Specific and Shared Targets of Ephrin A Signaling in Epidermal Keratinocytes

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Both ephrins (EFNs) and their receptors (Ephs) are membrane-bound, restricting their interactions to the sites of direct cell-to-cell interfaces. They are widely expressed, often co-expressed, and regulate developmental processes, cell adhesion, motility, survival, proliferation, and differentiation. Both tumor suppressor and oncoprotein activities are ascribed to EFNs and Ephs in various contexts. A major concern regarding the EFN/Eph system concerns their large number and functional redundancy given the promiscuous cross-activation of ligands and receptors and the overlapping intracellular signaling pathways. To address this issue, we treated human epidermal keratinocytes with five EFNAs individually and defined the transcriptional responses in the cells. We found that a large set of genes is coregulated by all EFNAs. However, although the responses to EFNA3, EFNA4, and EFNA5 are identical, the responses to EFNA1 and EFNA2 are characteristic and distinctive. All EFNAs induce epidermal differentiation markers and suppress cell adhesion genes, especially integrins. Ontological analysis showed that all EFNAs induce cornification and keratin genes while suppressing wound healing-associated, signaling, receptor, and extracellular matrix-associated genes. Transcriptional targets of AP1 are selectively suppressed by EFNAs. EFNA1 and EFNA2, but not the EFNA3, EFNA4, and EFNA5 cluster, regulate the members of the ubiquitin-associated proteolysis genes. EFNA1 specifically induces collagen production. Our results demonstrate that the EFN-Eph interactions in the epidermis, although promiscuous, are not redundant but specific. This suggests that different members of the EFN/Eph system have specific, clearly demarcated functions.

Ephrins (EFNs) and ephrin receptors (Ephs) are cell membrane-bound proteins that act as bidirectional, reciprocal ligands between adjacent cells (1). EFNs are classified into two subfamilies, EFNA and EFNB, based on their glycosylphosphatidylinositol-anchored versus transmembrane structure, respectively. In parallel, their receptors, Ephs, are classified into the EphA or EphB family depending on the preference for EFNA or EFNB ligands, respectively (2). Direct cell-to-cell contact is usually necessary for signaling, and to be recognized as ligands, EFNs and Ephs have to be physically clustered. Gene knock-out studies have demonstrated that the EFN/Eph system plays a major role in patterning the vertebrate neural system (3, 4). In addition, EFN/Eph signaling systems function in vascular system assembly, carcinogenesis, and tumor progression (5–7).

Ephs comprise the largest family of receptor tyrosine kinases with 14 members detected in humans (2). The intracellular domains of Ephs contain tyrosines that, when phosphorylated, serve as docking sites for signal transduction proteins, including SH2 and PTB domain proteins. Known signal transducers for Ephs are Src family kinases, the Jak/STAT3 pathway, Grb-2, Grb-10, Nck, PI3K, and Ras GTPase-activating protein (1, 8). Ena/vasodilator-stimulated phosphoprotein and Ephexin were identified as the links between ephrin receptors and the cytoskeleton (9–11). The intracellular signal transduction pathways downstream from EFNs include tyrosine phosphorylation, PDZ-binding proteins, and unrelated receptors, such as PDGF receptor (12, 13).

The role of the EFN/Eph system in healthy epidermis remains largely unexplored. Activation of EphA2 caused transient suppression of Erk1/2-MAPK but did not inhibit keratinocyte proliferation; actually, it increased desmosomal adhesion and terminal differentiation via the up-regulation of desmoglein 1, a desmosomal cadherin that maintains the integrity and differentiated state of suprabasal keratinocytes in the epidermis (21). These results suggest a role for the EFN/Eph proteins in the basal layer keratinocytes to respond to cues from their neighbors and regulate commencement of a terminal differentiation. When soluble EFN or Eph peptide mimetics were systemically delivered to mice, they caused increased keratinocyte proliferation (22). EphA2 expression is increased in keratinocytes in response to hypoxia or UV irradiation (23, 24). Our microarray studies suggest important epidermal function of the EFN/Eph system (Supplement 1). Specifically, we found that TNFα induces the expression of EFNA1 (25), and this induction is usually necessary on the activation of the NFκB tran-
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scription factor (26) but not on the MAPK kinases ERK, JNK, and p38 (27). In contrast, expression of EphA2 is induced by irradiation with UV light and by certain proinflammatory cytokines, e.g. Oncostatin M, which is dependent on the ERK and p38 signal transduction pathways (27–30).

Analysis of the gene expression patterns deposited with the Gene Expression Omnibus (GEO) data bank suggests that EFNA1, EFNA3, and EFNA4 as well as EphA1, EphA2, and EphA4, but no other EFNA/EphA gene, are expressed in human epidermis (Supplement 2). Interestingly, the three EFNA genes expressed in skin, EFNA1, EFNA3, and EFNA4, are encoded at Ch1q21–22 in the epidermal differentiation complex between SPPR and S100A11/12 genes (31). They are closely linked to other markers of keratinization, such as filaggrin, loricrin, and involucrin (and to the syntenic region of the mouse genome). The EFNA2 and EFNA5 genes, not expressed in skin, are located on chromosomes 19 and 5, respectively. We found EphA1 expressed in the basal layer of human epidermis (Supplement 3), which agrees with the published data (21, 32).

Given the large number of EFNs and Ephs and the promiscuous interactions within the A and B families, the most important and interesting questions regarding their biology are “why are there so many of them?” and “what are their individual and common functions?” (see Supplement 4). We can envision two extreme possibilities. First, because all EFNA3s can bind all EphAs, their functions are completely redundant: each EFNA causes the same changes in cells expressing any EphA, which means that they are individually distinguished only by their differential expression. Second, each EFNA and EphA is functionally unique and has specific roles and effects.

To answer these questions, we used large scale transcriptional profiling of EFNA-treated keratinocytes. Specifically, we treated cultures of human epidermal keratinocytes with EFNA1, EFNA2, EFNA3, EFNA4, or EFNA5; harvested the cells 24 h later; and identified the differentially expressed genes using Affymetrix HU133Av2 microarrays. We found that neither of the two extreme hypotheses is correct. EFNA3, EFNA4, and EFNA5 do have essentially identical transcriptional effects, i.e. they regulate the same set of genes, which makes them completely redundant in keratinocytes. However, the transcriptional effects of EFNA1 and of EFNA2 are distinct and unique, although they partly overlap both with each other and with the effects of the EFNA3,4,5 set. Although all five EFNAs promote keratinocyte differentiation, including induction of synthesis of differentiation markers, cessation of proliferation, and inhibition of migration, only EFNA1 and EFNA2 regulate the members of the ubiquitin-associated proteolysis genes, and EFNA1 alone induces fibrillar collagens. Our results implicate complex biological functions of EFNAs, some redundant for all, some shared by subsets, and yet others specific for individual members.

EXPERIMENTAL PROCEDURES

Human Keratinocyte Cultures and Treatment with EFNAs and EphAs—The growth and treatment of keratinocytes has been described (25–27, 33). Briefly, normal human neonatal foreskin epidermal keratinocytes were obtained from Dr. M. Simon (Living Skin Bank, Burn Unit, State University of New York, Stony Brook, NY) and grown in a defined serum-free keratinocyte growth medium (Keratinocyte-SFM, Invitrogen) supplemented with 2.5 ng/ml epidermal growth factor, 0.05 mg/ml bovine pituitary extract, and 1% penicillin/streptomycin (keratinocyte growth medium) at 37 °C in 5% CO2 incubator. Third passage cells were used at 70–80% confluence. We changed the medium to keratinocyte growth medium with 0.4 mM Ca2+ 24 h before EFN treatment to avoid the effects of the supplements in growth medium. Keratinocytes, grown on 100-mm plates, were then treated with human Fc-conjugated EFNA1, EFNA2, EFNA3, EFNA4, EFNA5, EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, and EphA8 (25 ng/ml; Sigma) or left untreated as controls. The cells were harvested by scraping 24 h after treatment. Cornified envelope ghosts were prepared as described before (27).

Western Blot Analyses—For preparation of the whole cell lysates, cells were washed with cold phosphate-buffered saline (PBS) 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 PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The lysates were clarified by centrifugation at 16,000 × g for 10 min at 4 °C. The protein concentration of each sample was determined with Bio-Rad protein assay reagent. For whole cell lysates, 20–50 μg of protein was loaded onto 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P) using a semidy transfer cell (Bio-Rad) and then blocked in 5% evaporated milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20). The membranes were incubated with primary antibody for 1 h at RT in 5% evaporated milk in TBST, washed extensively with TBST, then incubated with anti-rabbit or anti-mouse HRP-conjugated secondary antibodies, and visualized either using the ECL detection kit (Amersham Biosciences) or the Super Signal West Pico Chemiluminescent Substrate (Pierce). The equivalent loading of proteins in each well was usually ascertained using Ponceau staining of the membrane or reprobing with a GAPDH- or actin-specific antibody.

Immunofluorescence Staining—The keratinocytes were grown on Lab-Tek chamber slides (Nunc). At several time points after treatment, the cells were rinsed with PBS and then immediately fixed with 70% methanol for 10 min. After rinses with PBS, the cells were incubated with primary antibody diluted in PBS containing 1% bovine serum albumin. Following this, the cells were incubated with a dilution of FITC-labeled anti-IgG (Sigma) for 1 h at room temperature. The stained cells were observed under the microscope (Zeiss Axiophot), and images were captured with a digital photo camera (Sony DKC-5000). To visualize F-actin, we fixed the cells with 4% paraformaldehyde in PBS for 10 min at room temperature and used fluorescein-phalloidin (Molecular Probes, Eugene, OR) at a dilution of 1:40 after permeabilization of the cell membranes with 0.1% Triton X-100 in PBS.

In Vitro Wounding Scratch Assay—For the wounding scratch assay, cells were grown in a 12-well dish in 1 ml of keratinocyte growth medium. At 80% confluence, the medium was changed to keratinocyte basal medium. After 24 h, the medium was aspirated, and the cells were treated with mitomycin C (8 μg/ml) in
keratinocyte basal medium for 1 h in the dark at 37 °C. Mitomycin C prevents cell division and proliferation, which could confound our analysis. The mitomycin solution was removed, and the cells were washed three times with keratinocyte basal medium for 5 min each time. The medium (1 ml) was added, and the cell monolayer was scratched using a 200-μl pipette tip. The EFN or any other treatment agent was added at this point. Fresh medium with or without the treatment was replaced every 24 h, and the photographs were taken immediately after scratching and 24 and 48 h after the scratching.

Preparation of Labeled cRNA and GeneChip Hybridization—We isolated total RNA from treated and untreated keratinocytes with RNeasy kits (Qiagen) according to the manufacturer’s instructions. Approximately 5 μg of total RNA was reverse transcribed, amplified, and labeled as described (28). Fifteen micrograms of labeled cRNA was fragmented and hybridized to HU133Av2 arrays (Affymetrix). Arrays were washed, stained with anti-biotin streptavidin-phycocerythrin-labeled antibody, and scanned using the Agilent GeneArray scanner system (Hewlett-Packard) and GeneChip 3.0 software (Affymetrix) to determine the expression of each gene.

Array Data Analysis—Generally, we used the same data analysis approach as described in our previous publication (34). Intensity values from the chips were obtained using Microarray Suite version 5.0 (Affymetrix) and scaled by calculating the overall signal for each array. 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 eliminate genes exhibiting potential false positive differential expression, first we calculated the median intensity for all genes scored as “absent” on the chips and the standard deviation of this intensity, and then we selected for further analysis only genes that possessed relative signal intensity greater than 1 standard deviation above that median. We included in the analysis only those genes determined by the Affymetrix algorithm to be present in at least one sample. The eliminated genes are not expressed in keratinocytes or are expressed at such low levels that their measurements are unreliable. This selection usually eliminates approximately half of the genes represented on the chips. To improve reliability even more, we checked individually the absolute expression levels and detection p values at all time points. Differential expressions of transcripts at each time point were determined two ways: by using the standard GeneChip data mining software Affymetrix-DMT3.0 Microsuite 5.0 discrimination evaluation and by calculating the -fold change where genes were considered regulated if the expression levels differed more than 1.5-fold relative to untreated control. Thus, we combined two different and stringent selection criteria, one requiring a statistically significant difference in signal intensity as controls. Keratinocytes do not migrate into the denuded area if treated with EFNAs or with SP600125, whereas EGF enhances their migration. Unlike the EFNAs, EphA6 allows keratinocyte migration, whereas EGF promotes migration, resulting in significantly enlarged cleared areas.

FIGURE 1. EFNs and Ephs regulate keratinocyte motility. a, scratch assay of keratinocytes with all five members of the EFNA family. Untreated keratinocytes; cells treated with SP600125, which completely blocks keratinocyte migration (27); and cells treated with EGF, which promotes migration, serve
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and the second requiring a significant -fold change, which ensure that the genes we list as “regulated” are reliably affected by our experimental treatment. The hierarchical clustering was performed using Institute for Genomic Research MultiExperiment Viewer algorithms (35). Annotation and ontology of regulate genes was obtained using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (36). Raw data have been deposited in the Gene Expression Omnibus (GEO) database, record GSE26521.

We developed an extensive gene annotation table describing the molecular function and biological category of the genes present on the chip based on the Gene Ontology Consortium data.3 The regulated genes are classified according to this table. In addition, we used the L2L program to identify biological processes and cell components statistically over-represented in our lists of differentially expressed genes (37), oPOSSUM for identification of transcription factor binding sites (38), and LOLA programs for comparing lists of genes (39).

RESULTS

Ephrins and Ephrin Receptors Influence Keratinocyte Behavior and Regulate Gene Expression—Recent pioneering work demonstrated that EFNA1 peptide treatment of keratinocytes expressing EphA2 promoted epidermal cell colony stratification similar to keratinocyte differentiation (21). The increase in keratinocyte adhesion and differentiation was associated with up-regulation of desmoglein 1, a protein associated with the differentiated state of suprabasal keratinocytes in the epidermis. To explore whether other members of the EFN/Eph system have similar functional effects in keratinocytes, we used the scratch assay, reasoning that keratinocyte migration would be one of the most likely processes affected given the role of EFNs and Ephs in other systems. Indeed, we could clearly unambiguously demonstrate that all five EFNAS inhibit keratinocyte migration in this assay (Fig. 1a). The EFNAS inhibited keratinocyte migration just like the SP600125 control (27), EGF promoted migration, whereas EphA6 was without effect.

To demonstrate the effects of EFNs and Ephs on keratinocyte migration in another way, we used a cell motility kit; essentially, the kit measures the tracks cleared of fluorescent beads by the moving cells (Fig. 1b). We used JNK inhibitor SP600125 as an inhibitor of cell migration, EGF as an augmentor, and a set of Fc-conjugated EFN/Eph proteins. Qualitatively, the results parallel exactly those obtained with the scratch assays. The data are summarized in Table 1.

We examined the effects of EFNs and Ephs on the attachment of keratinocytes to their substratum because it has been hypothesized that detachment can of its own accord promote differentiation (40). Using cloning rings, we coated a section of the culture plates with a series of EFN/Eph proteins and plated keratinocytes onto such preconditioned surfaces. To compare keratinocyte attachment onto the treated and the bare surface, we fixed the plates, stained the cell nuclei with DAPI blue, and stained the coated surface with red anti-IgG Alexa Fluor 568

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3 M. Blumenberg and T. Banno, unpublished data.

TABLE 1
Specific members of EFNA/EphA families inhibit keratinocyte migration
ND, not done.

| Reagent | Scratch assay | Blue beads |
|---------|---------------|------------|
| EFNA    |               |            |
| 1       | Yes           | Yes        |
| 2       | Yes           | ND         |
| 3       | Yes           | ND         |
| 4       | Yes           | Yes        |
| 5       | Yes           | ND         |
| EphA    |               |            |
| 1       | Yes           | Yes        |
| 2       | No            | ND         |
| 3       | No            | ND         |
| 4       | No            | ND         |
| 5       | No            | ND         |
| 6       | No            | ND         |
| 7       | No            | ND         |
| 8       | No            | No         |

reagents. As shown in Fig. 2a, keratinocytes settled and attached equally to the coated and the uncoated portions of the plates, red and dark areas, respectively. However, while the cells dispersed on the uncoated surfaces and even many single cells could be seen, on the EFNA-coated surfaces, in contrast, the keratinocytes formed very compact, constricted colonies without any cell movement (Fig. 2b). In contrast, the control IgG Fc and EphA8, EphB2, and EphB6 allowed the colonies to spread and disperse as much as on uncoated plates. These data confirm our results shown above. Together they establish that EFNA proteins specifically inhibit keratinocyte migration. Importantly, they also show that the EFNA proteins do not inhibit (or promote) keratinocyte attachment to the substratum. Analogous results were obtained with HaCaT cells, a keratinocyte-derived immortal cell line (not shown).

EFNs and Ephs Regulate Gene Expression—Because the above results clearly demonstrated that EFNAS strongly affect keratinocyte motility, we hypothesized that these agents regulate gene expression as well. Therefore, we performed a series of microarray experiments with EFNA-treated keratinocytes. We treated a single large batch of keratinocytes with each of the five EFNAS and used an untreated culture as control. The cells were harvested 24 h later, and RNA was prepared for microarray analysis. We chose this time point because in preliminary experiments spanning 48 h we found that the 24-h time point showed the largest number of regulated genes (not shown).

The promiscuous binding of all EFNAS to all Ephs suggests a model in which all EFNAS could have identical effects on gene expression: they can all bind to all the EphA receptors expressed in a given cell type, and all could equally activate the downstream signaling pathways. Unexpectedly, we found that different members of the EFNA family regulate different sets of genes! This result clearly and unambiguously refutes the possibility that the EFNAS are redundant and interchangeable.

Instead, we found that only EFNA3, EFNA4, and EFNA5 produced identical transcriptional changes, forming a cluster of redundant signaling proteins. On the other hand, EFNA1 and EFNA2 produced changes that extensively over-
lap with those of the EFNA3,4,5 cluster and with each other. However, EFNA1 and EFNA2 also regulated nonredundant sets of genes, characteristic for each of these two ephrins (Fig. 3). We show these results in several ways. Spearman correlation clustering analyses demonstrate that the effects of EFNA3, EFNA4, and EFNA5 are, within experimental error, virtually identical, creating a large red square of high similarity (Fig. 3a). EFNA1 and EFNA2 regulate mutually very similar but clearly distinguishable sets of genes. Ctr, control. b, hierarchical clustering of the microarrays also showing virtually identical results in EFNA3, EFNA4, and EFNA5 samples and high similarity with EFNA1 and EFNA2. c, Venn diagrams comparing the genes regulated by EFNA3, EFNA4, and EFNA5. Note that, within selection parameters, an overwhelming majority of regulated genes are regulated by all three EFNAs (horizontal arrow). d, Venn diagrams comparing the genes regulated by EFNA1, EFNA2, EFNA3, and EFNA4. The single arrow points to a large set of genes regulated by all EFNAs; the triple arrow points to the genes specifically regulated by EFNA1, EFNA2, or both.

Characteristics of EFNA-regulated Genes—The functional categories of regulated genes are very interesting. For example, all EFNAs suppressed cell adhesion genes, especially integrins, and induce epidermal differentiation markers (Table 2). EFNA1 and EFNA2, but not the EFNA3,4,5 cluster, regulated the members of the ubiquitin-associated proteolysis genes. Ontological analysis of the EFNA-regulated genes showed that all EFNAs induced epidermis development, cornified envelope, and keratin genes while suppress-
pressed genes as well. This is not surprising given that the dermal development feature prominently in the set of suppressions (underlined in Table 4a). Importantly, skin expression and epidermal keratinocytes are flagged as statistically significant as well atins feature prominently in this set, intermediate filaments and thyosis” and “epidermis development” (Table 4a). Because keratin 18 homolog E3 ubiquitin protein ligase}

| Efna1  | Efna2  | Efna3  | Efna4  | Efna5  | Avg 1.2A  | Av 3.4V |
|--------|--------|--------|--------|--------|----------|---------|
| -0.37  | -0.26  | -0.84  | -0.97  | -0.75  | -0.32    | -0.85   |

| Gene Title | SYMBOL | Function |
|------------|--------|----------|
| leupaxin   | LPXN   | Adhesion |
| pixilluin  | PXN    | Adhesion |
| neurotrimin| HNT    | Adhesion |
| discoidin   | DCLBD2 | Adhesion |
| discoidin   | DCLBD2 | Adhesion |
| integrin, beta 6 | ITGB6 | Adhesion, integrin |
| integrin, beta 4 | ITGB4 | Adhesion, integrin |
| integrin, beta 4 | ITGB4 | Adhesion, integrin |
| integrin, beta 4 | ITGB4 | Adhesion, integrin |
| integrin, beta 4 | ITGB4 | Adhesion, integrin |

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**TABLE 2**

Selection of regulated genes

The adhesion- and the epidermal differentiation (Epid. Diff.-) associated genes are suppressed and induced, respectively, by all EFNAs: the ubiquitin-associated genes are regulated by EFNA1 and EFNA2 only. Red color marks the induced genes, and green color marks the suppressed genes. The blue color on the left indicates the relative level of expression of a given gene from highest (darkest blue) to lowest (light blue). Several genes are represented multiple times on the microarrays. Note that the -fold regulation is on a log2 scale, i.e., 1 = 2-fold induction, -2 = 4-fold suppression, etc. Avg/Av, average.

![Microarray image](image-url)

The ontological categories most prominent and statistically relevant in the set of genes induced by all five EFNAs are “ichthyosis” and “epidermis development” (Table 4a). Because keratin 18 homolog E3 ubiquitin protein ligase}

![Table 2](table-url)

| Gene Title | SYMBOL | Function |
|------------|--------|----------|
| ITGB6      | Adhesion, integrin |
| ITGB4      | Adhesion, integrin |
| ITGB4      | Adhesion, integrin |
| ITGB4      | Adhesion, integrin |
| ITGB4      | Adhesion, integrin |
| ITGB4      | Adhesion, integrin |

Focusing on the genes regulated by EFNA1, we found, in addition to epidermis development, very prominent induction of secreted extracellular proteins (Table 4c). Unique to EFNA1, experiments used epidermal keratinocytes. However, the induced and suppressed genes in these categories are quite distinct: although the induced genes comprise several differentiation-specific markers, keratins KRT1 and KRT10, E-FABP, and CRABP2, the suppressed genes are associated with cell-cell signaling (Table 4b).

Unexpectedly, we found that 24 of the suppressed genes are noted as targets of AP1 transcription factor (Table 4a). They are listed in Supplement 5. This is unexpected because functional AP1 sites have been found in several genes induced during epidermal differentiation (41–43). Many genes regulated by transcription factor IRF7 were also suppressed by all EFNAs. The results imply that AP1 and IRF7 are shut down by EFNAs in epidermal keratinocytes.

Focusing on the genes regulated by EFNA1, we found, in addition to epidermis development, very prominent induction of secreted extracellular proteins (Table 4c). Unique to EFNA1,
the fibrillar collagens seem induced. Interestingly, the suppressed genes include apoptosis regulators; predominantly inhibitors; and signaling proteins that promote angiogenesis, wound healing response, growth factors, cell mobility, and division. The targets of the AP1 transcription factor are particularly prominent among the genes suppressed by EFNA1. Targets of transcription factors NFκB, E47, BACH1,2, sex-determining region Y (SRY), and GATA6 are also overrepresented among the genes suppressed by EFNA1. Suppressed genes include regulators of apoptosis and proliferation.

Focusing on the genes regulated by EFNA2, we found sets of genes induced by several transcription factors and conversely sets suppressed notably by AP1, E47, and BACH2. Additional targets of specific transcription factors are among the suppressed genes (Table 4d). In common with EFNA1, the apoptosis and cell cycle genes were suppressed.

Focusing on the genes regulated by the EFNA3,4,5 set, we found epidermis development and secreted extracellular proteins (Table 4e). IRF targets were suppressed by these three EFNA. Genes involved in wound healing, signaling, and angiogenesis are prominent among genes suppressed by EFNA3,4,5. We note that apoptosis and proliferation genes are not highlighted in the EFNA3,4,5-regulated set; thus, the EFNA3,4,5-regulated genes are distinct from those regulated by EFNA1 and EFNA2.

**EFNAs Promote Epidermal Differentiation in Vitro**—We treated keratinocytes with each of the five EFNA in the keratinocyte growth medium developed by Lindberg and Rheinwald (44). The growth medium contained a small concentration of Ca^{2+} ions (0.4 mM), which allows long term cultivation and expansion of keratinocytes in a relatively slow growing mode (45). Under these conditions, the addition of each EFNA promoted epidermal differentiation (Fig. 4). For example, the epidermal differentiation markers were induced at the protein level (Fig. 4, a and b). Both EFNA tested induced the production of differentiation marker keratins KRT1 and KRT10, which we showed using AE2 antibody specific for these keratins (Fig. 4a), a gift from Cooper and Sun (46). Moreover, both induced the expression of TMG1, keratinocyte transglutaminase, the enzyme responsible for production of cornified envelopes by protein cross-linking, and involucrin (IVL), the canonical epidermal differentiation marker (Fig. 4b). Importantly, in the centers of the colonies, we observed piling up and stratification of keratinocytes (Fig. 4c, marked with arrows), which do not ordinarily occur in two-dimensional cultures. This is similar to what we saw in the SP6001125-treated cultures (27) and confirms the results of Lin et al. (21). EphAs did not have similar effects.

Finally, we showed that under the direction of EFNA keratinocytes completed their differentiation program by creating cornified envelopes, the final products of epidermal terminal differentiation (Fig. 4d). Together, these results unequivocally indicate that EFNA promote terminal differentiation of human epidermal keratinocytes.

**DISCUSSION**

Most importantly, the results presented here demonstrate that different members of the EFNA family have different and specialized effects on keratinocytes. In particular, the EFNA family can be subdivided into three characteristic subgroups: EFNA1, EFNA2, and the EFNA3,4,5 cluster. The results imply that similar subdivisions exist in the EphA, EFNB, and EphB families as well.

Analysis of the regulated genes indicated that all EFNA promote epidermal differentiation. This is in agreement with the data of others (21). Intriguingly, our results suggest a possible mechanism: many of the genes suppressed by EFNA are targets of the AP1 transcription factors, bringing up a testable hypothesis: EFNA cause epidermal differentiation in part by shutting down certain AP1 transcription factors. This will be the focus of our future studies. Although the AP1 proteins have been implicated in the induction of expression of certain epidermal differentiation markers (41, 47, 48), the AP1 proteins are indispensable for keratinocyte proliferation and also for the expression of basal cell markers (27, 49–51).

Our data suggest an interesting nexus between different signaling pathways affecting the AP1 transcription factors: inhibiting the JNK pathway and its downstream targets promotes epidermal differentiation (27), but inhibiting the parallel ERK and p38 pathways does not. The functional signaling via the JNK pathway by the Ephrin B family has been demonstrated (52), but no such data exist for the Ephrin A family at present. Interestingly, the transcriptional targets of IRF7 were also suppressed by EFNA. A role for IRF7 in epidermal differentiation or a nexus between IRF7 and ephrin signaling has not been reported (53).

Because we used epidermal keratinocytes in our experiments, it is not surprising that skin-specific genes predominate among both the induced and suppressed genes. However, discriminating between the two classes is very instructive: epidermal differentiation genes are induced, whereas secreted signaling proteins are suppressed. Apparently, when EFNA instruct keratinocytes to differentiate, they also instruct the cells to halt signaling to the underlying tissue. This notion raises interesting possibilities for using EFNA signaling in differentiation therapies in inflammatory diseases, such as psoriasis, or in squamous and basal cell carcinomas.

**TABLE 3**

Functional analysis of genes regulated by all EFNA

The gene ontology categories are given for the specifically induced biological processes, molecular functions, and cellular components, e.g. epidermis development (left), or those suppressed by all EFNA, e.g. response to wounding (right). Only the categories with probability (prob) values better that 10^-3 are listed. DN, suppressed; UP, induced.

| EFNAs-UP-Process | bin prob | EFNAs-Down-Process | bin prob |
|------------------|----------|--------------------|----------|
| keratinocyte transglutaminase | 2.8E-07 | regulation of cell cycle | 1.2E-07 |
| cell-matrix adhesion | 9.4E-06 | cell-matrix adhesion | 9.4E-06 |
| EFNA-UP-Function | bin prob | EFNA-Down-Function | bin prob |
| growth factor activity | 4.0E-08 | MAP kinase phosphatase activity | 2.0E-06 |
| EFNA-UP-Component | bin prob | EFNA-Down-Component | bin prob |
| cell surface | 1.3E-07 | integrin complex | 1.3E-05 |
| extracellular region | 3.8E-05 | extracellular region | 3.8E-05 |

* M. Blumenberg, manuscript in preparation.
### TABLE 4

**Ontological categories of genes regulated by various EFNAs**

| Gene Ontology Category | EFNA1 | EFNA2 | EFNA3, 4, 5 |
|------------------------|-------|-------|-------------|
| Epidermal development  |       |       |             |
| Suppressed             |       |       |             |
| Epidermal growth       |       |       |             |
| Suppressed             |       |       |             |
| Keratin differentiation|       |       |             |
| Suppressed             |       |       |             |
| TLR signaling          |       |       |             |
| Suppressed             |       |       |             |
| TNF signaling          |       |       |             |
| Suppressed             |       |       |             |

**Legend**

- **Suppressed** indicates genes whose expression is reduced in response to EFNA treatment.
- **Epidermal growth** refers to genes involved in the proliferation and differentiation of epidermal cells.
- **Keratin differentiation** includes genes involved in the production of keratin, a key constituent of skin.
- **TLR signaling** involves genes involved in the Toll-like receptor signaling pathway.
- **TNF signaling** includes genes involved in the tumor necrosis factor signaling pathway.

**Note:** The table provides a comprehensive overview of the ontological categories regulated by EFNA1, EFNA2, EFNA3, 4, and 5, highlighting the changes in gene expression associated with various signaling pathways in epidermal cells.
Additionally, EFNA1 shares many targets with other members of the EFNA family. However, the specific effects of EFNA1 include induction of collagen synthesis, suppression of collagen-degrading enzymes, and suppression of components of the cell cycle and apoptosis machineries. Both EFNA1 and EFNA2 shut down targets of AP1; this is a less prominent effect of the EFNA3,4,5 set, which seems to work more through inhibiting IRF7.

Importantly, although they promoted differentiation, EFNAs inhibited keratinocyte motility. Expression of several integrins was suppressed by EFNAs. Detachment of integrins from the substratum has been proposed as a signal to commence differentiation (40). We note, however, that EFNAs did not inhibit physical attachment of the keratinocytes; actually, the cells attached as well to the EFNA-coated surface as to plastic. There are several parallel mechanisms allowing keratinocyte attachment; decreasing the integrin-mediated component of attachment may have more of a signaling than a mechanical role in initiating epidermal differentiation.

Finally, our results begin to explain the existence of multiple members of EFN and Eph proteins, a large family of closely related proteins. Although characterized by promiscuous binding of multiple EFNs to multiple Ephs, these proteins have both specific and common functions. This allows specific fine tuning of cellular responses to specific signals. Future studies will define the various signal transduction pathways downstream from these cell surface sensors.

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