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Novel Genomic Effects of Glucocorticoids in Epidermal Keratinocytes

INHIBITION OF APOPTOSIS, INTERFERON-γ PATHWAY, AND WOUND HEALING ALONG WITH PROMOTION OF TERMINAL DIFFERENTIATION[*] [S]

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Glucocorticoids (GCs) have a long history of use as therapeutic agents for numerous skin diseases. Surprisingly, their specific molecular effects are largely unknown. To characterize GC action in epidermis, we compared the transcriptional profiles of primary human keratinocytes untreated and treated with dexamethasone (DEX) for 1, 4, 24, 48, and 72 h using large scale microarray analyses. The majority of genes were found to be regulated only after 24 h and remained regulated throughout treatment. In addition to regulation of the expected pro-inflammatory genes, we found that GCs regulate cell fate, tissue remodeling, cell motility, differentiation, and metabolism. GCs suppress the expression of essentially all IFNγ-regulated genes, including IFNγ receptor and STAT-1, an effect that was previously unknown. GCs also block STAT-1 activation and nuclear translocation. Unexpectedly, GCs induce the expression of anti-apoptotic genes and repress pro-apoptotic ones, preventing UV-induced keratinocyte apoptosis. Consequently, treatment with GCs blocked UV-induced apoptosis of keratinocytes. GCs have profound effect on wound healing by inhibiting cell motility and the expression of the proangiogenic factor, vascular endothelial growth factor. They play an important role in tissue remodeling and scar formation by suppressing the expression of TGFβ1 and -2 and MMP1, -2, -9, and -10 and inducing TIMP-2. Finally, GCs promote terminal epidermal differentiation while simultaneously inhibiting early stage differentiation. These results provide new insights into the beneficial and adverse effects of GCs in the epidermis, defining the participating genes and mechanisms that coordinate the cellular responses important for GC-based therapies.

GCs[5] play a key role in regulating diverse physiological processes, such as metabolism, salt, and water balance, cell proliferation, differentiation, inflammation, and immune response. Because of their systemic effects on multiple targets, GCs affect many tissues differentially. They are widely used for their anti-inflammatory effects in treating asthma, systemic lupus erythematosus, rheumatoid arthritis, transplant patients, psoriasis, etc., but the mechanism of their action in skin has not been fully understood. Topical GC therapy was introduced by Sulzberger and Witten in 1952 (see Ref. 1) and has been used since in the treatment of many dermatological diseases, including psoriasis, atopic and seborrheic dermatitis, intertrigo, and eczema.

The side effects of systemic GC therapy have been identified for many tissues and organs, including skin, and may result in what was clinically described as Cushing's syndrome (2). Corticosteroids with higher potency may cause severe side effects after topical application, including irreversible striae, skin atrophy, steroid acne, and perioral and periocular dermatitis. Delayed wound healing following steroid therapy is a well known side effect (3). Most of the known effects of GCs are thought to be dermal, including suppression of fibroblast proliferation, collagen turnover, and other ECM components (4–9). Epidermal keratinocytes also have important immunologic functions (10–12), many of which are affected by GCs (for a review, see Ref. 13).

At the molecular level, GCs act through "pluripotent" glucocorticoid receptors (GRs) that may be active in various cellular compartments: membrane, cytoplasm, and nucleus (13–15). In addition to operating as a transcription factor that directly binds promoter elements, GR also interacts with and affects the activity of a variety of transcription factors, thus affecting transcriptional potency of many signaling pathways, such as TNFα or epidermal growth factor (16). We have shown previously that GR represses the expression of epidermal keratin genes. This transcriptional regulation is mediated through a unique molecular mechanism that involves four GR monomers (17,

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 4–8.

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[5] The abbreviations used are: GC, glucocorticoid; ECM, extracellular matrix; GR, glucocorticoid receptor; TNF, tumor necrosis factor; STAT, signal transducers and activators of transcription; DEX, dexamethasone; RT, reverse transcription; TUNEL, terminal dUTP nick end labeling; MMP, matrix metalloproteinase; TGF, transforming growth factor; IFNγ, interferon-γ; IL, interleukin; C/EBP, CCAAT enhancer-binding protein; ERK, extracellular signal-regulated kinase.

18). In this conformation and DNA context, β-catenin and arginine methyltransferase (CARM-1), act as co-repressors of GR (19). We found GR inhibition to be dominant over epidermal growth factor receptor activation, leading to inhibition of keratinocyte migration and contributing to the inhibition of wound healing (20). The complex mechanism involving the transcriptional regulation of epidermal genes by GR is derived from the structure and the sequence of the response element, the conformation of the receptor and its modifications, the availability and the type of the interaction with co-regulators, and histone-modifying enzymes.

To identify the tissue-specific transcriptional effects of GCs on epidermis, we utilized a large scale microarray. We found that the initial response of keratinocytes to treatment with GCs (1–4 h) involved a small number of regulated genes and focused only on three processes: transcription/signaling, cell fate, and metabolism. After the first 24 h, the response is expanded to multiple functional groups of genes, and many cellular processes are affected, including inflammation, apoptosis, cell migration, metabolism, and differentiation. Specifically, GCs inhibit keratinocyte proliferation, migration, and early stages of differentiation while inducing late differentiation. Unexpectedly, GCs seem to have an anti-apoptotic effect on keratinocytes by inducing anti-apoptotic genes and suppressing pro-apoptotic genes. Another surprising finding regards the potent inhibition of the IFNγ response, resulting in suppression of IFNγ expression, the IFNγ receptor, and both expression and activation of STAT-1. To the best of our knowledge, such a profound effect of GCs on the IFNγ pathway has not previously been reported in any other tissue. Taken together, these results indicate that GCs affect a wide range of processes that include not only anti-inflammatory responses but also epidermal differentiation, remodeling, metabolism and cell fate, all of which have important clinical implications in treating dermatologic disorders.

EXPERIMENTAL PROCEDURES

Human Keratinocyte Cultures—Keratinocytes were maintained as previously published (20), in serum-free keratinocyte medium with epidermal growth factor (Gibco), bovine pituitary extract (Gibco), and antibiotic-antimycotic (Gibco) both in the presence and absence of 0.1 μM DEX (Sigma). This hormone concentration has been shown to saturate the receptor and have a potent transcriptional effect on keratinocytes (18, 20). The experiment was repeated twice using newly generated cultures. A paired set of treated and untreated cells was harvested for each time point, 1, 4, 24, 48, and 72 h, using 0.4% trypsin (Gibco) and stored in RNAlater (Ambion).

Total RNA Isolation—Total RNA was isolated using RNeasy (Qiagen). Northern blot analysis was done to assess the quality of mRNA isolated. 5 μg of total RNA was reverse-transcribed, amplified, and labeled according to the protocol (21). Labeled cRNA was hybridized to HGU95Av2 arrays (Affymetrix), and arrays were washed and stained with anti-biotin streptavidin-phycoerythin-labeled antibody using an Affymetrix fluidics station and then scanned using the Agilent GeneArray Scanner system (Hewlett-Packard).

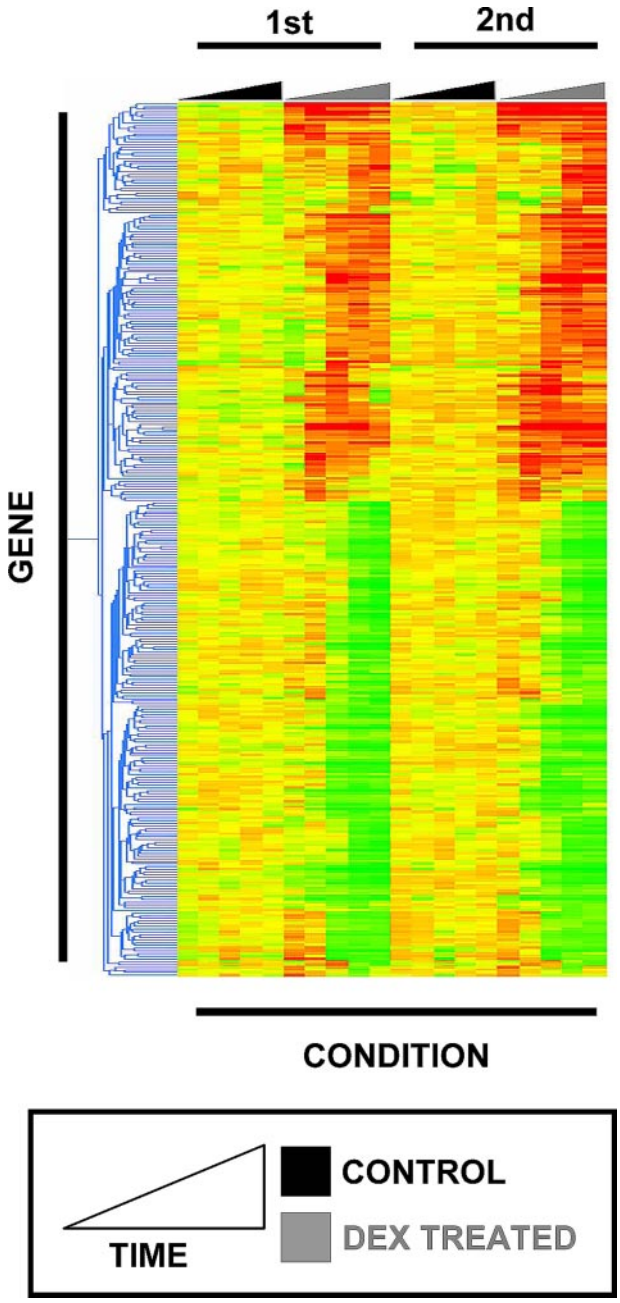


FIGURE 1. **Gene tree (a graphic presentation in which samples are grouped based on the similarity of gene expression profiles) showing gene expression patterns between two replicate experiments, indicating high reproducibility.** Primary keratinocyte cultures were generated independently, and cells were grown at separate times, followed by treatment. For each designated treatment time point, a paired, untreated control was harvested. Highly expressed genes are shown as *red lines*, low expressed genes are represented as *green*, and *yellow color* indicates intermediately expressed regulated genes.

Microarray—Microarray Suite 5.0 (Affymetrix) was used for data extraction and for further analysis. Data mining tool 3.0 (Affymetrix) and GeneSpring™ software 5.1 (Silicon Genetics) were used for normalization, -fold change calculations, and clustering. To compare data from multiple arrays, the signal of each probe array was scaled to the same target intensity value. The microarray experiments were repeated with high reproducibility Fig. 1. -Fold changes obtained from the first and second experiments were averaged. Genes were considered regu-

lated if expression levels differed more than 2-fold relative to untreated control at any time point. Using GeneSpring™, clustering was performed based on experiments or the expression profiles of individual genes. Functional annotation of regulated genes was performed as before (22, 23). The L2L program was used to identify the biological processes, molecular functions, and cell components of differentially expressed genes (24). The parameters of the program were calibrated using a set of identified NFκB-regulated genes (25).

RT-PCR—RNA isolation and purification was performed using Triazol (Invitrogen) extraction and subsequent Qiagen RNeasy kit column purification (Qiagen), followed by Northern blot as described (18). Reverse transcription was performed using the SuperScript™ first strand synthesis system for RT-PCR (Invitrogen). Primer sequences were as follows: TTCTCTCCCTTCCTCTCTCC (Bak1-fw), ACTCCCTACTCCTTTTCCC (Bak1-rev), TGTCTACACTTAGCCTCTATCC (IκB-fw), ATCAGCCCCACATTCAAC (IκB-rev), TTGATAGAGTGTGGGGTGGG (TRADD-fw), ATCATTGCTTAACATTCGGGG (TRADD-rev), CCAACCTGAAAACCCACAC (BCL6-fw), ACGAAAGCATCAACACTCC (BCL6-rev), TCTCTGCCCACAGTCTTTCC (SFRP1-fw), TCACCCAATTTCACAATTCACC (SFRP1-rev), GACAGCAAAAATGACCCACC (EEF1A1-fw), ACAGCAAAGCGACCCAAAG (EEF1A1-rev), ACACCTCGTCAAACTCCTC (STAT1-rev), ACTTTCTGCTGTTACTTTCCC (STAT1-fw), GAGCAAACACATCTGACCTAC (MMP1-fw), CAAAATGAGCATCCCCTCC (MMP1-rev), CACTACTGTGCCTTTGAGTCC (MMP9-), ATCGCCAGTACTTCCCATCC (MMP9-revfw), TGCCCACAAAATCTGTTCC (MMP10-fw), and AACCTGCTTGTACCTCATTTC (MMP10-rev).

Reverse transcription and amplification were carried out by incubation at 50 °C for 50 min. Initial PCR denaturation took place at 94 °C for 15 min, followed by 24–30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, initial polymerization at 72 °C for 1 min, and a final polymerization at 72 °C for 7 min. The numbers of cycles of amplification (24, 27, and 30) were selected to detect amplified products in the exponential phase. Samples were separated by electrophoresis on 2% agarose gels containing 0.5 μg/ml of ethidium bromide (Sigma) and were visualized under UV light.

Quantitative RT-PCR—Total RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen). The cDNA was amplified using SYBR-Green PCR Master Mix (Applied Biosystems) in an ABI 7900HT sequence detection system (Applied Biosystems). The primers used for PCR analysis were as follows: JAG1 (AATACATGTGGCCATTTCTGC, TGATTTCCTTGATCGGGTTC), TIMP1 (ACACTGTTGGCTGTGAGGAA, GTTTGCAGGGGATGGATAAA), IL-4R (GGGTCACAGTGGGAGAAGC, CAGGGCAAGAGCTTGGTAAG), LAMC2 (ACACATTAGACGGCCTCCTG, CCAGCCCCTCTTCATCTACA), FLNA (CAGTAGACTGCAGCAAAGCAG, ATGAACCCCCACCAGCAG), S100A7 (GGAGAACTTCCCCAACTTCC, ACATCGGCGAGGTAATTTGT), and EEF1A1 (CAAGCCCATGTGTGTTGAGA, CCACCGCAACTGTCTGTCT). The relative changes of gene expression were estimated and normalized to EEF1A1 by using the $2^{-\Delta\Delta C_T}$ method (26).

Histology and Immunocytochemistry—Keratinocytes were grown on coverslips to 70% confluence. Cells were incubated for 24 h in a basal serum-free medium (custom made without hydrocortisone) and treated as follows: 0.1 μM DEX (Sigma), INF-γ 100 ng/ml (Sigma), DEX and INF- γ simulatneously for 72 h, pretreated with DEX for 24 h, and treated with INF-γ for the next 48 h or pretreated with INF-γ for 24 h and treated with DEX for 72 h. Cells were fixed in acetone-methanol (1:1) for 2 min, permeabilized with 0.1% Triton X-100 for 10 min, and stained using STAT-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Human skin specimens were obtained from reduction mammoplasty following an approved protocol and treated for 24, 48, and 72 h as previously described (20). After incubation, skin biopsies were embedded in OCT compound (Tissue Tek) and frozen in liquid nitrogen. Five-micrometer-thick skin sections were cut with a cryostat (Jung Frigocut 28006; Leica) and stored at −80 °C. Slides containing frozen sections were fixed in a cold acetone and blocked with 3% bovine serum albumin diluted in 1× phosphate-buffered saline for 30 min. The following primary antibodies were as follows: monoclonal antibody against Filaggrin 1:1000 (Gift from Dr. Sun) (27) and Involucrin 1:400 (NeoMarkers) and polyclonal antibody against STAT-1 (1:500; Santa Cruz Biotechnology). These were used for overnight incubation at +4 °C. Signal was visualized using secondary fluorescein isothiocyanate anti-mouse or anti-rabbit secondary antibody 1:200 (Molecular Probes). Slides were mounted with mounting medium containing propidium iodide (Vector Laboratories). For staining with p65 antibody, 1:100 (Santa Cruz Biotechnology) skin samples treated with 0.1 μM DEX or 100 ng/ml TNFα (Sigma) or pretreated with DEX for 24 h and treated with TNFα for the next 24 h were embedded in paraffin and stained following a previously published protocol (19). All negative controls were prepared by substitution of the primary antibody with phosphate-buffered saline. Staining was analyzed using a Carl Zeiss microscope, and digital images were collected using the Adobe TWAIN_32 program. Three laboratory members blinded for the experiment performed quantification of the nuclei positive for STAT-1. The average and the S.D. values were calculated. All experiments were performed in triplicates, where 3–5 images/condition/time point were quantified.

Western Blotting—Keratinocytes were incubated with or without DEX (0.1 μM) for 0, 1, 24, 48, and 72 h, and total protein extracts were obtained for each time point using a standard protocol (28). 25 μg of each protein extract was electrophoresed in a 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (BioScience). The membrane was then incubated with Abs to STAT-1 (Santa Cruz Biotechnology) and β-tubulin (Santa Cruz Biotechnology). After the incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), the immune complexes were visualized using Super Signal West Pico Chemiluminescent substrate (Pierce) and exposed on x-ray film (Eastman Kodak Co. Bio Max MR-Film) according to the manufacturer's instructions.

Proliferation Assay—Keratinocytes were seeded in 0.75-cm^2 dishes at a concentration of 1100 cells/well and incubated in the keratinocyte basal media without hydorcortizone (Invitrogen)

24 h prior to treatment. Keratinocytes were incubated in the presence or absence of 0.1 μM DEX (Sigma) for 24, 48, and 72 h and harvested by trypsinization. Growth curves were established from triplicate experiments by three laboratory members blinded to the experiment by counting cell numbers per cm^2 at the each time point using a hemocytometer (Hausser Scientific). Statistical significance was determined using a standard *t* test.

Keratinocyte Treatment and TUNEL Assay—Cells were incubated for 24 h in a basal serum-free medium (custom made without hydrocortisone) before the experiment. On the day of the experiment, cells were incubated in the presence or absence of 0.1 μM DEX (Sigma) for 24 h (for the pretreatment condition), medium was removed from the cell cultures, and keratinocytes were irradiated with UVB irradiation (8 mJ/cm^2) (Stratagene 2000 illuminator, UV Stratalinker 24000) (21). Cells were then incubated for 48 h in the basal keratinocyte medium and fixed for the TUNEL assay. The TUNEL assay was performed following a commercial protocol for the *in situ* cell death detection kit (TMR red) (Roche Applied Science). Cells were then mounted on slides using Dako flourescent mounting medium (DAKO Corp.) and examined using a microscope (Carl Zeiss) and Adobe Photoshop TWAIN_32 program. All experiments were performed in triplicates. Three laboratory members blinded to the experiment counted apoptotic cells in 3–5 images/condition, and S.D. values were calculated.

RESULTS AND DISCUSSION

Global Transcriptional Changes after Glucocorticoid Treatment of Primary Human Keratinocytes—To identify the effects of GCs in epidermis, we treated primary human keratinocytes with 0.1 μM DEX for 1, 4, 24, 48, and 72 h, isolated and labeled mRNA, and hybridized it to Affymetrix HU95A chips. Of 12,653 total analyzed genes, 6,285 were found expressed in skin (49.7%). This is in agreement with results from other laboratories (21, 29). The majority of the GC-regulated genes were suppressed rather than induced. Of the 394 genes that were consistently regulated (6.3% of the total expressed in skin), 128 genes were induced, and 266 genes were suppressed. To compare experiments at different time points, cluster analysis was performed using Genespring™ 5.1, with each time point being a separate experiment. We found striking similarities among the 24, 48, and 72-h regulated genes. The most extensive regulation occurred at 24 h, where 172 genes were regulated. Most genes regulated by GCs at 24 h remained regulated until 72 h; after 48 h, only 15 of the 172 genes were not regulated, and 157 of these remained regulated even at 72 h of treatment. In addition, 125 and 74 new genes were regulated at 48 and 72 h, respectively.

Very few genes were affected at 1 and 4 h. GCs affected only 23 genes at 1 h; signal transduction, cell fate, and metabolism were the predominant functional gene groups regulated. Inhibitor of κB (*NFKBIA*) was one of the earliest induced genes at 1 h. The same functional groups were regulated at 4 h, yet the number of regulated genes increased to a total of 64 (Table 1).

To understand how GCs regulate cellular processes in keratinocytes, we summarized the microarray data in such a way that genes are clustered by their cellular functions (see below). Furthermore, we grouped several cellular functions into cellular processes, which resulted in the specific hierarchal tables summarized in Table 2. Overall, we found the genes that are involved in apoptosis, cell cycle, cornified envelope, cytoskeleton, DNA repair, ECM, interferon signaling, junctions, kinases, membrane protein, proteolysis, receptor, RNA metabolism, and secretion to be predominantly suppressed. We also found that genes involved in transcriptional regulation are among those induced by GC treatment (supplemental Tables 4–8).

We performed real time RT-PCR to evaluate the results obtained from microarrays and found that data generated by both methods are in agreement for all of the genes tested (Fig. 2). As expected, results obtained by real time RT-PCR followed the pattern of the microarray data but were more pronounced, a predictable result, considering the more sensitive method of mRNA detection.

Comparison of the lists of differentially expressed genes with their assigned ontology functions confirms the above analysis (Table 3). This is particularly apparent in the biological process category, where GCs induced the regulators of transcription, whereas they suppressed immune response and related processes. Similarly, the molecular functions overrepresented in the induced genes contain transcription factors and signaling proteins, whereas among suppressed genes the proteolysis inhibitors are significantly overrepresented. Correspondingly, the nuclear components are predominant in the induced set, whereas the extracellular matrix genes are suppressed by GCs.

We have described here the expression changes that glucocorticoid treatment exerts on the principal cellular components of epidermal tissue, primary human keratinocytes, thus providing a comprehensive view of the set of genes and cellular processes that are affected by GC treatment. Furthermore, we have described novel actions of GCs in epidermal keratinocytes, since we discovered a wide spectrum of genes affecting additional cellular processes that have not been associated previously with GCs. Specifically, we found that GCs inhibit apoptosis and block antigen presentation, tissue repair and remodeling, metabolism, keratinocyte migration and proliferation, differentiation, and cell fate control. Below, we focused on the analyses of specific gene groups and cellular processes regulated by GC treatment.

Inflammation and Innate Immunity—GCs are known as major anti-inflammatory agents, and this served as a control for our experiments. A summary of microarray results is presented in supplemental Table 4. We found that within the first 1 h of treatment and throughout all time points, GCs induce the expression of IkB, suggesting early effects on NFκB inhibition (30). We did not find either NFκB components (such as p65 or p50) or TNFα ligand and receptor to be regulated. The inhibitory effects of GCs on pro-inflammatory processes include repression of interleukin signaling, specifically the expression of IL-1β, IL-4 receptor, and IL-11 genes after 24 h. It was surprising that other than inducing IκB, GCs did not regulate transcription of other TNFα signaling molecules. There are several molecular mechanisms through which GR-mediated inhibition of NFκB may be accomplished, reflecting tissue-specific effects (31–43). Although the nongenomic effects of GCs on NFκB in

TABLE 1

List of genes regulated by GCs in the first 4 h of treatment

Immediate early genes regulated by GC after 1 and 4 h of treatment are shown. Numbers represent-fold change. The GC-induced genes are represented in red; the suppressed ones are represented in green. Shades of blue indicate the maximum signal intensities, which represent the absolute expression levels of mRNA, ranging from low (light blue) to high (dark blue).

1h-4h gene table

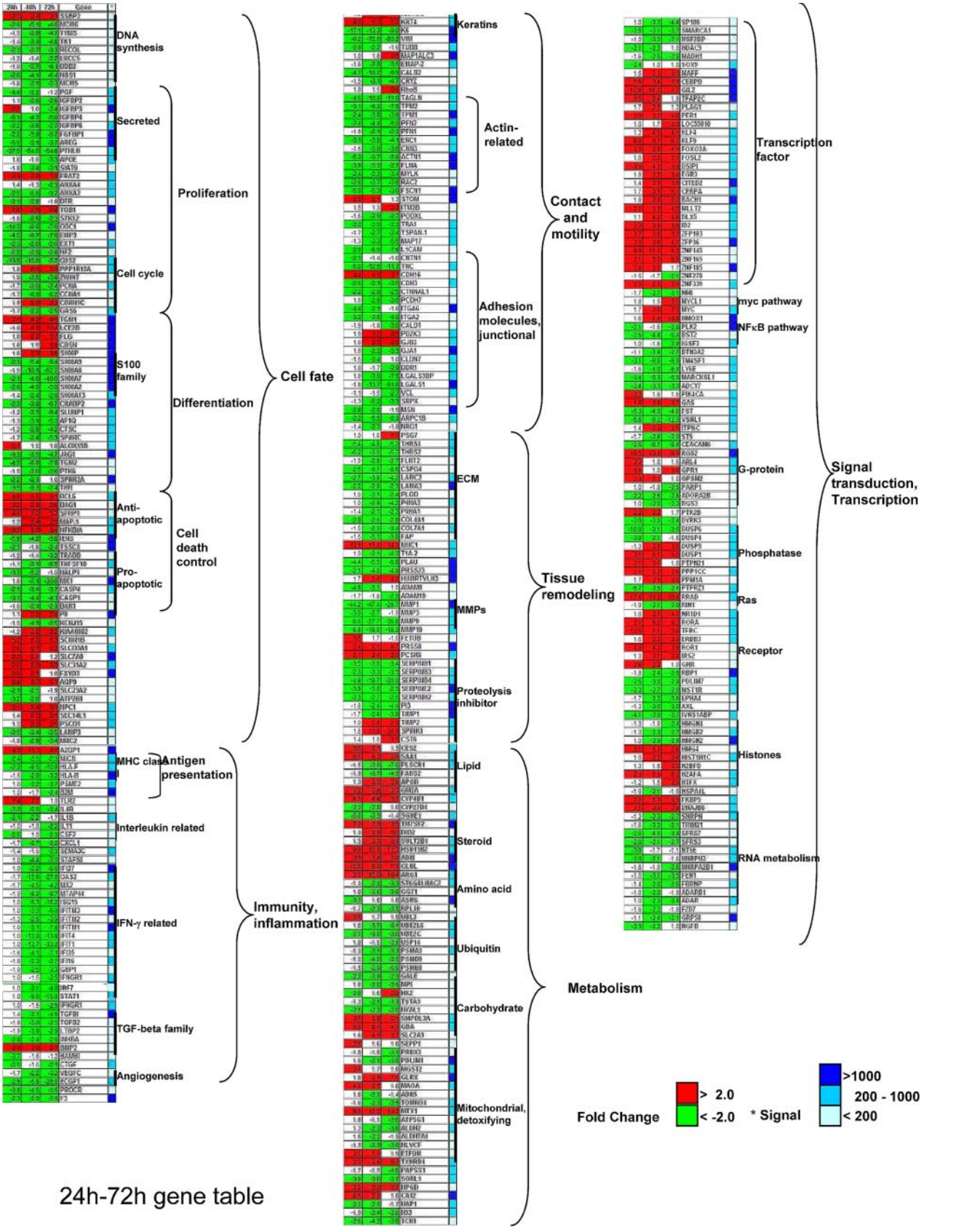
Fold Change: > 2.0 / < -2.0 * Signal: >1000 / 200 - 1000 / < 200

	1h	4h	GENE	*
Signal transduction, transcription factor	1.9	3.1	ITPKC	
	2.1	2.2	ZFP36	
	2.5	2.9	ARL4	
	4.2	3.5	DUSP1	
	4.3	9.8	RRAD	
	4.3	10.3	KLF9	
	4.6	7.9	DSIPI	
	9.6	36.6	GILZ	
	29.7	59.7	RGS2	
	2.5	1.6	PER1	
	-2.0	-1.2	ID2	
		22.1	ZNF145	
		2.3	ZFP103	
		2.9	CITED2	
		3.1	FOXO3A	
		2.9	ROR1	
		2.0	HMGB2	
		5.0	MYCL1	
		2.4	CEBPD	
		14.8	FKBP5	
		2.6	KLF4	
		-2.5	EGR3	
		-5.3	DUSP4	
		-2.7	DUSP5	
		-13.4	DUSP6	
		-3.0	FST	
		-2.0	EPHA4	
		-3.0	RGS3	
		-2.2	SOX9	
Cell fate	2.4	4.3	CDKN1C	
	2.5	2.4	NFKBIA	
	3.0	4.4	BCL6	
	-3.8	-4.0	DTR	
	-3.5	-13.2	IER3	
		-4.3	JAG1	
		-2.2	SIAT9	
		-4.8	PTHLH	
		-3.0	TSSC3	
		-3.6	ODC1	
		-2.2	FGFBP1	
		-6.5	AREG	
		-7.2	G0S2	
		-2.0	TNFSF10	
		2.2	SSBP2	
		2.3	CDSN	
		3.8	TGM2	
		3.6	SFRP1	
		5.4	FRAT2	
Metabolism	4.3	12.2	GLUL	
		2.4	SMPDL3A	
		2.4	MAOA	
		2.4	ARG1	
		2.2	GBA	
		2.2	UBL3	
		2.7	MTX1	
		3.0	CA12	
		2.5	HSD11B2	
		7.2	SAA1	

TABLE 2

Summary list of GC-regulated genes after treatment for 24–72 h

The epidermal genes regulated by GCs are arranged according to a hierarchical tree of related molecular and cellular functions. The coloring follows that of Table 1.



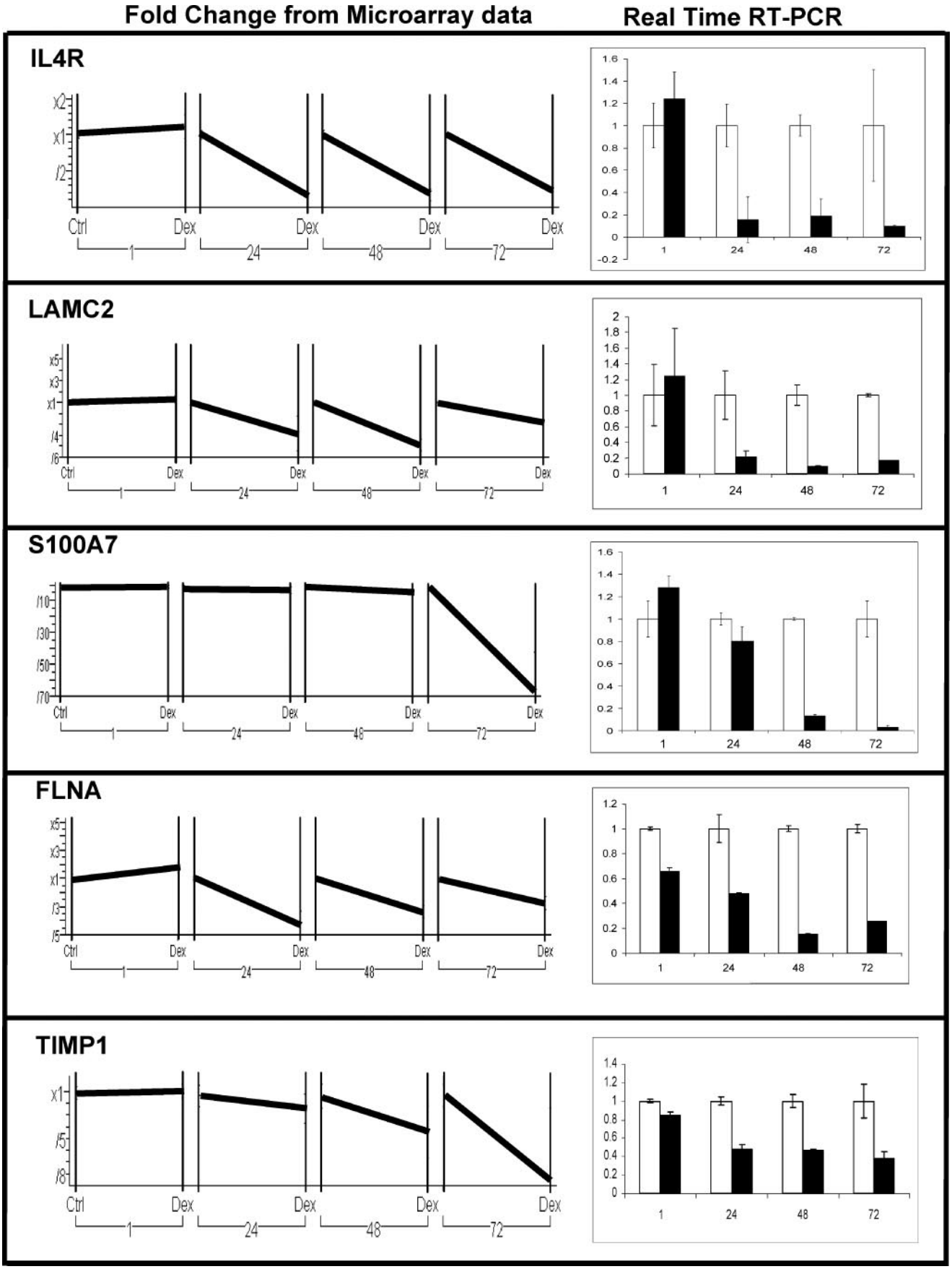


FIGURE 2. **Microarray data are confirmed by real time PCR.** The microarray data presenting five representative genes are shown at the *left*, and a *bar graph* represents the data obtained using quantitative real time PCR.

TABLE 3

Ontological categories of GC-regulated genes

The induced genes are on the left, and the suppressed ones are on the right. The *p* value shows the probability that the specific process, function, or component category is as overrepresented in a same-size list of random genes. #, the number of genes in each given category; Fold, the increase of regulated genes over the number expected at random. We present the categories with *p* values better than 0.005, or the top 10 scoring categories of each type.

Induced: Process			
list name	#	Fold	Prob.
regulation of transcription	28	2.1	3.9E-04
regulation of base, nucleoside, nucleotide and nucleic acid metabolism	28	2.0	4.7E-04
regulation of transcription, DNA-dependent	27	2.0	6.1E-04
transcription	28	2.0	7.1E-04
transcription, DNA-dependent	27	2.0	9.3E-04
nucleosome assembly	4	9.1	1.0E-03
regulation of metabolism	28	1.9	1.3E-03
transition metal ion homeostasis	3	12.2	1.9E-03
regulation of physiological process	28	1.8	2.8E-03
chromatin assembly/disassembly	4	6.5	3.4E-03

Suppressed: Process			
list name	#	Fold	Prob.
immune response	39	2.4	6.1E-07
defense response	40	2.3	2.2E-06
response to biotic stimulus	42	2.2	2.3E-06
response to external stimulus	53	1.9	1.7E-05
response to stimulus	60	1.8	3.4E-05
epidermis development	8	5.4	1.2E-04
ectoderm development	8	4.5	4.2E-04
organismal physiological process	55	1.6	5.2E-04
collagen catabolism	4	10.1	5.6E-04
morphogenesis	43	1.7	6.3E-04

Induced: Mollecular function			
list name	#	Fold	Prob.
DNA binding	33	2.2	3.4E-05
transcription factor activity	20	2.6	1.7E-04
transcription regulator activity	22	2.1	1.2E-03
nucleic acid binding	33	1.7	3.4E-03
MAP kinase phosphatase activity	2	16.2	6.5E-03
transition metal ion binding	13	2.2	8.6E-03

Suppressed: Mollecular function			
list name	#	Fold	Prob.
protease inhibitor activity	11	4.9	1.8E-05
endopeptidase inhibitor activity	11	4.9	1.8E-05
calcium ion binding	24	2.5	4.5E-05
insulin-like growth factor binding	5	9.1	1.9E-04
serine-type endopeptidase inhibitor activity	7	5.2	3.8E-04
enzyme inhibitor activity	13	3.0	4.5E-04
cytoskeletal protein binding	15	2.8	4.6E-04
endopeptidase activity	16	2.5	7.8E-04
actin binding	12	3.0	8.1E-04
growth factor binding	7	4.6	8.8E-04

Induced: Cell component			
list name	#	Fold	Prob.
nucleosome	4	9.7	7.6E-04
chromatin	5	5.0	3.4E-03
nucleus	38	1.6	4.4E-03

Suppressed: Cell component			
list name	#	Fold	Prob.
extracellular	40	2.0	5.0E-05
extracellular matrix	18	2.9	5.5E-05
extracellular space	18	2.8	1.2E-04
basement membrane	5	5.1	3.0E-03

epidermal keratinocytes remain to be elucidated, our results show that IκB is a transcriptional target for GCs.

Unexpectedly, we found *IRF7*, *IFNγ* receptor, *STAT-1*, and 12 other IFNγ-related genes to be suppressed at 48 and 72 h of GC treatment, indicating novel immunosuppressing properties of GCs in epidermal keratinocytes. We evaluated both the STAT-1 expression by RT-PCR and its regulation in cultured cells and in epidermis (Fig. 3*A*). We found that, indeed, GCs suppressed STAT-1 gene expression, and measuring STAT-1 protein levels using Western blots, we confirmed that GCs suppress STAT-1 on both mRNA and protein levels. To establish the physiological significance of this repression *in vivo*, we used immunohistochemistry and found that STAT-1 is suppressed in epidermis after topical treatment with GCs (Fig. 3*A*). The STAT-1 suppression occurred throughout the differentiating epidermal layers (demarcated as *D*), whereas the basal epidermal layer (demarcated as *B*) remained positive for STAT-1 (Fig. 3*A*).

To confirm the functional relevance of this inhibition, we tested the effects of GCs in the presence of IFNγ, by evaluating STAT-1 activation in three different conditions: 1) keratinocytes treated with DEX for 24 h to allow activation of glucocorticoid receptor and subsequently treated with IFNγ; 2) keratinocytes treated simultaneously with DEX and IFNγ; and 3) keratinocytes pretreated with IFNγ to allow activation of STAT-1 before subsequent DEX treatment. We found that pretreatment with GCs virtually abolishes STAT-1 activation, evidenced by the absence of nuclear translocation by IFNγ (Fig. 3, *B* and *C*). GCs also significantly reduced STAT-1 activation when added simultaneously with IFNγ. In contrast, pretreatment with IFNγ allowed full STAT-1 activation and a subsequent DEX addition had no significant effect. Therefore, we conclude that GCs block the IFNγ regulatory pathway by suppressing the expression of STAT-1 as well as by blocking its activation. Although it has been previously shown that GCs inhibit STAT-1 expression in macrophages (44), their anti-IFNγ effects in epidermis have not been documented before. Furthermore, the effect of blocking STAT-1 translocation to the nucleus seems unique to keratinocytes, suggesting a new aspect of tissue-specific GC regulation.

Taken together, the anti-inflammatory effects of GCs have a wide range and a specific timeline. The early effect is anti-TNFα (1 h after treatment), extending to anti-interleukin after 24 h and expanding to anti-ineterferon γ after 48 h. This is particularly relevant for the clinical use of GCs.

In addition to comprehensive anti-inflammatory effects, GCs also inhibit the expression of major histocompatibility antigens (*HLA-B*, *HLA-F*, and *B2F*), antigen-presenting (*MICB*), immunoproteasome assembly (*PSME2*), and antigen-processing proteases (*PSMB9*, *PSMB10*, and *PSME3*). These results suggest that GCs suppress antigen presentation on keratinocytes.

Effects of Glucocorticoids on Cytoskeleton, Cell Migration, and Tissue Remodeling—Of the ~80 genes encoding for molecules responsible for adhesion, cytoskeleton, cell junctions, ECM proteins, proteolysis, and proteolysis inhibition, 63 were

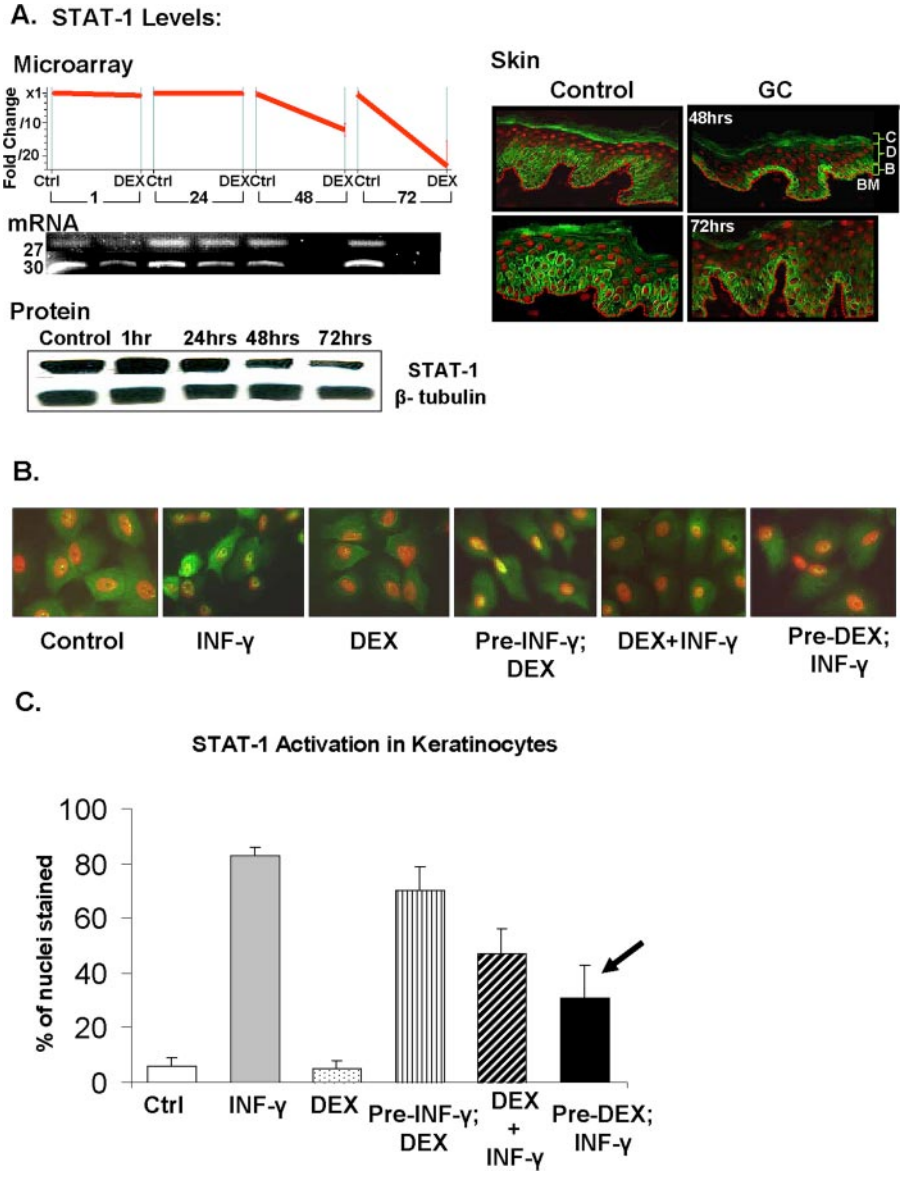


FIGURE 3. **GCs inhibit STAT-1 on genomic and nongenomic levels.** *A*, GCs suppress the expression of STAT-1 as evident from microarrays and RT-PCR. This suppression is also detected on the protein level by Western blot and immunohistochemistry of skin treated by topical GCs. Layers of skin are labeled on the *right*. A *dotted red line* demarcates the basement membrane (*BM*); *B*, basal keratinocyte layer; *D*, differentiating keratinocytes; *C*, cornified keratinocytes. *B*, primary human keratinocytes stained with STAT-1-specific antibody reveal that IFNγ-mediated activation and nuclear translocation of STAT-1 is blocked by GC in cells that are treated simultaneously with IFNγ and GC and even more effectively in cells pretreated with GCs for 4 h but not in cells pretreated with IFNγ for 2 h. *C*, quantification of STAT-1-positive nuclei for each condition for triplicate experiments.

down-regulated by GCs (supplemental Table 5). 20 of 23 cytoskeletal organizing genes, which include actin and keratin K6, were suppressed by GCs at 24 h and remained suppressed through 72 h. Other cytoskeleton-regulating genes, such as actin-related protein 2/3 complex, filamin A, neurofibromin 2, and profilin 1, were suppressed at each time point. They are involved in binding actin to the cell membrane, anchoring of the membrane proteins to the actin cytoskeleton, branching of actin filaments, actin polymerization in response to extracellular signals, and linkage of cytoskeletal components with the proteins in the cell membrane (45–49). We conclude that GCs affect cytoskeletal remodeling through the actin cytoskeleton, which affects the ability of the cells to form lamellipodial extensions (protrusions) and to migrate properly. Microarray analyses showed that other gene groups participating in keratinocyte migration were also repressed by GCs, including the extracellular matrix *LAMC2*, *LAMA3*, and hyaluronoglucosaminidase 1 genes, thus contributing to the inability of keratinocytes to migrate. Adhesion molecules were also suppressed at 24, 48, and 72 h, so that no adhesions could be established in order to foster cell movement.

Matrix metalloproteinases (MMPs) participate in degradation of ECM by cleaving a specific subset of matrix proteins, thereby helping keratinocytes to move over underlying dermis. MMP9, MMP3, and MMP10 were all found to be suppressed by GCs 15–18-fold in addition to MMP1, as previously described (50). We confirmed these findings by RT-PCR (Fig. 4). Upon treatment with GCs, keratinocytes shut down synthesis of MMPs, and as a consequence no matrix degradation necessary for keratinocyte migration can occur. Furthermore, tissue inhibitor of MMP, TIMP2, was induced at 48 and 72 h, providing a synergistic prevention of matrix degradation. Tenascin-C, found in migrating basal keratinocytes in the early phases of wound healing (51), was suppressed by GCs 17-fold at 72 h.

GCs are known inhibitors of the wound healing process. We have previously shown that they inhibit keratinocyte migration (20). We have also shown that GCs can effectively block the effects of epidermal growth factor and described a complex molecular mechanism through which they suppress the expression of keratin K6, one of the earliest markers of keratinocyte activation (20). Finally, we have also shown that GCs induce the expression of c-Myc, which also participates in the inhibition of keratinocyte migration (19). In this work, we also found that GCs suppress a plethora of genes that participate in cytoskeletal rearrangements and ECM remodeling, all necessary to support adequate cellular migration. These data clearly confirm that as potent inhibitors of keratinocyte migration and proliferation, GCs contribute to the inhibition of wound healing.

Vascular endothelial growth factor C, an angiogenic factor, was down-regulated by GCs at 24, 48, and 72 h, suggesting that

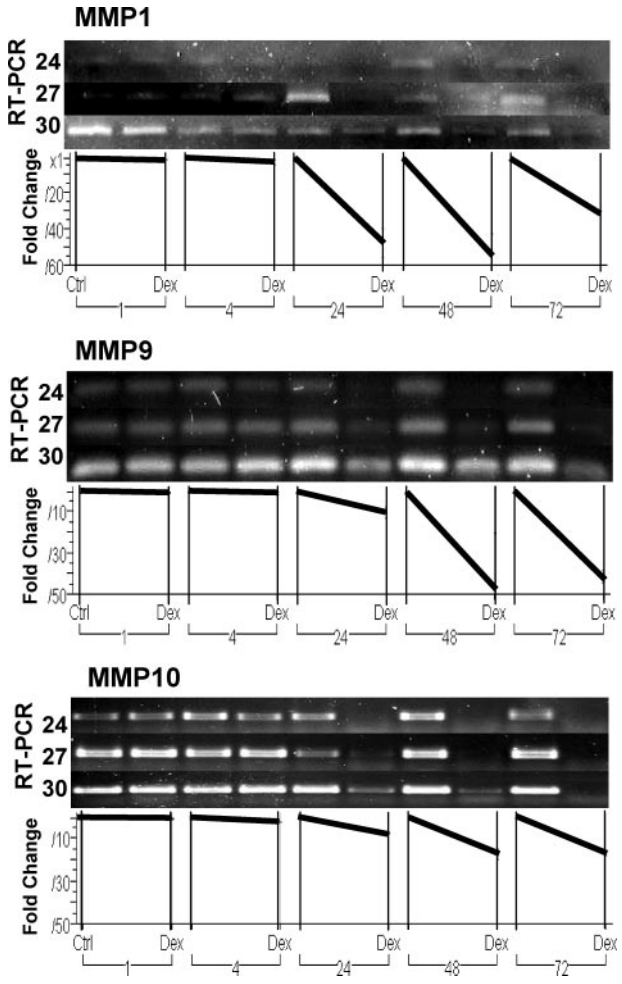


FIGURE 4. **GCs inhibit MMP1, −9, and −10.** The microarray data presenting MMP1, −9, and −10 (*graphs* shown *below*) were confirmed by RT-PCR performed for 24, 27, and 30 cycles (shown at the *top*) for each treatment time point.

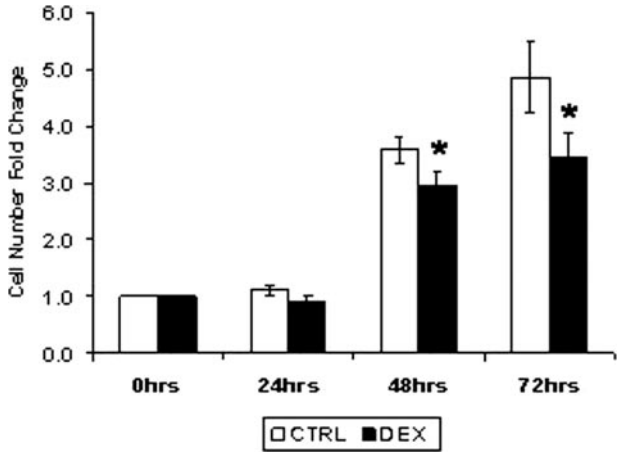


FIGURE 5. **GCs inhibit keratinocyte proliferation.** Quantification of triplicate experiments of cellular proliferation assay is shown. A statistically significant reduction in cell numbers was found after 48 and 72 h of DEX treatment. *, $p < 0.05$.

GCs may also affect angiogenesis, an essential process for successful wound healing.

We also found a specific effect on genes that participate in tissue remodeling/scar formation. GCs inhibit the expression of *TGFβ1*, *TGFβ2*, and *SMAD-1* (*MADH-1*) as well as collagens (*COL4A1* and *COL7A1*) in keratinocytes. GCs are used in the therapy of hyperthrophic scars and keloids, but their effects were thought to be primarily targeted on dermal fibroblasts (52). We found that GCs suppress the expression of *TGFβ1* and -2 but do not affect *TGFβ3*. Scarless (fetal-like) wound healing is associated with a lack of *TGFβ1* and -2 response during fetal wound healing, whereas *TGFβ3* activity remains intact (53). Therefore, our results would indicate that GCs may contribute to the reduction of scar formation by suppressing the expression of TGFβ1 and TGFβ2 but not of TGFβ3. Taken together, GC treatment affects cytoskeleton, ECM, and matrix remodeling, which results in inhibition of cellular migration and wound healing.

Control of Cell Fate: Growth, Differentiation, and Death—We analyzed the microarray data for genes contributing to proliferation, such as cell cycle-related or DNA synthesis (supplemental Table 6) and concluded that GCs may inhibit keratinocyte proliferation. To establish the functional relevance of this result, we tested GCs in a proliferation assay in which we quantified the cell number of untreated and DEX-treated keratinocytes at 0, 24, 48, and 72 h. We found a small but statistically significant ($p < 0.05$) decrease in keratinocyte proliferation after GC treatment at 48 and 72 h (Fig. 5). We concluded that GCs inhibit keratinocyte proliferation, confirming microarray data.

We also found that GCs have specific effects on keratinocyte differentiation. *TGM1* (transglutaminase 1), an enzyme that catalyzes the assembly of keratinocyte cornified envelope, was induced by GCs at 24, 48, and 72 h. In addition, *LCE2B* (late cornified envelope marker) as well as *FLG* (filaggrin), a cornified envelope precursor, were also induced by GCs 48 and 74 h after treatment. *CDSN* (corneodesmosin), a component of corneodesmosomes synthesized in the late stages of keratinocyte differentiation, was also found induced 72 h after treatment. Gene chip analysis revealed that the *SULT2B1* (sulfotransferase type 2 isoform B1) gene was up-regulated at 24, 48, and 72 h. The product of this gene sulfonates cholesterol, and it is also a critical regulator of terminal keratinocyte differentiation and desquamation as well as a mediator of barrier homeostasis. In addition, GCs induced expression of *KLF4* (Kruppel-like factor 4), which regulates terminal differentiation and barrier formation (54). This suggests that GCs promote the late stages of terminal keratinocyte differentiation.

In contrast, we found that GCs inhibit involucrin expression, which was confirmed using immunofluorescence (Fig. 6*A*). We found that after DEX treatment, skin exhibits induction of filaggrin and inhibition of involucrin when compared with untreated skin. Interestingly, involucrin suppression occurred only in lower suprabasal layers, whereas expression is maintained in the higher suprabasal (granular) layers. We also found that Jagged (*JAG1*), a Notch ligand that promotes early differentiation, was repressed at each time point observed. We confirmed the microarray data using real time quantitative RT-PCR (Fig. 6*B*). Suppression of JAG1 suggests that GCs may inhibit early stages of differentiation. Furthermore, we found that GCs induce expression of *THAP2C* (AP2-γ), which is the

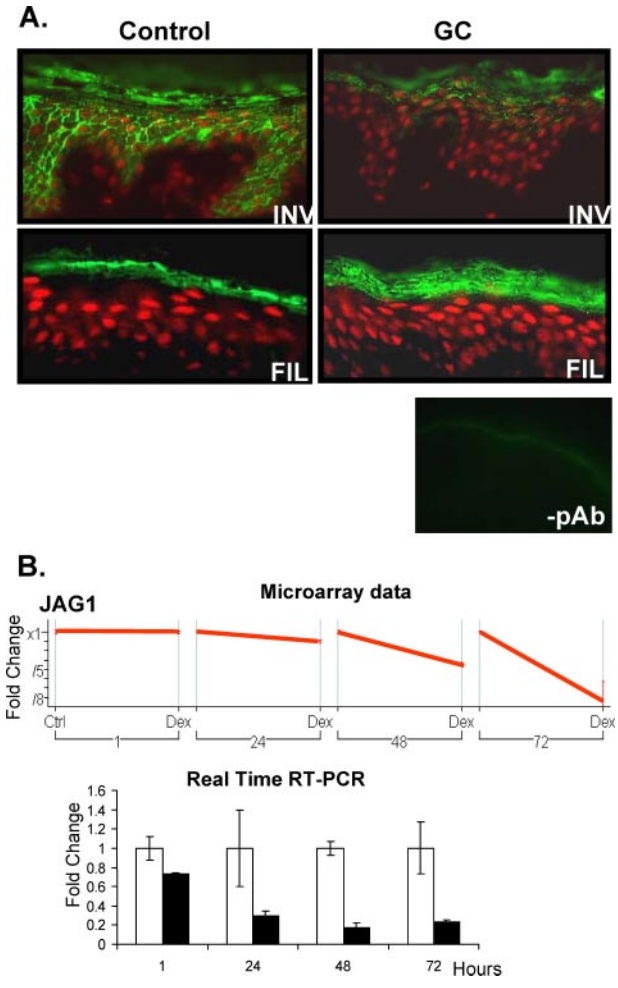


FIGURE 6. **GCs promote the terminal phase of epidermal differentiation, whereas they inhibit the early phase.** *A*, immunohistochemistry of skin treated with GCs reveals induction of filaggrin and restricted expression of involucrin to the cells undergoing the terminal phase of differentiation. *B*, quantitative real time RT-PCR shows suppression of Jagged-1, one of the regulators of early differentiation.

first *in vivo* target gene of TAp63α in embryonic epidermis, when commitment to stratification occurs, promoting the expression of basal-specific K14 gene (55). This suggests that GCs promote a basal keratinocyte phenotype rather than an early differentiating one.

GCs are widely used as therapeutic agents in treatment of premature babies to induce maturation of the lung epithelium and its barrier. It should not be surprising that GCs also induce terminal differentiation in epidermis. However, to the best of our knowledge, this has not been documented before. Another surprising component of our microarray data is that GCs may inhibit early epidermal differentiation by blocking Notch signaling, thus having a dual effect on the same process. By promoting the late stages and inhibiting the early stages of differentiation, GCs would decrease epidermal layers, which has been clinically observed as "thinning" of the epidermis. The microarray data presented here provides molecular explanation of this effect.

Unexpectedly, we found that GCs inhibit apoptosis. 12 anti-apoptotic genes, such as *IκB*, *BCL6*, *SFRP1*, *PTK2B*, and *BAG1*, were induced, whereas pro-apoptotic genes, such as *CASP1* and *-4*, *BAK1*, *TNFSF10*, *TSSC3*, *MX1*, and *TRADD*, were suppressed by GCs (Fig. 7). We confirmed that indeed, GCs induced anti-apoptotic genes, whereas they suppressed pro-apoptotic genes, by evaluating the mRNA levels of five different genes using both Northern and RT-PCR techniques (Fig. 7*A*). Finally, to test whether the anti-apoptotic genes induced by GCs have functional implications, we determined the effects of GCs in keratinocytes in which apoptosis was induced either by UV light or by Etoposide, a DNA-damaging agent. Keratinocytes were incubated in the presence or absence of DEX for 24 h to activate the GR transcriptional pathways and subsequently irradiated with 8 mJ/cm^2 of UVB. This particular UVB dose was chosen because it was previously shown to induce apoptosis in keratinocytes (21). We recorded gross apoptotic changes by photographing cells (Fig. 7*B*) and quantified the apoptosis using a TUNEL assay. As predicted by the microarray data, we found that GCs block apoptosis induced by either UV (Fig. 7*C*) or Etoposide (data not shown).

This finding has major clinical implications. GCs are known inducers of apoptosis in numerous cell types, including thymocytes, eosinophils, neutrophils, hippocampal neurons, and proliferative chondrocytes (56–60), and various malignancies of lymphoid origin and thus have become one of the most common therapeutic agents for leukemias and lymphomas (61). Interestingly, GCs have been described to exert an anti-apoptotic effect on epithelial ovarian or breast cancer cells, human glioblastoma, hepatoma, and fibroblasts (62–66). The anti-apoptotic effect of GCs on keratinocytes is achieved through the concerted suppression of pro-apoptotic and induction of anti-apoptotic genes. This effect is potent, since GCs effectively blocked UVB-mediated and DNA-damaging agent-mediated apoptosis in keratinocytes. This suggests that GCs protect keratinocytes from UVB-mediated apoptosis. We did not find the TNF receptor, *GITR*, regulated in our experiments. *GITR* expressed in mouse keratinocytes is under negative control of p21^{cip}/WAF1 and may also have a protective role from UV-induced apoptosis (67). *GITR* is not represented on the HU95A chip. We conclude that GCs have an anti-apoptotic effect on human keratinocytes and that they mediate this effect by regulating a specific subset of apoptosis-related genes in a very specific manner, suggesting a complex control mechanism in human epidermis.

GCs inhibit proliferation and may have a complex, dual effect on keratinocyte differentiation by inhibiting the early stages of differentiation while promoting the late stages.

Cellular Metabolism—A summary of the microarray data related to cellular metabolism is presented in supplemental Table 7. The ubiquitination-related *UBE2C* gene required for the destruction of mitotic cyclins, the *UBE2L6* gene, which catalyzes the covalent attachment of ubiquitin to other proteins, and *USP14*, a ubiquitin-specific protease gene, were suppressed at 24, 48, and 72 h. Proteolysis inhibitors *SRPINB1*, *SERPINB3*, *SERPINB4*, *SERPINE2*, and *SEPINH2* were also suppressed by GCs, suggesting that GCs in epidermal keratinocytes may block ubiquitination but promote proteolysis.

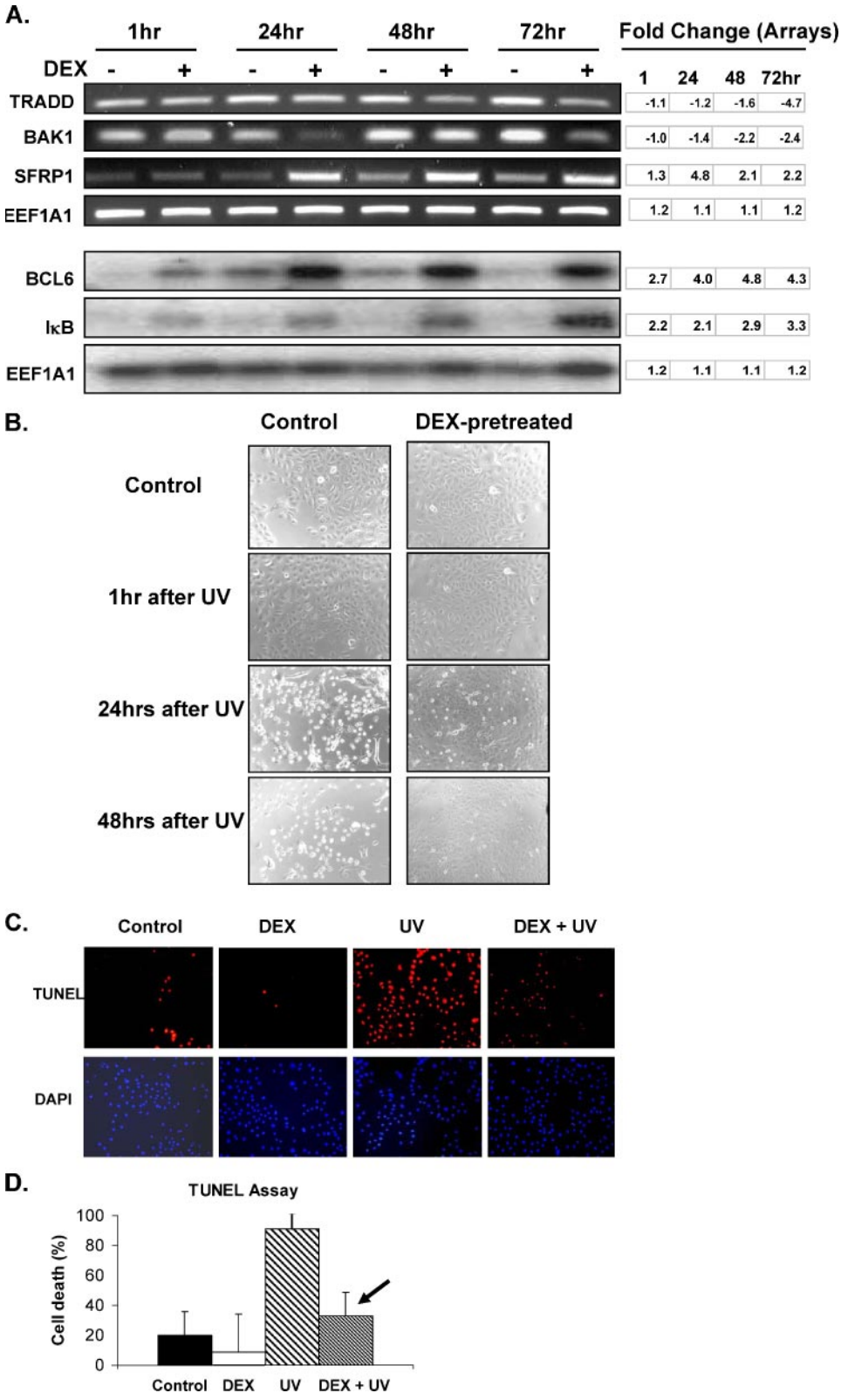


FIGURE 7. **GCs have an anti-apoptotic effect and protect keratinocytes from UVB-mediated apoptosis.** *A*, GCs suppress transcription of pro-apoptotic and induce expression of anti-apoptotic genes in keratinocytes. Microarray data (shown on the *right*) were confirmed using RT-PCR (*top*) or Northern blot (*bottom*). *B*, pretreatment with GCs prevented UVB-mediated apoptosis, as documented by phase microscopy and (*C* and *D*) TUNEL assay.

NLVCF, a structural constituent of mitochondrial ribosome, was repressed, whereas expression of metaxin (*MTX1*), a gene involved in protein transport into the mitochondria, was strongly induced. Proteins involved in detoxification, such as alkyl hydroperoxide reductase, alcohol, and aldehyde dehydrogenases, were suppressed as well.

GCs also affect lipid metabolism. They suppress the expression of fatty acid Δ-6-desaturase (*FADS2*), the rate-limiting enzyme in the synthesis of long-chain polyunsaturated fatty acids. This function includes the synthesis of arachidonic acid that is needed for synthesis of the eicosanoid biomediators that play central roles in inflammation (prostaglandins, leukotrienes, and thromboxanes). Enzymes involved in lipid and glycolipid catabolism, *CES2* and *GM2A* and *HPGD* (prostaglandin E2-metabolizing enzyme), were induced, as was apolipoprotein B, the main protein component of low density lipoproteins. GCs have minimal effect on amino acid and carbohydrate metabolism; they induce the expression of *GLUL* (glutamine synthase), *ARG* (arginase), *SMPDL3A* (sphingomyelin phosphodiesterase, acid-like 3A), *GBA* (intermediate in glycolipid metabolism), and *SLC2A3*, which facilitates glucose transfer, and suppress *HYAL1* (hyaluronidase 1) and *GALE* (galactose epimerase).

We found that GC treatment of cultured keratinocytes 8–14-fold up-regulates *HSD11B2* (hydroxysteroid (11-β)-dehydrogenase 2) at 24, 48, and 72 h. This gene encodes the enzyme that catalyzes the conversion of cortisol to the inactive metabolite cortisone, thus modulating the intracellular glucocorticoid levels (68). The iodothyronine deiodinase type II gene was also significantly up-regulated at 48 and 72 h; this enzyme activates the thyroid hormone by converting the prohormone thyroxine to bioactive triiodothyronine (69). GCs also induce adrenomedullin, a potent hypotensive and vasodilator peptide that at physiologically relevant doses also inhibits basal ACTH secretion. Recently,

Most mitochondrial and detoxifying genes were suppressed during GC treatment. It even appears that mitochondrial transcription is affected by the GC treatment. Expression of

adrenomedullin has been identified as a host defense peptide, and as such, it plays a role in the inflammatory response.

SAA1 (serum amyloid A1), the major acute phase reactant, was induced 3–9-fold at all time points. There are two reported immune-related functions of SAA (70). It can induce ECM-degrading enzymes, such as collagenase, stromelysin, and matrix metalloproteinases 2 and 3, which are important for repair processes after tissue damage. However, prolonged expression of SAA may play a role in degenerative diseases, such as rheumatoid arthritis. SAA can also act as a chemoattractant for such immune cells as monocytes, polymorphonuclear leukocytes, mast cells, and T lymphocytes.

Effects of GCs on Signaling and Transcription—In addition to affecting TNFα, interleukins, IFNγ, and TGFβ signaling, GCs regulate a number of genes that participate in transcriptional regulation and other signaling pathways (supplemental Table 8), such as several regulators of chromatin modulation members of SWI/SNF family (*SMARCA1* and *SMARCA2*) and *HDAC9*. In addition, *SOX9*, which is required for the formation of the hair stem cell compartment (71), was also suppressed. We found that GCs have a specific effect on the *C/EBP* family of transcription factors; the GCs induce *C/EBP*α, which is involved in mitotic growth arrest and *C/EBP*δ, but not *C/EBP*β, a mediator of IL-6 response. Coupled with the induction of *MAFF*, a repressor of transcription, and of *ID2*, an inhibitor of tissue-specific gene expression, this may suggest that the GCs inhibit transcription and proliferation. However, we found that GCs induce the expression of *PLAG-1*, which has been shown to induce expression of β-catenin and c-Myc (72), along with *MYCL1* and *MYC* (19). In addition, a Fos family member *FOSL2* and protein phosphatase 1 *PPP1CC*, essential for cell division, were also induced, suggesting a possible activation of proliferation. It is interesting to note that GCs regulate circadian regulators *PER1* and *EGR3* (73). Surprisingly, with the exception of suppression of *PLK2*, a serum-inducible kinase, and *MP1*, an adaptor protein that enhances the activation of *MAPK2*, GCs did not target the expression of any other kinase. In contrast, GCs regulate phosphatases in a very specific manner. The dual specificity phosphatases that target mitogen-activated protein kinases *ERK1* and *ERK*, *DUSP6* and *DUSP4*, were suppressed. Both *DUSP1*, which may play an important role in the human cellular response to environmental stress as well as in the negative regulation of cellular proliferation, and *DUSP5*, which inactivates *ERK1*, tyrosine-protein phosphatases *PTPN2* and *PPRZ1*, Ser/Thr protein phosphatases *PPM1A*, and *PP2C*, were induced. We also found specific effects of GCs on mRNA-related proteins. Essentially all mRNA processing, splicing, and maintenance-related genes were suppressed. It is interesting that proteins targeting mRNA turnover were not regulated. Instead, those regulating splicing (*SNRPN*, *SFRS7*, and *HNRPN3*), editing (*ADAR* and *ADARB1*), and cytoplasmic trafficking (*HNRPA3*) were suppressed.

The predominant effect of GCs on gene transcription is suppression. We have shown previously that GCs suppress the expression of epidermal keratin genes through an unconventional mechanism that involves four monomers of the GR, β-catenin and CARM-1 serving as co-repressors (18, 19). Although this may not be the only mechanism through which GC-mediated suppression occurs in epidermis, no epidermal defects are found in GRdim$^-$ mice, which have a mutation in the dimerization domain of GR that inhibits formation of homodimers and the "classical" mechanism of GC-mediated gene activation (74). Therefore, our microarray data support the notion that the majority of transcriptional effects of GCs in epidermis are mediated through transcriptional repression by GR monomers.

In summary, these data present a complete documentation and description of the transcriptional changes caused by GC treatment of primary human keratinocytes, demonstrating the complexity of the effects that GCs may have on epidermis. GCs inhibit inflammatory response in a specific time-related manner. Their initial effect is to block TNFα building up to block IL-1β, IL-4, and IL-11 after 24 h and inhibiting IFNγ signaling by 48 h. Furthermore, GCs inhibit apoptosis, keratinocyte migration, and proliferation and affect tissue remodeling in a manner that reduces scar formation. GCs also promote terminal differentiation and barrier formation.

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