biol. Med. 10, 442–457
5. Xia, Y., and Karin, M. (2004) Trends Cell Biol. 14, 94–101
6. Werner, S., and Smola, H. (2001) J. Invest. Dermatol. 115, 910–917
7. Segre, J. A., Bauer, C., and Fuchs, E. (1999) J. Biol. Chem. 274, 2896–2907
8. Yu, S. H., Kenncken, C., Sander, C., Mullen, R., Schlessinger, J., and Hengartner, M. O. (2001) Science 293, 541–545
9. Angel, P., Szabowski, A., and Schorpp-Kistner, M. (2001) Oncogene 20, 2233–2244
10. Yates, S., and Rayner, T. E. (2002) Wound Repair Regen. 10, 5–15
11. Weston, C. R., Wong, A., Hall, J. P., Goad, M. E., Flavell, R. A., and Davis, R. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 14114–14119
12. Kallunki, T., Su, B., Tsigelny, I., Sluss, H. K., Derijard, B., Moore, G., Davis, R., and Karin, M. (1994) Genes Dev. 8, 2996–3007
13. Dong, C., Yang, D. D., Wysk, M., Whittmarah, A. J., Davis, R. J., and Flavell, R. A. (1998) Science 282, 2092–2095
14. Kuan, C. Y., Yang, D. D., Samanta Roy, D. R., Davis, R. J., Rakic, P., and Flavell, R. A. (1999) Neuron 22, 667–676
15. Chen, N., Nomura, M., She, Q. B., Ma, W. Y., Holleran, W. M., Elias, P. M., Cunningham, J. M., and Jane, S. M. (2005) Science 308, 411–413
16. Angel, P., Szabowski, A., and Schorpp-Kistner, M. (2001) Oncogene 20, 2143–2153
17. Yates, S., and Rayner, T. E. (2002) Wound Repair Regen. 10, 5–15
18. Weston, C. R., Wong, A., Hall, J. P., Goad, M. E., Flavell, R. A., and Davis, R. J. (1994) Mol. Cell. Biol. 14, 8376–8384
19. Fisher, G. J., Talwar, H. S., Lin, J., Lin, P., McPhilips, F., Wang, Z., Li, X., Wan, Y., Kang, S., and Voorhees, J. J. (1998) J. Clin. Invest. 101, 1432–1440
20. D’Alessandro, M., Russell, D., Morley, S. M., Davies, A. M., and Lane, E. B. (2002) J. Cell. Sci. 115, 4341–4351
21. Freedberg, I. M., Tomic-Canic, M., Komine, M., and Blumenberg, M. (2001) J. Invest. Dermatol. 116, 633–640
22. Atabashi, H., Ibe, M., Nakamura, S., Ishida-Yamamoto, A., Hashimoto, Y., and Izuka, H. (2002) J. Invest. Dermatol. 109, 94–99
23. Sluss, H. K., Barrett, T., Derijard, B., and Davis, R. J. (1994) Mol. Cell. Biol. 14, 8376–8384
24. Fisher, G. J., Talwar, H. S., Lin, J., Lin, P., McPhilips, F., Wang, Z., Li, X., Wan, Y., Kang, S., and Voorhees, J. J. (1998) J. Clin. Invest. 101, 1432–1440
25. D’Alessandro, M., Russell, D., Morley, S. M., Davies, A. M., and Lane, E. B. (2002) J. Cell. Sci. 115, 4341–4351
26. Freedberg, I. M., Tomic-Canic, M., Komine, M., and Blumenberg, M. (2001) J. Invest. Dermatol. 116, 633–640
27. Assets, Z., Garmyn, M., Bouillon, R., Merlevede, W., Vandenhedde, J. R., and Agostinis, P. (1997) J. Invest. Dermatol. 108, 886–891
28. Ramaswamy, N. T., Ronai, Z., and Pelling, J. C. (1998) Oncogene 16, 1501–1505
29. Silbers, A. L., Bachelor, M. A., and Bowden, G. T. (2003) Neoplasia 5, 319–329
30. Adachi, M., Gazel, A., Pintucci, G., Shuck, A., Shifteh, S., Ginsburg, D., Rao, L. S., Kaneko, T., Freedberg, I. M., Tamaki, K., and Blumenberg, M. (2003) DNA Cell Biol. 22, 665–677
31. Zhang, J. Y., Green, C. L., Tao, S., and Khvapari, P. A. (2004) Genes Dev. 18, 17–22
32. Zeiger, M. E., Chi, Y., Schmidt, T., and Varani, J. (1999) J. Cell. Physiol. 180, 271–284
33. Li, W., Henry, G., Fan, J., Bandyopahdyay, B., Pang, K., Garner, W., Chen, M., and Woodley, D. T. (2004) J. Invest. Dermatol. 123, 622–633
34. Dashti, S. R., Efimova, T., and Eckert, R. L. (2001) J. Biol. Chem. 276, 8089–8096
Merchant, A., Chau, K., and Tomic-Canic, M. (2005) *J. Invest. Dermatol.* **124**, 1034–1043

62. Sandelin, A., Alkema, W., Engstrom, P., Wasserman, W. W., and Lenhard, B. (2004) *Nucleic Acids Res.* **32**, D91–D94

63. Janes, S. M., Ofstad, T. A., Campbell, D. H., Watt, F. M., and Prowse, D. M. (2004) *J. Cell Sci.* **117**, 4157–4168

64. Harvey, R. P. (1996) *Dev. Biol.* **178**, 203–216

65. Jang, S. I., and Steinert, P. M. (2002) *J. Biol. Chem.* **277**, 42268–42279

66. Sun, T.-T., and Green, H. (1976) *Cell* **9**, 511–521

67. Rossi, A., Jang, S. I., Ceci, R., Steinert, P. M., and Markova, N. G. (1998) *J. Invest. Dermatol.* **110**, 34–40

68. Welter, J. F., Crish, J. F., Agarwal, C., and Eckert, R. L. (1995) *J. Biol. Chem.* **270**, 12614–12622

69. Mehic, D., Bakiri, L., Ghannadan, M., Wagner, E. F., and Tschachler, E. (2005) *J. Invest. Dermatol.* **124**, 212–220

70. Yamada, K., Yamanishi, K., Kakizuka, A., Kibe, Y., Doi, H., and Yasuno, H. (1994) *Biochem. Mol. Biol. Int.* **34**, 827–836

71. Karin, M. (1996) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **351**, 127–134